MICROBIOTEST, INC
The Microbiology and Virology Laboratory

Volume ___

FINAL REPORT - COVER PAGE

AOAC USE DILUTION TEST

AXENOHL

Data Requirements
40 CFR 158.640, Guideline 91-2

Author
Donna B. Suchmann

Study Completion Date: 11/09/99

Performing Laboratory

MicroBioTest, Inc.
105B Carpenter Drive
Sterling, Virginia 20164

Laboratory Project Identification

438-101

CERTIFIED COPY
Signature 11/18/99 Date

Submitted to: ETI H2O, INC.
Route 14, Box 1517
Lake City, FL 32054

STATE OF NO DATA CONFIDENTIALITY

Title: AOAC USE DILUTION TEST

Performed by MicroBioTest, Inc.
105B Carpenter Drive
Sterling, Virginia 20164

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B) or (C).

Company Agent Edwin A Woodson 11/18/99
Date

COMPLIANCE STATEMENT

This study meets the requirements for 40 CFR Part 160 with the following exception(s):

- information on the synthesis, purity analysis, composition and other characteristics of the test product remain with the sponsor.

The following personnel participated in this study:

Lorraine P. Donahue, Erica L. Eaton, Charlie Swearingen, Alice A. Suchman, Angela L. Hollingsworth

Study Director: MICROBIOTEST, INC.

Deborah C. Draghi 11/9/99

Deborah C. Draghi — Date

Submitter:

_____ _____

Name — Title

_____ _____

Signature — Date

Sponsor: ETI H2O, INC.

/ Edwin A. Woolson — Sponsor Monitor

Name — Title

Edwin A Woolson — 11/18/99

Signature — Date

QUALITY ASSURANCE UNIT STATEMENT

TITLE OF STUDY: AOAC Use Dilution Test

The Quality Assurance Unit of MicroBioTest, Inc. has inspected the Project Number 438-101 in compliance with current Good Laboratory Practice regulations, (40 CFR 160) to assure the integrity of the data.

The dates that inspections were made and the dates that findings were reported to management and to the study director are listed below.

PHASE INSPECTED	DATE OF INSPECTION	DATE REPORTED TO STUDY DIRECTOR	DATE REPORTED TO MANAGEMENT
Protocol	10/06/99	10/07/99	10/11/99
In-Process	10/06/99	10/07/99	10/11/99
Final Report	11/05/99	11/05/99	11/07/99

Kathy A. Kenepp 11/08/99

Kathy A. Kenepp
Manager, Quality Assurance Unit

Date

TABLE OF CONTENTS

TEST SUMMARY

TITLE: AOAC USE DILUTION TEST

TEST AGENT: See Project Sheet(s) (Appendix I)

STUDY DESIGN: See signed protocol (Appendix II)
See Project Sheet(s) (Appendix I)

TEST MATERIALS SUPPLIED BY THE SPONSOR OF THE STUDY:

1. AXENOHL, Lot 8995, received at MicroBioTest, Inc. 10/06/99, and assigned DS No. 4467
2. AXENOHL, Lot AG-006, received at MicroBioTest, Inc. 09/16/99 and 10/07/99, and assigned DS No. 4468
3. AXENOHL, Lot 8991, received at MicroBioTest, Inc. 10/06/99, and assigned DS No. 4469
4. 5% Citric Acid in Water, no lot number given, received at MicroBioTest 09/16/99 and 10/06/99, and assigned DS No. 4470

NEUTRALIZER EFFECTIVENESS CONTROL:

Three sterile carriers (for each challenge microorganism) were exposed to Lot No. 8995 in the same manner as the test for the 10-minute contact time. The carriers were inoculated into individual tubes of 10 mL Letheen Broth containing 0.3% $Na_2S_2O_3$ and 1.5% Polysorbate 80. To each tube, fewer than 10 colony forming units (CFU) per mL of the challenge microorganism were added and the count of the bacteria inoculated into these tubes was confirmed.

SPONSOR: ETI H2O, INC.
Route 14, Box 1517
Lake City, FL 32054

TEST CONDITIONS

Challenge Microorganisms:

Salmonella choleraesuis, ATCC 10708
Staphylococcus aureus, ATCC 6538
Pseudomonas aeruginosa, ATCC 15442

Active Ingredient in Test Products:

Silver citrate

Neutralizer Used:

Letheen Broth containing 0.3% $Na_2S_2O_3$ and 1.5% Polysorbate 80

Contact time: 10 minutes

Contact temperature:

20±1C

Use Dilution:

One Gram of active test agent was added to 199 Gram of 5% (w/w) citric acid solution to achieve a 200-Gram final weight use dilution of test agent.

