



HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

**HILL TOP RESEARCH, INC.**

**APPENDIX IV**

**COPY OF PROTOCOL**

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation



HILL TOP RESEARCH, INC.

*Hill Top Research Confidential*  
PROTOCOL FOR

ASSESSMENT OF RAPID GERMICIDAL (TIME KILL)  
ACTIVITY FOR HAND PRODUCT

For: Bayer Chemicals Corporation

HTR Ref.: 03-122096-106

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

**TABLE OF CONTENTS**

1.0	INTRODUCTION .....	1
2.0	PURPOSE.....	1
3.0	STUDY SPONSOR AND SPONSOR REPRESENTATIVE.....	1
4.0	TEST FACILITY AND INVESTIGATIVE PERSONNEL.....	1
5.0	RESEARCH STANDARDS .....	1
6.0	EXPERIMENTAL DESIGN .....	2
7.0	PROPOSED EXPERIMENTAL STARTING AND COMPLETION DATES .....	2
8.0	TEST ARTICLE IDENTIFICATION .....	2
9.0	TEST ARTICLE CHARACTERIZATION.....	2
10.0	TEST SYSTEM JUSTIFICATION .....	2
11.0	TEST SYSTEM IDENTIFICATION .....	2
12.0	TEST PROCEDURE.....	3
13.0	STATISTICAL METHOD .....	5
14.0	REPORT .....	6
15.0	DATA RETENTION.....	6
16.0	NOTICE.....	6
17.0	PROTOCOL APPROVAL FORM.....	7
	Appendix I/Materials and Reagents.....	8
	Appendix II/Test Organisms.....	10

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

1.0 **INTRODUCTION**

This procedure is used to obtain information that allows an assessment of how rapidly an antibacterial agent produces its effects.

2.0 **PURPOSE**

To measure the ability of an antibacterial agent to rapidly reduce a known population of bacteria.

3.0 **STUDY SPONSOR AND SPONSOR REPRESENTATIVE**

Bayer Chemicals Corporation  
100 Bayer Rd.  
Building 14  
Pittsburgh, PA 15205-9741

Telephone No.: (412) 777-3934  
Fax No.: (412) 778-4473

REPRESENTATIVE: Kevin Ajoku

4.0 **TEST FACILITY AND INVESTIGATIVE PERSONNEL**

Hill Top Research, Inc.  
Main and Mill Streets  
Miami, Ohio 45147

Telephone No: (513) 831-3114  
Fax No.: (513) 831-1217

Study Director: Kathleen A. Baxter, B.S.  
Study Manager: Jane M. Young, B.S.  
Study Coordinator: Patricia M. Schario, B.S.

5.0 **RESEARCH STANDARDS**

This study will be run according to Good Laboratory Practice Standards (21 CFR Part 58). The In-life Phase and the Report will be audited by the Quality Assurance Unit of Hill Top Research, Inc.

July 21, 2003  
Page 1 of 12

August 13, 2003  
Page 47 of 60

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

6.0 **EXPERIMENTAL DESIGN**

A dilution/aliquot of the test material is brought into contact with a known population of test bacteria for a specified period of time. The antibacterial active is neutralized at the end of specified exposure periods. A sample is then plated to enumerate the surviving bacteria. The percent reduction and the log<sub>10</sub> reduction from the original population are calculated.

7.0 **PROPOSED EXPERIMENTAL STARTING AND COMPLETION DATES**

Proposed Experimental Starting Date: July 24, 2003  
Proposed Experimental Termination Date: August 4, 2003  
Proposed Completion Date: August 18, 2003

8.0 **TEST ARTICLE IDENTIFICATION**

Two test articles, received prior to testing and identified by the sponsor as 3554-194 and 3354-196, will be used for testing. The test articles will be assigned Hill Top Research codes for generation of the test data.

9.0 **TEST ARTICLE CHARACTERIZATION**

The sponsor will assume responsibility for physical and chemical characterization studies of test and control articles (21 CFR Part 58.105).

