



# **Kappa Laboratories, Inc.**

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## **STUDY TITLE**

**Modified Time Kill Study**

## **REPORT TITLE**

**Emuaid**

**Formula #DC-137**

**Date Received: 10/13/2015**

**Report Date: 11/18/2015**

## **PROJECT NUMBER**

**Laboratory Number 27181**

**Log #832415**

## **AUTHOR**

**Dr. Peter J. Kmieck**  
**Director, Kappa Laboratories, Inc.**

## **PERFORMING LABORATORY**

**Kappa Laboratories, Inc.**  
**2577 NW 74<sup>th</sup> Avenue**  
**Miami, Florida 33122**

## **SPONSOR**

**Emuaid**  
**Attn.: Amy Nicolo**  
**5821 North Andrews Way**  
**Ft. Lauderdale, Florida 33309**

**GOOD LABORATORY PRACTICE STATEMENT**

The study referenced in this report was conducted in general compliance with the U.S. Food and Drug Administration, Good Laboratory Practice (GLP) regulations set forth in 21 CFR Part 58 modified in certain of the procedures.

The studies not performed by or under the direction of Kappa Laboratories, Inc. are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the compound (s).

Submitter: Emuaid Date: 10/13/2015  
Sponsor: Emuaid Date: 10/13/2015  
Study Director: Dr. Peter J. Kmiecik Date: 10/13/2015

**Kappa Laboratories – Cross-referenced Materials:**

Thermometer, Records/Incubation Temperature Log  
Microbiological Organisms - Stock Culture Log.

Staphylococcus aureus	(ATCC No. (6538)
Pseudomonas aeruginosa	(ATCC No. (9027)
E. coli	(ATCC No. (8739)
Candida albicans	(ATCC No. (10231)
Aspergillus niger	(ATCC No. (16404)

**Test Record/Notes – Sample Log Book (SpecMic) SAMPLE MEDIA CONTROL DATA**

Media:

	FCD Modified Neutralizer	Lot # 151013C	Exp.: 01/13/16
	Phosphate Buffer	Lot # 151111	Exp.: 02/01/16
	TSA	Lot # 733788	Exp.: 11/24/15
	PDA	Lot # 74916	Exp.: 12/21/15
Control G/NG	TSA	Lot # 733788	Exp.: 11/24/15
Control G/NG	PDA	Lot # 749116	Exp.: 12/21/15
	FCD Modified Neutralizer	Lot # 151013C	Exp.: 01/13/16
	Phosphate Buffer	Lot # 151111	Exp.: 02/01/16

Media Log Book: FCD broths and buffer solutions per date/lot#.  
Sterilization Log: According to date/spore strip verification.

**METHOD AND RESULTS SUMMARY**

**Modified Time Kill Study Assay:** Formula #DC-137, W/O Waxes, MFG: 10/08/2015

**Quality Assurance Unit:**

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of non-clinical laboratory studies. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the date(s) listed below. Studies are inspected at time intervals to assure the integrity of the study. The findings of these inspections have been reported to management and the Study Director.

**Phase Inspected:****Staphylococcus aureus**

▪ Test Set Up – MTK Study	Date: 10/27/15	LD/VRP/PJK
▪ Transfer MTK Study	Date: 10/27/15	LD/VRP/PJK
▪ Platings MTK Study	Date: 10/28/15	LD/VRP/PJK
▪ Platings MTK Study	Date: 10/29/15	LD/VRP/PJK
▪ Platings MTK Study	Date: 11/04/15	LD/VRP/PJK
▪ Platings MTK Study	Date: 11/11/15	LD/VRP/PJK
▪ Readings MTK Study	Date: 10/29/15	LD/VRP/PJK
▪ Readings MTK Study	Date: 10/30/15	LD/VRP/PJK
▪ Readings MTK Study	Date: 11/05/15	LD/VRP/PJK
▪ Readings MTK Study	Date: 11/16/15	LD/VRP/PJK
▪ Final Report	Date: 10/29/15	LD/VRP/PJK
▪ Study Director Review	Date: 10/29/15	PJK
▪ Management Review	Date: 10/29/15	DMK/PJK
▪ Report Date	Date: 11/18/15	PJK/DMK

**Professional personnel involved**

▪ Laboratory Director:	Dr. Peter J. Kmieck
▪ Study Director:	Dr. Peter J. Kmieck
▪ Laboratory Supervisor:	Lorena Duarte
▪ Laboratory Supervisor:	Valerie Rodriguez Purcell
▪ Research Assistant:	Juana R. Rodriguez
▪ Quality Assurance Deputy:	Denise M. Kmieck

**Project: Modified Time Kill Assay****Report Date: 11/18/2015****Sponsor:**

A certified copy of the original final report and all raw data pertinent to this study will be stored at Kappa Laboratories, Inc., 2577 NW 74<sup>th</sup> Avenue, Miami, Florida 33122. The test substance will be discarded per Sponsor's request following study completion.

