



**US Environmental Protection Agency  
Office of Pesticide Programs**

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

**Standard Operating Procedure for  
Neutralization Confirmation Procedure for  
Products Evaluated with the AOAC Use Dilution Method  
and the AOAC Germicidal Spray Products as Disinfectants Test  
(Staphylococcus aureus, Pseudomonas aeruginosa,  
and Salmonella enterica)**

**SOP Number: MB-17-01**

**Date Revised: 02-04-09**

EPA/OPP MICROBIOLOGY LABORATORY  
ESC, Ft. Meade, MD

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Effective Date: \_\_\_/\_\_\_/\_\_\_

Controlled Copy No.: \_\_\_\_\_

Withdrawn By: \_\_\_\_\_ Date: \_\_\_/\_\_\_/\_\_\_

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1.0 SCOPE AND APPLICATION:

- 1.1 The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in efficacy testing. A neutralizing agent is used to inactivate the product's active ingredients, a process essential to achieving the desired contact time. In addition, the neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbes. Bacteriostatic activity may bias the outcome of an efficacy evaluation.
- 1.2 This SOP describes methodology used to determine the effectiveness of neutralizers specified for disinfectant efficacy testing. The Neutralization Confirmation Procedure is a carrier-based method (sterile carriers) which simulates the test conditions, but is designed to quantitatively assess the effectiveness of neutralizers across a broad range of microbe concentrations.
- 1.3 *Staphylococcus aureus* (ATCC No. 6538), *Pseudomonas aeruginosa* (ATCC No. 15442), and *Salmonella enterica* (ATCC No. 10708) are the test microbes in disinfectant efficacy evaluation, and are used in the neutralization testing.
- 1.4 General Description of the Assay.
  - 1.4.1 Follow the test conditions specified for product testing (e.g., H<sub>2</sub>O hardness, Use Dilution, pH, Organic Soil, Neutralizer, Contact Time, Temperature) for the neutralization confirmation assay. Record the test information on the appropriate Neutralization Confirmation Assay: Test Information Sheet (see 16.0).
  - 1.4.2 This assay is designed to simulate the conditions of the AOAC UDM and AOAC GSPT; however, sterile carriers are used instead of inoculated carriers. Diluted *S. aureus*, *P. aeruginosa*, or *S. enterica* inoculum is added directly to the various sets of subculture media tubes (see Table 1). The inoculum is quantified by plating on a suitable agar such as TSA. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer × disinfectant interactions.
- 1.5 This method can also be used to determine the effectiveness of an alternative neutralizer, one not specified in the test parameters.
- 1.6 Preferably, perform the neutralization assay concurrently with product testing; however, an independent, stand-alone assay may also be performed in the event that the potential success of the neutralizer is questionable.

2.0 DEFINITIONS:

- 2.1 AOAC = AOAC INTERNATIONAL
- 2.2 AOAC UDT = AOAC Use Dilution Test
- 2.3 AOAC GSPT = AOAC Germicidal Spray Products as Disinfectants Test
- 2.4 Bacteriostatic = Capable of inhibiting or controlling the growth or reproduction of bacteria without killing the cells
- 2.5 CFU = Colony Forming Unit
- 2.6 PBDW = Phosphate Buffered Dilution Water
- 2.7 TSA = Tryptic Soy Agar
- 2.8 DI = Deionized Water

3.0 HEALTH AND SAFETY:

- 3.1 All manipulations of the test organisms are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.
- 3.2 Disinfectants may contain a number of different active ingredients, such as quaternary ammonium compounds, halogens, phenolics, aldehydes, peroxides, and heavy metals. Personal protective clothing or devices are recommended during the handling of these items for the purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine the best course of action.

4.0 CAUTIONS:

- 4.1 To ensure the stability of the test disinfectant solution, perform testing within 3 hours of preparation.
- 4.2 Strict adherence to the protocol is necessary for validity of test results.
- 4.3 Use appropriate aseptic techniques for all test procedures involving the

manipulation of test organisms and associated test components.

5.0 INTERFERENCES:

5.1 For each neutralizer and medium tested per study, use one batch (preparation) of neutralizer and medium for all treatment and control groups. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS: None

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not specify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing or as specified in the product test parameters. Follow SOP MB-22, Disinfectant Sample Preparation, for dilution of products.

10.0 PROCEDURE AND ANALYSIS:

10.1 Preparation, Dilution, and Enumeration of Inoculum.

10.1.1 For Assay Simulating the AOAC Use Dilution Method. The inoculum is prepared according to SOP MB-05, AOAC Use Dilution Method for Testing Disinfectants, sections 10.2 and 10.3. In section 10.3, do not proceed past the point where a minimum of four tubes of inoculum are prepared (i.e., do not inoculate carriers).