10.0 **TEST SYSTEM JUSTIFICATION**

The test system has been used historically for this type of study.

11.0 **TEST SYSTEM IDENTIFICATION**

The test organisms to be used in this study will be 24±2 hour agar plate or slant cultures that have been passed through three but no more than five consecutive transfers for the bacteria. The inocula are prepared by washing the plates or slants with phosphate buffer dilution water and spectrophotometrically adjusting to a concentration of approximately 1x10<sup>8</sup> CFU/mL. The organisms to be used in testing are outlined in

July 21, 2003  
Page 2 of 12

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

11.0 **TEST SYSTEM IDENTIFICATION CON'T.**

Appendix II.<sup>1</sup> These organisms will be assigned unique codes to provide for the correct generation of data and will be propagated in medium appropriate for each organism.

12.0 **TEST PROCEDURE**

- 12.1 The test articles will be tested at a 75% concentration (75 mL of product plus 25 mL of sterile purified water) with exposure times of 30 seconds and 60 seconds at 20-25°C. Sterile purified water, with the same exposure time as the test articles, will be substituted for the test article to serve as the numbers control. All tests involving the test articles will be conducted in triplicate.
- 12.2 A seventy-five (75) mL aliquot of the test article will be placed in a sterile glass jar, flask, or beaker containing a magnetic spin bar at 20-25°C, on a magnetic stirring plate, stirring at a speed that ensures adequate mixing. An additional 25 mL of sterile purified water will be added to the test article, just prior to testing, and the resulting solution will be mixed thoroughly prior to adding the inoculum. [The setting on the stir plate will be recorded in the study records.] The diluted test article will then be inoculated with 1.0 mL of the test organism in phosphate buffer dilution water, adjusted to approximately  $10^8$  CFU/mL.<sup>2</sup> The inoculum and the test article are mixed for the entire exposure time of 30 and 60 seconds. At the appropriate exposure times, a 1 mL aliquot of the test/organism mixture will be transferred to 99 mL of D/E Broth to provide a  $10^{-2}$  dilution.
- 12.3 A subsequent one (1) mL aliquot will then be removed from the D/E Broth ( $10^{-2}$  dilution) and 10-fold serial dilutions (containing 9 mL of D/E Broth for the test article and for the numbers control) will be prepared to  $10^{-6}$ <sup>3</sup> for the test article and to  $10^{-7}$ <sup>4</sup> for the numbers control.
- 12.4 An inoculum count will be determined for the test organism by 10-fold serial dilutions to  $10^{-8}$ <sup>3</sup> using tubes containing 9 mL of AOAC Phosphate Buffer Dilution Water.
- 12.5 The dilutions will be plated in duplicate by the Spread or Pour Plate Technique using the

<sup>1</sup> Growth requirements of some organisms may necessitate the use of incubation conditions and culture media other than those specified in the protocol. When such changes are necessary they will be documented in the study records.

<sup>2</sup> The  $10^8$  inoculum may not be attainable for some of the test organisms. In these cases, the highest attainable level will be noted in the study records.

<sup>3</sup> The number of dilutions for some organisms may differ than those specified in the protocol. Appropriate dilutions will be plated in order to capture a countable range.

<sup>4</sup> Ibid.

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; Hill Top Research Confidential

12.0 **TEST PROCEDURE CON'T.**

appropriate agar according to Appendix II. The plates will be incubated in an inverted position at  $35 \pm 2^\circ\text{C}$  for  $48 \pm 2$  hours (bacteria) or as required for propagation [see footnote 1, page 3]. Following incubation, colony forming units (CFU) per mL will be counted and numbers of surviving organisms will be calculated.