**Test Material:** Formula #DC-137, W/O Waxes, MFG: 10/08/2015**Control Material:**

Experimental Controls per Method

**Characterization:**

Sponsor did not provide the identity, strength, stability, solubility, purity and chemical composition to Kappa Laboratories, Inc.

**Objective:**

The objective of this study was to evaluate the Time Kill Kinetics of Antimicrobial activity of the Germicide Product against Gram Negative E. coli and Pseudomonas aeruginosa and Gram Positive Staphylococcus aureus. As well as Mold Aspergillus niger and Yeast Candida albicans

**Test Facility:** Kappa Laboratories, Inc.  
2577 NW 74<sup>th</sup> Avenue  
Miami, Florida 33122

- Date Sample Received                      Date: 10/13/2015
- Study Initiation Date                      Date: 10/27/2015
- Experimental State Date:                Date: 10/27/2015
- Study Completion Date:                 Date: 11/16/2015

**References:**

Time Kill Study and Modified Time Kill Study Protocol CFR, Vol. 59, No. 116, Section 333.470  
Official Methods of Analysis (15<sup>th</sup> Ed.), Association of Official Analytical Chemists, Arlington, VA 1990.

## Method and Results Summary

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### Experimental Design

#### Culture Media:

1. Trypticase Soy Broth (Accumedia):  
Prepare according to manufacturer's directions.
2. Trypticase Soy Agar (Remel):  
Prepare according to manufacturer's directions.

#### Subculture Media:

1. Trypticase Soy Agar (Remel):  
Prepare according to manufacturer's directions.
2. FCD Broth (Kappa):  
Prepare according to directions.

## Reagents and Apparatus

### 1. Phosphate Buffer Stock (0.25M)

Dissolve 34.0 g  $\text{KH}_2\text{P}_04$  in 500 ml of Laboratory Purified water. Adjust to pH 7.2 with 1N NaOH and dilute to 1L.

### 2. Phosphate Buffer Dilution Water

Add 1.25 ml of 0.25M phosphate buffer stock to 1L of Laboratory Purified water and mix. Dispense in 99 ml portions in milk dilution bottles. Autoclave for 20 minutes at 121 degrees centigrade.

### 3. Glassware

50 ml beakers with magnetic stir bars. Cover with aluminum foil and sterilize for 20 minutes at 121 degrees centigrade. For modified study, use sterile disposable 50 ml centrifuge tubes (Baxter Cat. No. C3920-50A or equivalent).

### 4. Petri Dishes

Sterile disposable petri dishes. 15 X 100mm for pour plate method. Prepared media may be used for spread plate method.

### 5. Pipettes

Sterile disposable 1, 2, 2 and 5 ml pipettes.

### 6. Transfer Loops

Transfer loops held in a suitable holder with a 4 mm diameter of Platinum-rhodium, 1 inch long and bent at a 30-degree angle would be used in situations where the volume transferred is critical (i.e. volume transfer during testing). Suitable metal or plastic disposable transfer loops can be used in situations where the volume is required.

### 7. Magnetic Stir Plate

## Test Systems

Staphylococcus aureus	(ATCC No. (6538)
Pseudomonas aeruginosa	(ATCC No. (9027)
E. coli	(ATCC No. (8739)
Candida albicans	(ATCC No. (10231)
Aspergillus niger	(ATCC No. (16404)

Maintained on nutrient agar slants by weekly transfers. From the stock culture inoculate a plate of PDA incubated at 25 degrees +/- 2 degrees centigrade before using culture for testing. Use 5 day culture of organism grown on PDA and harvest aseptically. Vortex and let settle for 30 minutes prior to testing.

### Operating Technique – Time Kill Study

1. Place 20 ml of antibacterial test product in 50 ml beaker and place on magnetic stir plate. Adjust speed of mixer for rapid mixing without creating air bubbles.
2. Add 0.2 ml of test organism to antibacterial test product.
3. After each desired exposed time (such as 15, 30 or 60 seconds), remove 1.0 ml of inoculated antibacterial test product and subculture into 99 ml of FCD Broth. This represents a 10 to the -2 dilution. Subculture again from first bottle of FCD Broth to second bottle of FCD Broth. This represents a 10 to the -4 dilution.
4. Enumerate by serial dilutions and pour plate technique. (For antibacterial test product, 10 to the -2, 10 to the -3, 10 to the -4 should be plated in duplicate.)
5. For each test organism tested, initial test organism numbers must be determined. This is accomplished by replacing 10 ml of antibacterial soap with 10 ml phosphate buffer and repeating steps 1-4 with the exception of exposure time. (Plate 10 to the -5, 10 to the -6 in triplicate for # control.)

