STUDY TITLE
Non-GLP Suspension Time-Kill ASTM E2315

Product Identity
Hand Sanitizer

Test Microorganisms
S. aureus ATCC 6538
K. pneumoniae ATCC 13883
C. albicans ATCC 10231

Authors
Monica Roberts, B.S.

Testing Facility
Microchem Laboratory
1304 W. Industrial Blvd.
Round Rock, Texas 78681

Study Sponsor
Gennady Sirota
StellaLife®, Inc.
2875 NE 191 Street,
Suite 500
Aventura, FL 33180

PERSONNEL INVOLVED IN THE STUDY

Name: Hannah Klug, M.S.
Title: Team Lead, Medical Device Efficacy

Name: Brooke Lewis, B.S.
Title: Associate Analyst

TABLE OF CONTENTS

TEST ARTICLE INFORMATION

Test Substance:	Hand Sanitizer
Test Substance Manufacturer:	StellaLife
Special Handling Requirements:	None
Test Substance Dilution:	None

TEST PARAMETERS

Contact Time(s):	30 seconds
Test Temperature:	Ambient
Testing Replicates:	Double Replicate
Control Substance (Volume):	Phosphate Buffered Saline (10.0mL)
Incubation Temperature:	See Table 1
Test Substance volume:	10.0mL
Inoculum Target Concentration:	≥1 x 10^6 CFU/mL
Inoculum Volume:	0.500mL
Plate Wash Media (Volume):	PBS (10.0 mL)
Serial Dilution Media (Volume):	PBS (0.900 mL)
Test plate dilutions:	1, 2, 3
Control plate dilutions:	5, 6, 7
Neutralizer Used (Volume):	Dey-Engley Broth (D/E Broth) (9.0 mL)
Neutralization Inoculum Volume:	0.100 mL
Neutralization Inoculum Target Concentration:	10-100 CFU
Neutralization Dwell Time:	≥10 minutes
Volume Plated for Neutralization Plates:	1.000 mL
Growth/Plating Media:	See Table 1

Table 1: Growth Media and Incubation Conditions.

Test Microorganism	Temperature	Incubation Conditions	Growth Media
S. aureus ATCC 6538	36±1°C	Aerobic	Tryptic Soy Agar/Broth (TSA/TSB)
K. pneumoniae ATCC 13883			
C. albicans ATCC 10231	30±1°C		Potato Dextrose Agar (PDA)

TEST METHOD

Preparation of Test Substance
Prior to test initiation, 10.0 mL of test substance was placed in individual sterile conical tubes. The number of contact times, replicates, and the number of test microorganisms was used to determine the number of sterile conical tubes prepared for testing.

Preparation of Control Substance for Microbial Population
Prior to test initiation, 10.0 mL of sterile Phosphate Buffered Saline (PBS) was placed in individual sterile conical tubes to be used for the microbial population controls. The number of replicates and test microorganisms was considered to determine the number of sterile conical tubes to prepare for testing.

Preparation of Test Culture and Test Inoculum
The test cultures were initiated from the monthly working stock cultures by transferring a loop full of the target microorganism to the appropriate growth media. The test cultures were incubated at the appropriate temperature.
To prepare the test inoculum for *C. albicans*, the test culture plate was washed with 10.0mL sterile Phosphate Buffered Saline (PBS). The cultures initiated in broth did not require any further preparation.

Inoculation of Test Substance
The individual test substance aliquots were inoculated and a digital timer was started. The inoculated test substance was vortex mixed and allowed to dwell for the appropriate contact time. Following the completion of the contact time, 1.0 mL of the inoculated test substance was neutralized with sterile D/E Broth and serially enumerated by 1:10 dilutions in sterile PBS. The appropriate dilutions were plated to the appropriate agar.

Inoculation of Control Substance for Microbial Population Control
The microbial population control was taken at the start of testing for time zero for each test microorganism. The same volume of test inoculum used in testing was added to 10.0 mL of sterile PBS. Following inoculation, 1.0 mL of the time zero solutions were immediately neutralized with sterile D/E Broth. The neutralized solutions were serially enumerated by 1:10 serial dilutions in sterile PBS and appropriate dilutions were plated to the appropriate growth agar.

TEST METHOD (cont.)

Neutralization Control Test A – Neutralization Effectiveness

Test microorganisms were diluted to a final suspension that contained a target concentration of 10-100 CFU/mL by 1:10 serial dilutions in sterile PBS. For each test microorganism, the same volume of neutralization media used in testing was inoculated with the dilute test microorganism. A volume of test substance equal to the volume harvested in testing (1.0 mL) was added to the inoculated suspension and vortex mixed. The inoculated neutralization suspension was left to dwell at ambient temperatures for >10 minutes and then 1.0 mL aliquots were plated in duplicate to Tryptic Soy Agar (TSA).

Neutralization Control Test B – Neutralizer Toxicity

Test microorganisms were diluted to a final suspension that contained a target concentration of 10-100 CFU/mL by 1:10 serial dilutions in sterile PBS. For each test microorganism, the diluted microorganism was used to inoculate a volume of sterile neutralization media equal to that used in testing. The inoculated suspension was vortex mixed and a volume of sterile PBS equal to the volume of test substance harvested for testing (1.0 mL) was added to the inoculated neutralization suspension. The suspension was left to dwell at ambient temperatures for >10 minutes and then 1.0 mL aliquots were plated in duplicate to the appropriate growth agar.