- 12.6 Both the percent reduction in numbers and the  $\log_{10}$  reductions will be reported. A percent reduction, as compared to the numbers control, will be determined for each test organism against the average result of the triplicate replicates for each exposure period. It will be determined as illustrated below.

$$\% \text{ Reduction} = \frac{[\text{NC}(\text{CFU}/\text{mL}) - \text{TA}(\text{CFU}/\text{mL})]}{\text{NC}(\text{CFU}/\text{mL})} \times 100$$

NC = Average Numbers Control population  
TA = Average Test Article population

- 12.7 The neutralizer used for this study will be D/E Neutralizing Broth as listed in the Protocol in Appendix I. Neutralizer effectiveness for the test article will be determined concurrently with the time kill portion of the study. If the neutralizer cited in the protocol is found to be ineffective for the test article, other neutralizer systems will be tested and the testing will be repeated at additional cost to the sponsor.
- 12.7.1 Neutralizer/Toxicity Blanks used will be as follows in conjunction with the test article and controls:
- 1) One (1) mL of the test article will be used.
  - 2) A 1.0 mL portion of the test organism inoculum, further diluted in phosphate buffer dilution water to approximately  $10^4$  CFU/mL, will be used to deliver  $10^2$  CFU/mL in the final dilution.
  - 3) Ninety-nine (99) mL D/E Neutralizing Broth will be used.
  - 4) Ninety-nine (99) mL AOAC Phosphate Buffer Dilution Water will be used.

July 21, 2003  
Page 4 of 12

August 13, 2003  
Page 50 of 60

HTR Ref: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

## 12.0 TEST PROCEDURE CON'T.

12.7.2 The test organisms used for this neutralizer test will be *Staphylococcus aureus*, ATCC 6538, *Corynebacterium minutissimum*, ATCC 23347, *Streptococcus pneumoniae*, ATCC 6303, and *Enterococcus faecalis*, ATCC 29212 propagated as described in the Protocol.

### 12.7.3 Neutralizer Toxicity

Each bottle containing 99 mL of the blanks (12.7.1, items 3,4) and 1 mL of sterile purified water will be equilibrated to ambient room temperature and 1.0 mL of the adjusted test organism suspension will be added to each blank and thoroughly mixed. Immediately following mixing, three, one (1) mL aliquots and three, 0.1 mL aliquots will be removed from each bottle.

The aliquots will be immediately plated by the pour or spread plate technique using Tryptic or Trypticase Soy Agar or other appropriate agar without neutralizer. The plates will be incubated in an inverted position at  $35 \pm 2^\circ\text{C}$  for  $48 \pm 2$  hours. Following incubation, colony forming units (CFU) per milliliter will be calculated. This procedure will be repeated after 20 minutes from the same tubes.

### 12.7.4 Neutralizer Effectiveness

Each bottle containing the 99 mL blanks (12.7.1, items 3 & 4) will be equilibrated to ambient room temperature and then 1 mL of the test article will be added to each blank and thoroughly mixed. Immediately following mixing, 1 mL of the adjusted test organism suspension will be added to each blank and mixed again. After this mixing, three, one (1) mL aliquots and three, 0.1 mL aliquots will be removed from each bottle. The aliquots will be immediately plated by the pour or spread plate technique using Tryptic or Trypticase Soy Agar or other appropriate agar without neutralizer. The plates will be incubated in an inverted position at  $35 \pm 2^\circ\text{C}$  for  $48 \pm 2$  hours. Following incubation, colony forming units (CFU) per milliliter will be calculated. This procedure will be repeated after 20 minutes from the same tubes.

12.7.5 Neutralizer toxicity is evident if more than a 50% difference is observed in recovery of numbers of test organism in the neutralizer used versus recovery from the AOAC Phosphate Buffer Dilution Water. Similarly, neutralizer effectiveness is evident if less than a 50% difference is observed in the numbers of test organism recovered when comparing counts in the neutralizer with and without the test article added.

## 13.0 STATISTICAL METHOD

No statistical analysis is required to interpret the results of this study.

July 21, 2003  
Page 5 of 12

August 13, 2003  
Page 51 of 60

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

14.0 **REPORT**

The report will include (but not be limited to) identification of the test organism, test procedure, protocol modification (if any), identification of the test material, solvent (if any), test concentration, subculture media, results, and summary.

