US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Glass Washing and Detergent Residues Test

SOP Number: QC-03-08

Date Revised: 08-04-17

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Title	Glass Washing and Detergent Residues Test
Scope	This protocol describes procedures to determine the potential presence of detergent residues on glassware washed in lab dishwashers. Detergents used in washing glassware may leave residues which are bacteriostatic. If residues are present, glassware may require additional rinsing to remove them (see section 15).
Application	To verify that detergent residue is not present in laboratory glassware when cleaned using the dishwashers.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

TABLE OF CONTENTS

1. Definitions	Abbreviations/definitions are provided in the text.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Not Applicable.
5. Sample Handling and Storage	1. Use and store detergents according to manufacturer's instructions. 2. Refer to section 10 for cautions associated with handling of Petri dishes.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Inspect all glassware prior to use. Discard items with chips and etched surfaces. 2. Ensure dishwashers are working properly prior to commencing the assay (e.g., scheduled preventive maintenance, if available). 3. Petri plates should be analyzed within 48 hours after sterilization.
8. Non-conforming Data	1. Management of non-conforming data will be consistent with SOP ADM-07, Non-Conformance Reports. 2. Any deviation from the protocol will be documented. If the regular wash procedure (Group A plates) is found to be inadequate for removal of inhibitory detergent residues, then the wash procedure will be adjusted and the detergent residue test repeated.
9. Data Management	1. Data will be archived consistent with SOP ADM-03, Records and Archives. 2. The SOP forms and spreadsheets used for data collection during the inhibitory residue analysis should be completed and archived in the Glass Washing and Detergent Residues Test records.
10. Cautions	1. Ensure plates are thoroughly dried after washing and properly sterilized prior to conducting the test. 2. For this assay, dishwasher runs should only be conducted with the glass Petri dishes to be used in the detergent residue test.
11. Special	1. Test microbe: *Enterobacter aerogenes* (ATCC No. 13048)

Apparatus and Materials	a. Frozen culture prepared according to the Attachment 1. 2. Dishwashers a. Miele Thermal Disinfector/Laboratory Glassware Washer Model G7783 serial number 16/18344823 located in room C206. b. Lancer1600 UP Laboratory Glassware Washer serial number 9G050714 located in room B206. c. Lancer1600 XLP Laboratory Glassware Washer serial number 6G073992 located in room B206. 3. Detergents a. Powder Detergent for Miele dishwasher – Alcojet Low-Foaming Powdered Detergent. b. Liquid Detergent for Lancer dishwashers – Lancer Clean Detergent LCD-P. 4. Materials a. Glass Petri Dishes – Group A (20 × 100 mm) – used for dishwasher treatments. b. Disposable plastic Petri dishes – Group B (20 × 100 mm) – used for the control (reference point) treatment. 5. Culture Media a. *Cryoprotectant solution (TSB with 15% glycerol).* Suspend 7.5 g Tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, boil to homogenize. Dispense into bottles and autoclave for 15 min at 121°C. Solution is used in the preparation of frozen stock cultures, see Attachment A. b. *Tryptic soy agar (TSA).* Prepare according to manufacturer's instructions. Agar is kept at 45-50˚C. Used for pour plating – for recovery of *E. aerogenes*. c. *Tryptic Soy Broth (TSB).* Purchased broth from a reputable source and prepared according to manufacturer's instructions. Media is used in the preparation of test cultures. d. *Nutrient Broth (NB).* Purchase broth from a reputable source and prepare according to manufacturer's instructions. Media is used in the preparation of frozen stock cultures.