Testing supplies:

Nutrient Agar
Nutrient Broth
Phenol stock solution, 5.0%
Letheen Broth (LB)
Letheen Broth containing 0.3% 3% $Na_2S_2O_3$ and 1.5% Polysorbate 80 (LB+)
Phosphate Buffered Saline (PBS)
Sterile Deionized Water
Asparagine solution, 0.1%
NaOH, 1N
Tryptic Soy Agar
Gram stain reagents

STUDY DATES AND FACILITIES

The laboratory phase of this test was performed at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164, from 10/06/99 to 10/17/99. The study director signed the protocol 09/22/99. The study completion date is the date the study director signed the final report.

All changes or revisions of the protocol were documented, signed by the study director, dated and maintained with the protocol.

RECORDS TO BE MAINTAINED

All testing data, protocol, protocol modifications, test material records, the final report, and correspondence between MicroBioTest, Inc. and the sponsor will be stored in the archives at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

RESULTS

Results and data are presented in Tables 1 - 5. The challenge microorganisms were confirmed by Gram stain to be Gram positive cocci for *S. aureus* and Gram negative bacilli for *S. choleraesuis* and *P. aeruginosa*.

CONCLUSIONS

When tested as described, AXENOHL passes the AOAC Use Dilution Test when *S. aureus*, ATCC 6538; *S. choleraesuis*, ATCC 10708 and *P. aeruginosa*, ATCC 15442 are exposed to the test material for ten minutes at 20±1C. All of the sterility and viability control cultures met the resistance required to meet the criteria for a valid test. These conclusions are based on the observed data.

APPENDIX I
PROJECT SHEET(S)

RESULTS

Table 1

Test Results

Results Expressed as Number of Positive Carriers / Total Number of Carriers

Lot No.	Challenge Microorganism	No. + / Total No.
8995	*S. choleraesuis*	0/60
	S. aureus	0/60
	P. aeruginosa	0/60
AG-006	*S. choleraesuis*	1/60
	S. aureus	0/60
	P. aeruginosa	0/60
8991	*S. choleraesuis*	0/60
	S. aureus	0/60
	P. aeruginosa	0/60

Table 2

Inoculum Confirmation Counts

Results Expressed as Average Colony Forming Units (CFU) Recovered per mL

Challenge Microorganism	Date tested: 10/06/99	Date tested: 10/14/99
S. choleraesuis	1.1×10^8	3.9×10^8
S. aureus	4.2×10^8	3.8×10^8
P. aeruginosa	1.1×10^8	NA

Table 3

Phenol Resistance

Results Expressed as Growth (+) / No Growth (-)

Challenge Microorganism	Phenol Dilution	Contact Time (min)		
		5	10	15
S. choleraesuis	1:90	+	–	–
	1:100	+	+	+
S. aureus	1:60	+	–	–
	1:70	+	+	+
P. aeruginosa	1:80	+	–	–
	1:90	+	+	+

RESULTS (continued)

Table 4

Neutralizer Effectiveness Control

Results Expressed as Growth (+) / No Growth (-)

Challenge Microorganism	Average CFU/mL Added per Tube	Replicate	+ / -
S. choleraesuis	34	1	+
		2	+
		3	+
S. aureus	26	1	+
		2	+
		3	+
P. aeruginosa	78	1	+
		2	+
		3	+

Date Issued: 10/06/99 Project Sheet No. 1 Page No. 1 Laboratory Project Identification No. 438-1

STUDY TITLE: AOAC Use Dilution Test	**STUDY DIRECTOR:** Deborah C. Draghi Deborah C. Draghi 10/6/99 Signature		Date
TEST MATERIAL(S):	**LOT NO.**	**DATE RECEIVED:**	**DS N**
AXENOHL	8895 DD 11/7/99	09/16/99 & 10/06/99	4467
AXENOHL	AG-006	09/16/99	4468
AXENOHL	8891 DD 11/7/99	09/16/99 & 10/06/99	4469
5% Citric Acid In Water	NA	09/16/99 & 10/06/99	4470
PERFORMING DEPARTMENT(S): Applied Microbiology Laboratory	**STORAGE CONDITIONS:** Location: B2 ■ Dark ■ Ambient Room Temperature ☐ Desiccator ☐ Freezer ☐ Refrigerator ☐ Othe		

PROTECTIVE PRECAUTION REQUIRED: MSDS ■Yes / ☐ No

PHYSICAL DESCRIPTION: ☐ Solid ■ Liquid ☐ Aerosol ☐ Other:

PURPOSE: See attached protocol. **AUTHORIZATION:** See client signature.