15.0 **DATA RETENTION**

The final report will be sent to the sponsor following completion of the study. All records that would be required to reconstruct the study and demonstrate adherence to the Protocol will be maintained. The raw data and the original of the final report will be on file at the testing facility for a period of not less than two years. Permanent records will be in the form of microfilm.

Upon completion of testing, the test articles will be retained for a period of thirty (30) days, and then destroyed. The test articles will be returned to the sponsor only if the sponsor so requests and agrees to pay the cost of shipping.

16.0 **NOTICE**

If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study.

Similarly, the sponsor will be notified as soon as is practical whenever an event occurs that is unexpected and may have an effect on the validity of the study.

July 21, 2003  
Page 6 of 12

August 13, 2003  
Page 52 of 60

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; Hill Top Research Confidential

17.0

**PROTOCOL APPROVAL FORM  
MICROBIOLOGICAL SERVICES DIVISION  
HILL TOP RESEARCH, INC.**

PROTOCOL TITLE  
Assessment of Rapid Germicidal  
(Time Kill) Activity for Handwashes

REFERENCE CODE  
DISF\PROVTK\BAYC

**PROTOCOL APPROVED FOR: HILL TOP RESEARCH, INC.**

BY: Kathleen A. Baxter 7/22/03  
Kathleen A. Baxter, B.S. Date  
Study Director  
Microbiological Services Division

Protocol Approved By (Sponsor):

Kendra L. Lark 07/22/03  
Signed Date

\_\_\_\_\_  
Signed Date

\_\_\_\_\_  
Bayer Chemicals Corporation

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Address

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

HTR Ref: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

**Appendix I**  
**Materials and Reagents**

July 21, 2003  
Page 8 of 12

August 13, 2003  
Page 54 of 60

HTR Ref.: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

## Appendix I

### 1.0 MATERIALS AND REAGENTS

- 1.1 AOAC Nutrient Agar or AOAC Synthetic Agar (see footnote 1, page 3)
- 1.2 BBL Trypticase or Difco Tryptic Soy Agar (see footnote 1, page 3)
- 1.3 Brain Heart Infusion Agar (see footnote 1, page 3) with and without Sheep Blood
- 1.4 D/E Neutralizing Broth
- 1.5 AOAC Phosphate Buffer Dilution Water
- 1.6 Sterile purified water
- 1.7 Sterile magnetic spin bars
- 1.8 Sterile jars, flasks or beakers
- 1.9 Transfer loop, 4 mm id
- 1.10 Sterile Serological Pipets, 1.0, 5.0 and 10.0 mL
- 1.11 Incubators, 37±2°C, 35±2°C; aerobic and 5% CO<sub>2</sub>
- 1.12 Magnetic stir plate capable of adequately mixing test article

### 2.0 REFERENCES

- 2.1 Official Methods of Analysis of AOAC International, 17th Edition, 2000, Chapter 6, Section 6.3.03 A.
- 2.2 ASTM Standard: E 1054-91, "Standard Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectant, Sanitizer, Antiseptic, or Preserved Products.

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

HTR Ref: 03-122096-106  
Rapid Germicidal (Time Kill)  
Activity Protocol; *Hill Top Research Confidential*

**Appendix II**  
**Test Organisms**

July 21, 2003  
Page 10 of 12

August 13, 2003  
Page 56 of 60

HTR Ref.: 03-122096-106  
 Rapid Germicidal (Time Kill)  
 Activity Protocol; Hill Top Research Confidential