10.1.2 For Assay Simulating the AOAC Germicidal Spray Products as

Disinfectants Test. The inoculum is prepared according to SOP MB-06, AOAC Germicidal Spray Products as Disinfectants Test, section 10.2. In section 10.2, do not proceed past the point where at least two tubes of inoculum are prepared (i.e., do not inoculate carriers).

- 10.1.3 If the product test conditions include the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum as per SOP MB-05, section 10.3.4 or SOP MB-06, section 10.3.10. Otherwise, the inoculum should be prepared without the addition of an organic soil load.
- 10.1.4 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the inoculum into 9 mL of PBDW. Use four dilutions, ( $1.0 \times 10^{-5}$ ,  $1.0 \times 10^{-6}$ ,  $1.0 \times 10^{-7}$ , and  $1.0 \times 10^{-8}$ ) to inoculate the neutralizer and subculture media tubes described below. The target number of cells to be delivered (section 10.3.9) is 5-100 CFU/mL; this level should be seen in one of the two highest dilutions.
- 10.1.5 To estimate CFU/mL, plate 0.1 mL of each of the four dilutions in duplicate on TSA. Briefly vortex each dilution tube prior to plating. See 10.1.7 for spread plate method.
- 10.1.6 Record the dilution and plating information on the appropriate Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form and the Neutralization Confirmation Assay: Inoculum Enumeration Form (see 16.0).
- 10.1.7 Spread plate method: allow refrigerated plates to come to room temperature prior to use. Use a glass spreading rod and plate spinner to spread dilutions evenly over the surface of the agar.
- 10.1.8 Incubate plates at  $36 \pm 1^\circ\text{C}$  for 24-48 hours. Count colonies with aid of a plate counter. Plates that have colony counts over 300 can be estimated or labeled TNTC. Record the counts on the Neutralization Confirmation Assay: Inoculum Enumeration Form (see 16.0).

## 10.2 Product Sample Preparation.

- 10.2.1 Follow guidelines for disinfectant sample preparation provided in SOP MB-22.

- 10.3 Performing the Assay. The following instructions apply to the analysis of one neutralizer with one carrier type, and one test organism.
- 10.3.1 Each assay requires four sterile carriers per organism. Use the carrier type required for the specific test. Prepare carriers according to SOP MB-05 (for stainless steel penicylinders) or SOP MB-06 (for glass slide carriers).
- 10.3.1.1 For liquid products: Remove sterile stainless steel carriers from the water, place onto filter paper in sterile glass Petri dishes, and dry for 40 minutes per SOP MB-05.
- 10.3.1.2 For spray products: Before conducting the neutralization assay, the analyst must practice applying the spray product to sterile glass slides to determine the product's level of dispersion. If the product beads up and rolls off of the slide rather than completely covering the glass slide as it would in a typical efficacy evaluation (with inoculated slides), the analyst should apply 10  $\mu$ L of an organic material (e.g., broth used for culturing test organism, 5% horse serum in sterile deionized water) onto the surface of each sterile glass slide and spread it with a sterile loop and dry the slides for 40 minutes per SOP MB-06. Once drying is complete, apply the spray product again to verify that product dispersion is improved, and conduct the neutralization assay. Based on previous observations in the laboratory, the addition of an organic substance to the surface of the slide increases product dispersion on glass slides. If no organic substance is deemed necessary, no drying of the sterile glass slides is necessary prior to conducting the assay.
- 10.3.2 Apply the product to the sterile carriers according to specific instruction provided in the test parameters (e.g., use dilution, spray distance, spray period, contact time).
- 10.3.3 Per test, one test per organism, expose four of the carriers to the disinfectant for the specified contact time in the same manner as product efficacy testing. Record the carrier transfer information on the Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers.

- 10.3.4 After the last carrier of a set (4 total carriers) has been treated with the disinfectant, and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Drain excess liquid from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) will represent the **Neutralizer-Primary Subculture Treatment**. Each tube is inoculated with 0.1 mL of each of the four inoculum dilutions as indicated in Table 1 and section 10.3.9.

Note: For spray products, the amount of neutralizer is 20 mL per tube (38 × 100 mm Bellco tubes) compared to 10 mL (20 × 150 mm tubes) used in the test method for liquid products.

- 10.3.5 Following the last carrier transfer into the neutralizer tube, incubate the neutralizer tubes at  $36 \pm 1^\circ\text{C}$  for a minimum of 30 minutes. Then transfer each carrier in order into a culture tube containing the secondary subculture medium. This portion of the assay is not timed, but the transfers should be made as soon as possible after the end of the 30 minute incubation. This set of tubes (4 total tubes) will represent the **Secondary Subculture Treatment**. Each tube is inoculated with 0.1 mL of each of the four inoculum dilutions as indicated in Table 1 and section 10.3.9.