PROPOSED EXPERIMENTAL START DATE: 10/06/99 **TERMINATION DATE:** 10/10/99

CONDUCT OF STUDY: ☐ FDA ■ EPA ☐ R&D ■ GLP ☐ GCP ☐ Other:

SPONSOR: ETI H20, Inc. Route 14 Box 1517 Lake City, Fl 32054	**CONTACT PERSON:** Telephone No. FAX No.	Ed Woolson (217)963-2143 ext. 12 (217)963-2283

TEST CONDITIONS:

Challenge organism(s): Salmonella choleraesuis, ATCC 10708
Staphylococcus aureus, ATCC 6538
Pseudomonas aeruginosa, ATCC 15442

Active ingredient(s): Silver Citrate

Neutralizer(s): Letheen Broth + 0.3% $Na_2S_2O_3$ + 1.5% Polysorbate 80

Contact Time(s): 10 minutes Contact Temperature(s): 20 ± 1C

Diluent(s): 5% citric acid in water (DS#4470)

Dilution(s): 1/200 with 5% (w/w)citric acid in water

Serum: ☐ Yes / ■ No

Incubation Time(s): 48 ± 2 hr Incubation Temperature: 37± 2C

Comments: 1 g of active ingredient will be added to 199 g of the 5% citric acid solution in order to achieve a 200 g final weight

Date Issued: 10/06/99 Project Sheet No. 2 Page No. 1 Laboratory Project Identification No. 438-10			
STUDY TITLE: AOAC Use Dilution Test	**STUDY DIRECTOR:** Deborah C. Draghi Deborah C. Draghi 10/6/99 Signature		Date
TEST MATERIAL(S): AXENOHL AXENOHL AXENOHL 5% Citric Acid In Water	**LOT NO.** 8895 AG-006 8891 NA	**DATE RECEIVED:** 09/16/99 & 10/06/99 09/16/99 09/16/99 & 10/06/99 09/16/99 & 10/06/99	**DS N(** 4467 4468 4469 4470
PERFORMING DEPARTMENT(S): Applied Microbiology Laboratory	**STORAGE CONDITIONS:** Location: B2 ■ Dark ■ Ambient Room Temperature ☐ Desiccator ☐ Freezer ☐ Refrigerator ☐ Othe		
PROTECTIVE PRECAUTION REQUIRED: MSDS ■Yes / ☐ No			
PHYSICAL DESCRIPTION: ☐ Solid ■ Liquid ☐ Aerosol ☐ Other:			
PURPOSE: See attached protocol. **AUTHORIZATION:** See client signature.			
PROPOSED EXPERIMENTAL START DATE: 10/06/99 **TERMINATION DATE:** 10/10/99			
CONDUCT OF STUDY: ☐ FDA ■ EPA ☐ R&D ■ GLP ☐ GCP ☐ Other:			
SPONSOR: ETI H20, Inc. Route 14 Box 1517 Lake City, Fl 32054	**CONTACT PERSON:** Telephone No. FAX No.	Ed Woolson (217)963-2143 ext. 12 (217)963-2283	

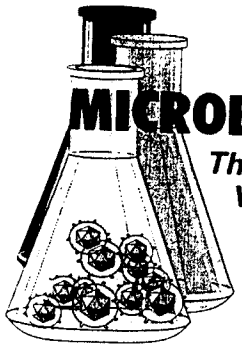
EXPLANATION:

This project sheet was issued to document the following:

Protocol Amendment:

1. In accordance with MBT SOP 1009.4(0) and in order to demonstrate the CFU/mL of inoculur inoculum confirmation counts will be performed by serially diluting each challenge organism Phosphate Buffered Saline blanks (PBS). Selected dilutions will be plated in duplicate in Nutrie Agar (NA) pour plates. The plates will be incubated along with the test. After incubation, the plate wil' be enumerated and the CFU/mL calculated.