Appendix II  
 Test Organisms

Organism	ATCC#	Incubation for Growth		Growth Medium	Incubation for Recovery		Recovery Medium*
		Time(±2 hrs)	Temp (± 2°C)		Time (±2 hrs)	Temp (± 2°C)	
<i>Corynebacterium minutissimum</i>	23347	24	35	BHI	48	35	BHI
<i>Enterococcus faecalis</i>	29212	24	37	TSA w/ SB	48	35	TSA w/SB
<i>Enterococcus faecium</i> (VRE)	51559	24	37	TSA	48	35	TSA
<i>Staphylococcus aureus</i>	6538	24	37	TSA	48	35	TSA
<i>Staphylococcus aureus</i>	29213	24	37	TSA	48	35	TSA
<i>Staphylococcus epidermidis</i>	12228	24	37	TSA	48	35	TSA
<i>Staphylococcus aureus</i> (MRSA)	33591	24	37	TSA	48	35	TSA
<i>Staphylococcus aureus</i> (clinical isolate/MRSA)	2857	24	37	TSA w/SB	48	35	TSA w/SB
<i>Streptococcus pneumoniae</i>	6303	24	37**	BHI	48	37**	BHI
<i>Streptococcus pyogenes</i>	19615	24	37**	BHI	48	37**	BHI

\*The agar will contain 25mL/L AOAC Stock Neutralizer except TSA w/SB \*\*5% CO<sub>2</sub>  
 Key: TSA = Tryptic Soy Agar  
 BHI = Brain Heart Infusion Agar  
 TSA w/SB = Tryptic Soy Agar with Sheep Blood

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

HILL TOP RESEARCH, INC.

MAIN AND MILL STREETS  
MIAMIVILLE, OHIO 45147

PROTOCOL AMENDMENT #1      Assessment of Rapid Germicidal (Time Kill)  
Activity For Hand Product

HTR STUDY NO.:                      03-122096-106

SPONSOR & ADDRESS:              Bayer Chemicals Corporation  
100 Bayer Rd.  
Building 14  
Pittsburgh, PA 15205-9741

SPONSOR'S REPRESENTATIVE:    Kevin Ajoku

**PROTOCOL MODIFICATIONS:**

The protocol incorrectly states in section 8.0 the test article identification of the second sample received as 3354-196. The correct test article identification is 3554-196.

APPROVED FOR: HILL TOP RESEARCH, INC.

BY:

*Kathleen A. Baxter*      *July 25, 2003*  
Kathleen A. Baxter, B.S.      Date  
Study Director  
Microbiology Business Unit

HTR Study No. 03-122096-106  
Bayer Chemicals Corporation

HILL TOP RESEARCH, INC.

MAIN AND MILL STREETS  
MIAMIVILLE, OHIO 45147

**PROTOCOL DEVIATION #1**                      Assessment of Rapid Germicidal (Time Kill)  
Activity For Hand Product

**HTR STUDY NO.:**                              03-122096-106

**SPONSOR & ADDRESS:**                      Bayer Chemicals Corporation  
100 Bayer Rd.  
Building 14  
Pittsburgh, PA 15205-9741

**SPONSOR'S REPRESENTATIVE:**          Kevin Ajoku

**PROTOCOL DEVIATION:**

The testing conducted on July 24, 2003, which included testing against *Staphylococcus aureus*, ATCC 6538 and *Streptococcus pneumoniae*, ATCC 6303, was incubated for 44 hours. Testing conducted on July 25, 2003 against *Staphylococcus aureus*, ATCC 33591 was incubated 44.75 hours. The protocol required an incubation time of  $48 \pm 2$  hours. This deviation did not have an effect on the results of the study in the opinion of the Study Director.

**APPROVED FOR: HILL TOP RESEARCH, INC.**

BY: Kathleen A. Baxter                      8.13.03  
Kathleen A. Baxter, B.S.                      Date  
(Study Director  
Microbiology Business Unit

**HILL TOP RESEARCH, INC.**

RECORD RETENTION AND PUBLICATION NOTICE

Hill Top Research, Inc., submits this report with the understanding that the Sponsor may use the Study report for its own purposes. The Sponsor agrees, however, not to use the name **Hill Top Research, Inc.**, or any derivation thereof, in advertising or marketing without the prior written consent of Hill Top.

Unless otherwise agreed in writing by the parties, Hill Top shall maintain Study records on microfilm or in other readily retrievable form for a minimum of two years after the completion of the Study.