## Operating Technique – Modified Time Kill Study

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Place 20 ml of the test substance into a 50 ml centrifuge tube. Allow the test substance in the tube to equilibrate to test temperature. Add 0.2 ml of a 24-hour broth culture to 10 ml of the test substance and vortex vigorously for 10 seconds. Fifteen seconds after adding the suspension remove 1 ml of the test substance/culture suspension mixture with a 1 ml syringe and transfer to 9 ml of neutralizer (a 10 to the –1 dilution). Repeat the same procedure for the 30-second exposure. Transfer 1 ml from the initial neutralizer (a 10 to the –2 dilution). Both 10 to the –2 and 10 to the –3 dilutions from the second neutralizer tube are the plated by adding 1 ml and 0.1 ml respectively to separate petri plates. Dilute 1 ml of the 10 to the –2 into 99 ml of phosphate buffered dilution water (PBDW) to result in a 10 to the –4 dilution. Add 1 ml of the 10 to the –4 dilution to a petri plate. Use pour plate or spread plate technique with the subculture medium for enumeration of survivors. Enumerate inoculum numbers by adding 0.2 ml of the 24-hour broth culture to 10 ml of PBDW, Vortex missing, then serially diluting in PBDW. Enumerate using pour plate or spread plate technique 10 to the –5 and 10 to the –6 dilutions. Incubate all plates at 35 degrees centigrade +/-2 degrees centigrade. Incubate all plates at 35° centigrade for 24 to 48 hours for bacteria and at room temperature for 5 to 7 days for mold and yeast.

### Calculation

I = Initial Bacterial Suspension Count

S = Survivors (Test Substance) Count

$$\%R = I-s/I \times 100$$

Results should be reported as a % reduction in relationship to exposure time.



**Project: Modified Time Kill Assay****Report Date: 11/18/2015****TEST Product – Formula #DC-137, W/O Waxes, MFG: 10/08/2015****RESULTS**

Staphylococcus aureus	(ATCC No. (6538))
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**SAMPLE DATA RESULTS**

Percent Reduction indicated for test time points.

Note: NG = No Growth Detected at 48 hours incubation

G = Growth Detected at 48 hours incubation

Average Initial Bacterial Counts =  $3.15 \times 10^9$  cts per 0.2 ml

Time Point	Initial Population	Average Survival (CFU)	Average Percent Reduction
30 seconds	$3.15 \times 10^9$	$2.9 \times 10^6$	99.999078%
1 minute	$3.15 \times 10^9$	$3.35 \times 10^4$	99.999989%
5 minutes	$3.15 \times 10^9$	$1.2 \times 10^3$	< 99.99999%
10 minutes	$3.15 \times 10^9$	<1	< 99.99999%
15 minutes	$3.15 \times 10^9$	<1	< 99.99999%
Positive Controls	G	G	G
Negative Controls	NG	NG	NG

A "Kills on Contact" product will reduce 99.999% or greater bacterial challenges in less than one (1) Minute.

**Project: Modified Time Kill Assay****Report Date: 11/18/2015****TEST Product – Formula #DC-137, W/O Waxes, MFG: 10/08/2015****RESULTS**

<i>Pseudomonas aeruginosa</i>	(ATCC No. (9027))
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**SAMPLE DATA RESULTS**

Percent Reduction indicated for test time points.

Note: NG = No Growth Detected at 48 hours incubation

G = Growth Detected at 48 hours incubation

Average Initial Bacterial Counts =  $6.3 \times 10^9$  cts per 0.2 ml

Time Point	Initial Population	Average Survival (CFU)	Average Percent Reduction
30 seconds	$6.3 \times 10^9$	$1.6 \times 10^4$	> 99.99999%
1 minute	$6.3 \times 10^9$	$2.0 \times 10^3$	> 99.99999%
5 minutes	$6.3 \times 10^9$	$1.0 \times 10^3$	> 99.99999%
10 minutes	$6.3 \times 10^9$	$8 \times 10^2$	> 99.99999%
15 minutes	$6.3 \times 10^9$	<10	> 99.99999%
Positive Controls	G	G	G
Negative Controls	NG	NG	NG

A "Kills on Contact" product will reduce 99.999% or greater bacterial challenges in less than one (1) Minute.

**Project: Modified Time Kill Assay**

**Report Date: 11/18/2015**

**TEST Product – Formula #DC-137, W/O Waxes, MFG: 10/08/2015**

**RESULTS**

E. coli	(ATCC No. (8739))
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**SAMPLE DATA RESULTS**

Percent Reduction indicated for test time points.