APPENDIX II
SIGNED PROTOCOL



MICROBIOTEST, INC
The Microbiology and Virology Laboratory

MicroBioTest Protocol

AOAC USE DILUTION TEST

Prepared for:

ETI H_2O, INC.
Route 14 Box 1517
Lake City, FL 32054

September 2, 1999

OBJECTIVE:

This test is designed to substantiate disinfectant effectiveness claims for a product registered with the Environmental Protection Agency. It measures the potential of the test material to disinfect hard surfaces contaminated with various bacteria. The test follows *Official Methods of Analysis*, Fifteenth edition, 1990, AOAC; is required by EPA DIS/TSS 1 & 2; and will be conducted under EPA GLP regulations (40 CFR 160).

TESTING CONDITIONS:

A total of sixty replicates per organism per lot of test material are evaluated using three lots of the test material, one of which is at least sixty days old. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella choleraesuis* cultures dried on stainless steel penicylinders, will be exposed to the test material at the temperature and for the contact time stipulated by the sponsor. The carriers will be removed from the test solution, neutralized and cultured.

MATERIALS

A. Test agents supplied by the sponsor: see last page.

The test substance is tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or specialized storage conditions must by specified by the sponsor prior to the initiation of testing.

The sponsor assures MicroBioTest, Inc. testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

All unused test materials will be retained by MicroBioTest for a period of three months after completion of the test, then discarded in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest, Inc., including, but not limited to:

1. Challenge organisms, required by EPA DIS/TSS 1:

   a. *Salmonella choleraesuis*, ATCC 10708
   b. *Staphylococcus aureus*, ATCC 6538
   c. *Pseudomonas aeruginosa*, ATCC 15442

2. Media and reagents:

   a. Nutrient Agar Plates (NA)
   b. Nutrient Broth (NB)
   c. Phenol Stock Solution, 5% (PSS)
   d. Asparagine Solution, 0.1%
   e. Sodium Hydroxide Solution, 1N (NaOH)
   f. Recovery Broth (LB, NB or Fluid Thioglycollate Medium)
   f. Letheen Broth (LB)
   g. Recovery Broth with Neutralizer(s)(if required)
   h. PBS with 1% Polysorbate 80 (PBS+) (If carrier counts required)
   i. Heat Inactivated Horse Serum (if required)

3. Laboratory equipment and supplies including polished stainless steel penicylinders

EXPERIMENTAL DESIGN:

A. Inocula preparation:

Bacteria from stock cultures will be transferred into NB using a 4-mm loop and incubated at 37±2C. Daily transfers will be made for at least three consecutive days (but no more than 30). Tubes of 10-mL NB will be inoculated with one loopful of inoculum per tube and incubated at 37±2C. After 48-54 hours, cultures will be used for contaminating the carriers. The pellicle formed in the culture of *Pseudomonas aeruginosa* will be removed prior to carrier contamination. For each organism, the NB cultures will be pooled into a sterile flask. If requested by the sponsor, serum will be added to the cultures to achieve an organic load of 5%.

A. Inocula preparation (cont.):

Each inoculum will be agitated on a Vortex-type mixer for 3-4 seconds. The inoculum will be allowed to sit for ten minutes then decanted into a sterile flask, leaving all residue in the original flask. Twenty-mL aliquots will be transferred into 25x150 mm sterile tubes, with mixing of the inoculum between transfers.

B. Carrier preparation:

The carriers will be soaked overnight in 1N NaOH, rinsed with tap water until a neutral pH is reached, then rinsed twice with deionized water (DI). Cleaned carriers will be placed in multiples of 10 into 32x200 mm tubes, covered with 0.1% asparagine solution, steam-sterilized for 20 min at 121C, cooled and stored at room temperature until use.

The carriers will be placed into the broth and remain in contact with the inocula (20 carriers per tube of 20-mL inocula) for 15 min at ambient temperature; then they will be removed from the broth and placed into sterile, Petri dishes matted with filter paper, and dried at 37±2C for 40 min.

C. Test material preparation:

The disinfectant will be prepared according to the sponsor's specifications and dispensed in 10-mL aliquots into sterile 25x150 mm test tubes. The tubes will be placed in a water bath and allowed to come to test temperature for at least ten minutes before testing.

D. Test:

Tubes containing the test material will be maintained at 20±1C throughout the test. One contaminated carrier will be added to each tube; the tube swirled to mix; and the carrier allowed to remain in contact with the test agent for a time specified by the sponsor of the study. After the contact time, the carriers will be removed, transferred to recovery broth with neutralizer(s) and the tubes will be thoroughly shaken. All tubes will be incubated at 37±2C for 48±2 hr and the results recorded as + (visible growth) or - (no visible growth).