- 10.3.6 Repeat the assay for the second test organism, if required.

- 10.3.7 Inoculated Controls.

The **Neutralizer-Primary Inoculated Control** contains four tubes of fresh, unexposed (to disinfectant) neutralizer-primary media.

The **Secondary Subculture Inoculated Control** contains four tubes of secondary subculture media.

It is highly desirable that the preparation (media preparation number) of each medium be the same as used in the treatments. Each tube will be inoculated with one of four inoculum dilutions as indicated in Table 1 and section 10.3.9.

- 10.3.8 Uninoculated Controls.

**Neutralizer-Primary and Secondary Subculture Uninoculated**

**Controls.** One tube each of uninoculated neutralizer and secondary subculture media will be included in the test and incubated with the other tubes. Sterility of carriers must be confirmed either in advance or concurrently with testing by adding the carrier to a tube of 10 mL fluid thioglycollate medium and incubating at 36±1°C for 5-10 days.

10.3.9 Inoculating the Tubes. Inoculate treatment and control tubes with 0.1 mL of the diluted inoculum as indicated in Table 1. **Inoculate the media following the transfer of all carriers into the secondary subculture media.**

Table 1. Inoculation of Treatment and Control Groups with Dilutions of the Test Organism\*

Treatments/Controls	Dilutions Added**				Treatment Description
	1.0×10 <sup>-4</sup>	1.0×10 <sup>-5</sup>	1.0×10 <sup>-6</sup>	1.0×10 <sup>-7</sup>	
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL	These tubes represent the mock efficacy test with sterile carriers
Secondary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL	
Neutralizer-Primary Inoculated Control	0.1 mL	0.1 mL	0.1 mL	0.1 mL	These tubes represent media performance controls
Secondary Subculture Inoculated Control	0.1 mL	0.1 mL	0.1 mL	0.1 mL	
Neutralizer-Primary Uninoculated Control	N/A	N/A	N/A	N/A	These tubes represent media sterility controls
Subculture Media Uninoculated Control	N/A	N/A	N/A	N/A	

\*1×10<sup>-4</sup> through 1×10<sup>-7</sup>; based on an approx. starting suspension of 10<sup>7</sup> to 10<sup>8</sup> CFU/mL

\*\*Accounting for the volume added to each tube (0.1 mL), the final dilution in each tube is 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup>, and 10<sup>-8</sup>, respectively.

10.3.10 Incubate tubes for 48±2 hours at 36±1°C.

10.4 Results are recorded as + (growth) or 0 (no growth). Record results on the appropriate Neutralization Confirmation Assay Results Form (see 16.0). Confirmation testing of the growth will be performed as listed in section 10.5.

10.5 Identification and Confirmation Testing.

10.5.1 Presumptively confirm a minimum of one positive tube per treatment and control, if available, using Gram staining. If further confirmation is deemed necessary, selective media and VITEK™ analysis may be used.

For each treatment and control group, select the tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered) and conduct confirmation testing on a sample of

the growth.

- 10.5.2 Record confirmation results on the Neutralization Confirmation Assay: Microbe Confirmation Sheet (see 16.0).

10.6 Interpretation of Results.

- 10.6.1 Plate count data. One of the four dilutions plated should provide counts within the approximate target range, 5-100 CFU/mL.

Note: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum is added to the subculture tubes.

- 10.6.2 Controls. Growth in the **Secondary Subculture Inoculated Control** verifies the presence of the test microbe, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.* Growth in the **Neutralizer-Primary Inoculated Control** should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media.

There may be cases when the neutralizer (primary tubes) is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

The **Neutralizer-Primary Uninoculated Control** and **Secondary Media Uninoculated Control** tubes are used to determine sterility, and must show no growth for the test to be valid.

- 10.6.3 Inoculated Treatments. The occurrence of growth in the **Neutralizer-Primary Subculture** and **Secondary Subculture Treatment** tubes are used to assess the effectiveness of the neutralizer. The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the*

*neutralizer to be deemed effective, growth must occur in the **Secondary Subculture Treatment** tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).*

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Plate counts are enumerated and CFU/mL is calculated based on the average of countable plates.

11.2 The evaluation of the data to determine whether a bacteriostatic effect is present is described in Section 10.6.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in secure file cabinets in D217. Only authorized personnel have access to the secure files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocol must be recorded on the form and an explanation for the deviation given.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 2006. 18<sup>th</sup> Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Methods 955.15, 964.02, and 955.14).