Note: NG = No Growth Detected at 48 hours incubation

G = Growth Detected at 48 hours incubation

Average Initial Bacterial Counts =  $3.05 \times 10^6$  cts per 0.2 ml

Time Point	Initial Population	Average Survival (CFU)	Average Percent Reduction
30 seconds	$3.05 \times 10^6$	$1.2 \times 10^3$	> 99.9996%
1 minute	$3.05 \times 10^6$	$1.9 \times 10^2$	> 99.9999%
5 minutes	$3.05 \times 10^6$	$2.5 \times 10^2$	> 99.9999%
10 minutes	$3.05 \times 10^6$	$3.1 \times 10^1$	> 99.9999%
15 minutes	$3.05 \times 10^6$	$1.1 \times 10^1$	> 99.9999%
Positive Controls	G	G	G
Negative Controls	NG	NG	NG

A "Kills on Contact" product will reduce 99.999% or greater bacterial challenges in less than one (1) Minute.

**Project: Modified Time Kill Assay****Report Date: 11/18/2015****TEST Product – Formula #DC-137, W/O Waxes, MFG: 10/08/2015****RESULTS**

Candida albicans	(ATCC No. (10231))
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**SAMPLE DATA RESULTS**

Percent Reduction indicated for test time points.

Note: NG = No Growth Detected at 48 hours incubation

G = Growth Detected at 48 hours incubation

Average Initial Bacterial Counts =  $4.05 \times 10^6$  cts per 0.2 ml

Time Point	Initial Population	Average Survival (CFU)	Average Percent Reduction
30 seconds	$4.5 \times 10^6$	$6 \times 10^4$	99.9867%
1 minute	$4.5 \times 10^6$	$2 \times 10^3$	<99.9995%
5 minutes	$4.5 \times 10^6$	$4 \times 10^2$	99.9999%
10 minutes	$4.5 \times 10^6$	$2 \times 10^2$	99.99995%
15 minutes	$4.5 \times 10^6$	$5 \times 10^1$	99.99999%
Positive Controls	G	G	G
Negative Controls	NG	NG	NG

A "Kills on Contact" product will reduce 99.999% or greater bacterial challenges in less than one (1) Minute.

**Project: Modified Time Kill Assay**

**Report Date: 11/18/2015**

**TEST Product – Formula #DC-137, W/O Waxes, MFG: 10/08/2015**

**RESULTS**

Aspergillus niger	(ATCC No. (16404))
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**SAMPLE DATA RESULTS**

Percent Reduction indicated for test time points.

Note: NG = No Growth Detected at 48 hours incubation

G = Growth Detected at 48 hours incubation

Average Initial Bacterial Counts =  $2.5 \times 10^5$  cts per 0.2 ml

Time Point	Initial Population	Average Survival (CFU)	Average Percent Reduction
30 seconds	$2.5 \times 10^5$	$1 \times 10^2$	99.9996%
1 minute	$2.5 \times 10^5$	<10	< 99.9999%
5 minutes	$2.5 \times 10^5$	<10	< 99.9999%
10 minutes	$2.5 \times 10^5$	<10	< 99.9999%
15 minutes	$2.5 \times 10^5$	<10	< 99.9999%
Positive Controls	G	G	G
Negative Controls	NG	NG	NG

A "Kills on Contact" product will reduce 99.999% or greater bacterial challenges in less than one (1) Minute.

## SUMMARY

The Formula #DC-137, W/O Waxes, MFG: 10/08/2015, product appeared to be consistently effective in exerting On-Contact Bactericidal Activity against Staphylococcus aureus, Pseudomonas aeruginosa and E. coli bacterial organisms. As well as Candida albicans and Aspergillus niger which are yeast and mold. The product appeared to be a "Kills on Contact" product.

## Controls

Negative Controls for all Medias were performed by incubation of uninoculated media. Positive Controls were performed for all organisms by plating directly onto the appropriate media employed for the assay. Broths were tested for Positive growth by inoculation with the appropriate organism.

Test Facility: Kappa Laboratories, Inc.  
2577 NW 74<sup>th</sup> Avenue  
Miami, Florida 33122

Kappa Laboratories has been inspected and previously recognized by the U.S. Department of Agriculture (USDA Microbiology-#0093, Chemistry-#1282); Registered with the U.S. Food and Drug Administration (FDA-#1039389) and is an FDA Accepted Laboratory for Import Testing. Kappa Laboratories is currently a Contract Laboratory to the U.S. Centers for Disease Control (CDC), Atlanta, Georgia; Vessel Sanitation Program and is U.S. Dept. of Homeland Security, U.S. Coast Guard Recognized Facility.

Signed:   
Dr. Peter J. Kmiec  
Director, Kappa Laboratories, Inc.