E. Controls:

1. Neutralizer effectiveness:

Three tubes containing ten mL of one of the test material lots will be allowed to equilibrate to 20±1C for at least 10 min. A single sterile carrier will be added to each tube and held for the same time as the test carriers. After the contact time, each carrier will be added to a tube containing recovery broth with neutralizers and fewer than 100 CFU of the challenge organism will be added to each tube. The CFU added to each tube will be confirmed.

This procedure will be repeated for each challenge organism. All tubes and plates will be incubated with the test.

2. Carrier counts:

If serum is added to the inocula, the average CFU per carrier will be determined for each organism on three carriers. Dried carriers will be placed individually into tubes containing 10 mL PBS + 1% polysorbate 80. The tubes will be subjected to ultrasound for 5 min in a cleaning (not cavitating) sonicator. Serial ten-fold dilutions of each suspension will be performed in PBS blanks. Duplicate one-mL aliquots from selected dilutions will be plated in Nutrient Agar pour plates. All plates will be incubated with the test and the average CFU/carrier determined.

3. Phenol resistance:

Five percent phenol stock solution will be diluted with sterile DI to give final phenol dilutions of 1:60, 1:70, 1:80, 1:90 and 1:100. All tubes will be maintained at 20±2C. A 0.5-mL aliquot of each prepared inocula will be added to five mL of the appropriate dilution tube and mixed thoroughly. After 5, 10, and 15 min, a standardized loopful (4 mm) of mixture will be removed from each dilution tube and placed into a tube of LB. Each LB tube will be thoroughly agitated and incubated at 37±2C for 48±2 hours and the results recorded as + (growth) or – (no growth) of the challenge organism.

E. Controls (cont.):

4. Viability controls:

Two inoculated carriers for each challenge organism will be inoculated into tubes of recovery broth with neutralizers and incubated with the test to serve as comparison for the test cultures. Fewer than 100 CFU of each challenge organism will be added to two tubes of LB and incubated with the test to serve as comparison for the phenol control cultures.

5. Bacteriostasis control:

If, after two days incubation, no growth is observed in any tube for one challenge organism, all tubes will be streaked onto TSA and incubated for 24±2 hr at 37±2C. No growth on these plates will negate bacteriostasis as the cause for lack of growth in the test tubes.

6. Sterility controls:

One tube of recovery broth with neutralizers containing a single sterile carrier and one tube of LB will be incubated with the test.

7. Confirmation of challenge microorganisms:

Gram stains will be performed on at least 20% of tubes showing growth to verify growth of the challenge microorganisms.

PRODUCT EVALUATION CRITERIA:

According to EPA, the compound passes the test if visible growth is observed in one out of sixty of the subculture broths for any organism for any lot of test material and the controls meet the stipulated resistance criteria.

DATA PRESENTATION:

The final report will include the following information in tabular form:

- The number of positive carriers per organism per lot.
- The results for the phenol resistance and other controls.
- The average colony-forming units per carrier, if performed.

RECORDS:

All raw data, protocol, protocol modifications, test material records, final report, and correspondence relevant to this study, between MicroBioTest and the sponsor will be stored in the archives at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

All changes or revisions of the approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of the change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; and the type of neutralizer(s) to be employed in the test will be addressed in a project sheet issued separately for each study.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The broth cultures must meet the phenol resistance requirements as outlined by AOAC. An exception may be possible if the microorganisms show greater phenol resistance than required.
- In the presence of an organic load, the carrier counts should be at least 10^4 CFU/carrier.

REPORT FORMAT:

MicroBioTest employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test material identification
- Type of test and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements

STUDY DATES:

The anticipated date of study initiation (date when the study director signs the protocol) is upon receipt of test material and letter of authorization with a purchase order number and a signed protocol. The date for submission of the final report to the sponsor will be within one month of laboratory phase completion. The date the study director signs the final report is the study completion date.

MISCELLANEOUS INFORMATION:

The following information is to be completed by sponsor before initiation of study:

A. Name and address: ETI H_2O, INC.
Route 14 Box 1517
Lake City, FL 32054

B. Test agent: AXENOHL

1st Lot No: 8995 2nd Lot No: 8991

3rd Lot No (≥ 60 days old): AG-006

C. Precautions/storage conditions: See MSDS

REPORT HANDLING:

This information is to be: ☐ submitted to California DHS ☒ submitted to the EPA
☐ submitted to the FDA ☐ used for internal purposes only

PROTOCOL APPROVAL:

Sponsor: E A Worthington Date: 9/14/99

Study Director: Michael C. Vlagdi Date: 9/22/99