16.0 FORMS AND DATA SHEETS:

16.1 Neutralization Confirmation Assay: Time Recording Sheet for Carrier Inoculation Steps

16.2 Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers

- 16.3 Neutralization Confirmation Assay: Test Information Sheet
- 16.4 Neutralization Confirmation Assay: Results Form
- 16.5 Neutralization Confirmation Assay: Test Microbe Confirmation Sheet
- 16.6 Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form
- 16.7 Neutralization Confirmation Assay: Inoculum Enumeration Form

16.1

Neutralization Confirmation Assay: Time Recording Sheet for Carrier Inoculation Steps  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____	
Test Date	
Type of Test	
Product Reg. No.	
Product Name	
Sample No.	
Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Loading Time*+		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/

\* Recorded from laboratory clock/and timer.

+ Use to record time for application of organic material to sterile glass slides.

16.2

Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No.	
Organism	
Neutralizer	
Carrier Type	
Test Type	

Initials/Date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time for first carrier into the disinfectant		Carrier Drop End Time for last carrier into the neutralizer		First Carrier Transfer (into secondary media)
			Clock	Timer	Clock	Timer	Start Time <sup>1</sup>
	1-4	1-4					
Comments: Carriers dropped/sprayed by: _____. Carriers transferred by: _____.							

<sup>1</sup>=Carrier transfer into secondary subculture (time elapsed after last carrier dropped in primary); taken from clock

16.3

**Information Sheet for the Neutralization Confirmation Assay**  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
EPA Reg. No.		SOP	
Product Name		Test Date	
Sample No.		Neutralizer	
Lot No.		Comments:	
Expiration Date		Test Type:	

TEST PARAMETERS/Confirmed by: _____					
H <sub>2</sub> O Hardness (CaCO <sub>3</sub> ) ppm	Specified	Titrated (Buret)/Date/Init		HACH/Date/Init	
		/ /		/ /	
Use Dilution	Specified	As Prepared/Date/Initials			
		/ /			
Organic Soil	Specified	As Prepared/Date/Init.			
Neutralizer	Specified				
Temperature (°C)	Specified	Chiller Unit Display		Test Tube Waterbath	
		Before:	After:	Before:	After:
Contact Time	Specified	As Tested			
Other Parameters	Specified				
Carriers (not inoculated)	Type			Preparation #	

TEST MICROBE INFORMATION/Confirmed by: _____				
Test Microbe		48-54 Hour Culture		
Org. Control No.		Date/Time	Initiated	Harvested
Avg. CFU/Carrier	Not Applicable			

REAGENT/MEDIA INFORMATION/Confirmed by: _____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

16.4

**Neutralization Confirmation Assay: Results Form**  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments:	
Test Type			

TEST RESULTS*: Date Recorded/Initials: _____				
Treatments/Controls	Final Inoculum Dilutions			
	$1.0 \times 10^{-5}$	$1.0 \times 10^{-6}$	$1.0 \times 10^{-7}$	$1.0 \times 10^{-8}$
Neutralizer-Primary Subculture Treatment				
Secondary Subculture Treatment (with Carrier)				
Neutralizer-Primary Inoculated Control				
Secondary Subculture Inoculated Control				
Neutralizer-Primary Uninoculated Control Tube				
Subculture Media Uninoculated Control Tube				
*+=growth, 0=no growth				

SUMMARY OF RESULTS: Date/Initials: _____	
Bacteriostatic Effect Observed?	Yes _____ No _____
Comments:	

16.5

Neutralization Confirmation Assay: Test Microbe Confirmation Sheet  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.		Carrier Type	

Source: Tube/Plate ID	Date/ Initials	Stain Results <sup>1</sup>	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)

<sup>1</sup> Record Gram Stain results as GPC=gram positive cocci or GNR=gram negative rods

16.6  
 Neutralization Confirmation Assay Serial Dilution/Plating Tracking Form  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer(s)	
Sample No.		Organism Control #	

Confirmed by: _____	Dilution Tube						
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>
Starting volume of diluent	9 mL	9 mL	9 mL	9 mL	9 mL	9 mL	9 mL
Volume added to serial dilution tube (1 mL)	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Volume plated (0.1 mL)	N/A	N/A	N/A	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Final dilution factor (used for calculations) <sup>1</sup>	N/A	N/A	N/A	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>
Number of Plates per Dilution	N/A	N/A	N/A	2	2	2	2
Plating medium							
Comments:							

REAGENT/MEDIA INFORMATION/Confirmed by: _____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

16.7

Neutralization Confirmation Assay Inoculum Enumeration Form  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
EPA Reg. No.		Test Date	
Product Name		Organism	
Sample No.		Test Type	

RESULTS: Date/Initials: _____			
Plating Method			
	CFU per Dilution Plate		Average CFU per mL
Dilution	Plate 1	Plate 2	
$1.0 \times 10^{-5}$			
$1.0 \times 10^{-6}$			
$1.0 \times 10^{-7}$			
$1.0 \times 10^{-8}$			
TNTC = Too Numerous To Count			
Comments:			

REAGENT/MEDIA INFORMATION/Confirmed by: _____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.