	e. *Nutrient Agar (NA).* Purchase broth from a reputable source and prepare according to manufacturer's instructions. Media is used in the preparation of frozen stock cultures. *f.* *Levine EMB Agar*. Purchase pre-made plates from a reputable source. Media is used for confirmation of the test microbe during preparation of frozen stock cultures. 6. Reagents a. *Phosphate buffer saline (PBS) stock solution – 10X*. Stock solution has a pH of 7.2±0.2. Used to prepare 1X phosphate buffered saline (PBS). b. *Phosphate buffered saline (PBS – 1X).* PBS 1X with a pH of approximately 7.0±0.5 is desirable. Used for dilution blanks. 7. Apparatus a. Calibrated micropipettes (e.g., 1000 µL) with appropriate tips. Used for serial dilutions and plate inoculation.
12. Procedure and Analysis	Perform the detergent residues test once every 24 months or when a new lot or different type of detergent is used. 1. The procedure includes one group (set) of six washed Petri dishes per dishwasher, this group is designated Group A (i.e., Group – A – Miele, Group – A – Lancer 1600 UP and Group – A – Lancer 1600 XLP). 2. In addition, Group B corresponds to a group of 6 sterile plastic Petri dishes used as a reference point (i.e., controls) 3. Conduct the analysis (detergent inhibitory residue test) within 48 hours after plate sterilization. 4. Fill out a preparation sheet for each set of plates corresponding to Group A (i.e. three preparation sheets for each dishwasher set of plates). See SOP QC-15, Media Prep and Sterilization Run Numbers.
12.1 **Washing Procedure for Miele Thermal Disinfector/Laboratory Glassware Washer (Model G7783)**	Group – A: a. Place six glass Petri dishes in the dishwasher facing down and spaced evenly so the water will run out of the dish. Place three Petri dishes in the lower compartment and three Petri dishes in the upper compartment. b. A total of 4 scoops of powdered detergent are used. Fill the detergent compartment on the door with 2 scoops of powder detergent and close the detergent cover. Place one scoop of detergent directly on the washer door and a second one on the base of the dishwasher. Record

	the amount of detergent used on the media prep sheet. c. Press the button for Program E, the Universal wash program, and then press the Start button (diamond with vertical bar symbol). This program includes a pre-wash and heated main wash (85°C), two tap water rinses and two DI water rinses, one unheated and one heated (70°C). This is the normal treatment that all machine-washed laboratory glassware receives in this dishwasher.
12.2 **Washing Procedure for Lancer 1600 UP and Lancer 1600 XLP Laboratory Glassware Dishwashers**	Liquid detergent is dispensed automatically through a metering pump. Group – A: a. Place six glass Petri dishes in the dishwasher facing down and spaced evenly so the water will run out of the dish. Place three Petri dishes in the lower compartment and three Petri dishes in the upper compartment. b. Enter 10 on the keypad to select Cycle 10 and press the Start button. Cycle 10 is the designated standard laboratory wash program.
12.3 **Petri Plate Preparation and Sterilization**	a. Remove each set of glass Petri dishes from each dishwasher. b. Record group designation and dishwasher type (Miele – Group – A, Lancer 1600 UP – Group – A and Lancer 1600 XLP– Group – A) on each bin. c. Allow plates to dry overnight after washing in open bins. d. Sterilize plates using a gravity cycle for 25 min. e. Record the sterilization run number on the appropriate Laboratory Detergent Residue Test Form (see section 14).
12.4 **Preparation of the Test Organism**	a. Refer to Attachment 1 for preparation of the frozen stock cultures for *Enterobacter aerogenes* (ATCC #13048). b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only. c. Add 100 µL of defrosted stock culture to 10 mL TSB, briefly vortex mix and incubate for 18-24 h at 36±1°C d. In addition, inoculate an agar plate (e.g., TSA, TSA with 5% sheep blood) with a loopful from the inoculated tube and streak for isolation. Incubate plate with the test culture and examine for purity. Record results of purity check on microbe tracking sheet (see section 14 e. Briefly vortex the 18-24 h culture and transfer to a 15 mL centrifuge tube.

	f. Centrifuge the 18-24 h broth cultures at ~5,000 g_N for 20±5 min. g. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in 10 mL of PBS.
12.5 **Detergent Residue Test**	a. Use test culture prepared in step 12.4, serially dilute the test culture by transferring 1 mL into 9 mL PBS in a dilution tube. Dilute out to 10^{-7}. Volume in dilution tube may be adjusted according to the number of plates to be inoculated. For example, based on the number of glass Petri dishes (18 total) and controls (6 total), the 10^{-7} should have a final volume of at least 27 mL. b. Add 1 mL of 10^{-7} dilution to each of the 18 glass Petri plates (Group A). c. Add 1mL of the 10^{-7} dilution to each of the six plastic Petri dishes (Group B - controls). d. Add 25 mL of molten TSA tempered to 45-50ºC, to each Petri plate (glass and plastic) and gently swirl to thoroughly mix. e. Allow plates to harden. f. Invert plates and incubate plates at 36±1ºC for 24±2 hours.
12.6 **Results and Confirmation Procedures**	a. Count colonies and record results. Record colony counts in excess of 300 on plates as Too Numerous to Count (TNTC). If no colonies are present, record as zero. b. Inspect the growth on the plates for purity and typical characteristics of the test microbe (see Table 1). c. If isolated colonies are present, prepare a Gram stain on one representative colony. Record stain results and the purity of isolated colonies on the Test Microbe Confirmation Sheet. d. If additional confirmatory analyses are necessary, perform a streak isolation from a plate representing Group A and B onto a BAP and a Levine EMB Agar. Incubate all plates at 36±1°C for 24±2 h. e. See Table 1 – General Diagnostic characteristics for *Enterobacter aerogenes*.

Table 1. General and Selective media and diagnostic characteristics for *Enterobacter aerogenes* (see ref. 15.4)

Typical Microscopic Characteristics of *Enterobacter aerogenes*	
Cell appearance	Straight or slightly curved rods, single polar flagella. 0.6-1.0 micrometers by 1.2-3.0 micrometers. Motile with flagella.
General Media	
BAP	Small, round, smooth grayish white colonies
Selective Media	
Levine EMB Agar	Large mucoid purple small colonies
Confirmatory Stain	
Gram Stain	Negative

13. Data Analysis/ Calculations

1. Use a spreadsheet to calculate the average CFU per treatment (Group A and B) and to calculate the Percent Difference between Group A and B per dishwasher (see section 14).
2. Differences in averaged counts between Groups A and B plates should be within 25% of each other if there are no toxic or inhibitory residues.
3. A difference of more than 25% between replicate plates (six per group) is associated with the presence of inhibitory detergent residues.
4. The increased percent difference (>25%) may be due dishwasher performance, plating inconsistencies and other factors. In this case the test results are unacceptable and the test should be repeated.

14. Forms and Data Sheets

Test Sheets. Test sheets are stored separately from the SOP under the following file names:

Detergent Residue Test: Organism Culture Tracking Form	QC-03-08_F1.docx
Detergent Residue Test: Test Microbe Confirmation Sheet (Quality Control)	QC-03-08_F2.docx
Detergent Residue Test: Processing Form for Machine Washed Items	QC-03-08_F3.docx
Detergent Residue Test: Test Culture Dilution Scheme, Plate Count Data and Results Form	QC-03-08_F4.docx

	Detergent Residue Test spreadsheet QC-03-08_F5.xlsx
15. References	1. Bordner, R. H., J. A. Winter and P. V. Scarpino. eds. 1978. Microbiological Methods for Monitoring the Environment, Water and Wastes. EPA-600/8-78-017, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. 2. Eaton, A. D., Clesceri, L. S., Rice, E. W., Greenberg, A. E. and Franson, M. A. H. eds. 2017. Standard Methods for the Examination of Water and Wastewater, 23rd Edition. American Public Health Association, Washington, DC. 3. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011. 4. Holt, J.G, Sneath, P.H.A, Krieg, N.R., Staley, J.T, Williams, S.T. Bergey's Manual of Determinative Bacteriology, 9th Edition. 1994.

Attachment 1

Procedures for Maintenance of Vegetative Bacterial Cultures – Preparation of Frozen Stock Cultures for *Enterobacter aerogenes*

Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Enterobacter aerogenes* from ATCC at least every 18 months.

b. Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of Nutrient Broth (NB), aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36±1ºC for 24±2 h.

c. After incubation, streak a loopful of the suspension on Nutrient Agar (NA) to obtain isolated colonies. Incubate the plates for 24±2 h at 36±1°C.

   i. For QC purposes, perform a streak isolation of the 24±2-hour broth culture on a BAP and Levine EMB Agar. Incubate all plates at 36±1°C for 24±2 h.

d. Select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of NB. Spread plate 0.1 mL of the suspension on each of 6-10 NA plates. Incubate the plates for 24±2 h at 36±1°C.

   i. For QC purposes, perform a streak isolation of the 1 mL NB + isolated colonies on a BAP and Levine EMB Agar. Incubate all plates at 36±1°C for 24±2 h.

e. Following the incubation of the agar plates from d, place approximately 5 mL sterile cryoprotectant solution on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

   i. For QC purposes, perform a streak isolation of the pooled culture on a BAP and Levine EMB Agar. Incubate all plates at 36±1°C for 24±2 h.

f. Immediately after mixing, dispense 0.5-1 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

g. Store the cryovials at -70ºC or lower for a maximum 18 months then reinitiate with a new lyophilized culture.

Note: New stock culture may be initiated one time using an existing, unexpired frozen stock culture.

h. Following the incubation period (see e.i), record the colony morphology as observed on the BAPs and selective media plates.

i. Perform a Gram stain from growth taken from the TSA plates according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion, see section 12.7 for confirmation information)).

j. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control).