Neutralization Control Test C- Test Organism Viability

Test microorganisms were diluted to a final suspension that contained a target concentration of 10-100 CFU/mL by 1:10 serial dilutions in sterile PBS. For each test microorganism, the diluted test microorganism was used to inoculate a volume of sterile PBS equal to the volume of neutralization media used in testing. A volume of sterile PBS equal to the amount of test substance harvested in testing (1.0 mL) was added to the inoculated suspension and vortex mixed. The suspension was left to dwell at ambient temperatures for >10 minutes and then 1.0 mL aliquots were plated in duplicate to the appropriate growth agar.

Sterility and Viability Controls

Sterility and viability controls were performed for all media used in testing. Controls included the following:

A plate containing sterile agar was incubated along with test materials to confirm sterility. A plate containing a 0.100 mL aliquot of PBS used as culture diluent and testing controls was incubated along with test materials to confirm sterility. A plate containing a 0.100 mL aliquot of serial dilution media was incubated along with test materials to confirm sterility. A plate containing a 0.100 mL aliquot of the D/E Broth was incubated along with test materials to confirm sterility. A plate containing a purity streak of each test microorganism was incubated along with test materials to confirm purity and viability of test culture. Any additional media used in the study was plated and incubated along with test materials to confirm sterility.

Incubation of Test and Controls

All test and control plates were incubated for 18-48 hours at the conditions listed in Table 1. The selected incubation time allowed for growth of the surviving test microorganisms, without overgrowth of colonies.

STUDY SUCCESS CRITERIA

The experimental success (controls) criteria follow:

- The initial microbial population demonstrates a starting concentration of at least $1x10^6$ CFU/mL.
- For a qualitative NV, tests A, B, C must demonstrate growth for neutralization to be considered.
- For a quantitative NV the average CFU from test A and B must all be ≥50% of Test C for neutralization to be considered effective.
- The media sterility controls demonstrate no growth.
- The test microorganism purity control plate demonstrates the presence of the target microorganism and absence of contaminant microorganisms.

PASSING CRITERIA

ASTM International does not specify performance criteria, therefore it may be established by the Study Sponsor.

CALCULATIONS AND STATISTICAL ANALYSIS

[(Plate count 1 + plate count 2) / 2] • dilution factor = CFU/ml

Log_{10} Reduction=LR=Log_{10}(A) – Log_{10}(B)
Where:
LR= mean log_{10} of surviving microbial population
A= the mean CFU/mL recovered from the microbial population controls
B= the mean CFU/mL recovered from the test substance at the contact time

Percent Reduction = $100*(1-10^{-LR})$
Where:
PR = Percent Reduction versus microbial population control

RESULTS

Table 2: *S. aureus* ATCC 6538 Percent Reduction and Log_{10} Reduction Compared to Time Zero.

Test Microorganism	Contact Time	Test Substance	Replicate	CFU/ml	Average CFU/ml	Percent Reduction compared to Time Zero	Log Reduction Compared to Time Zero
S. aureus ATCC 6538	Time Zero		1	5.50E+07	3.33E+07	N/A	
			2	1.15E+07			
	30 seconds		1	<5.00E+00*	<5.00E+00	>99.99998%	>6.82
			2	<5.00E+00*			

*Limit of detection. Values fell below the limit of detection <5.00E+00

Table 3: *K. pneumoniae* ATCC 13883 Percent Reduction and Log_{10} Reduction Compared to Time Zero.

Test Microorganism	Contact Time	Test Substance	Replicate	CFU/ml	Average CFU/ml	Percent Reduction compared to Time Zero	Log Reduction Compared to Time Zero
K. pneumoniae ATCC 13883	Time Zero		1	1.55E+07	3.35E+07	N/A	
			2	5.15E+07			
	30 seconds		1	<5.00E+00*	<5.00E+00	>99.99998%	>6.82
			2	<5.00E+00*			

*Limit of detection. Values fell below the limit of detection <5.00E+00

Table 4: *C. albicans* ATCC 10231 Percent Reduction and Log_{10} Reduction Compared to Time Zero.

Test Microorganism	Contact Time	Test Substance	Replicate	CFU/ml	Average CFU/ml	Percent Reduction compared to Time Zero	Log Reduction Compared to Time Zero
C. albicans ATCC 10231	Time Zero		1	4.50E+07	3.43E+07	N/A	
			2	2.35E+07			
	30 seconds		1	<5.00E+00*	1.00E+02	>99.99998%	>6.83
			2	<5.00E+00*			

*Limit of detection. Values fell below the limit of detection <5.00E+00

RESULTS (Cont.)

Table 5: Neutralization Verification against *S. aureus* ATCC 6538.

Test Microorganism	Test Substance	Test	Replicate	Neutralization Validation plate counts (CFU)		Average CFU	Percent Recovery Neutralization	Neutralization Verified
S. aureus ATCC 6538	N/A	C	1	3.50E+01	4.60E+01	3.15E+01	N/A	
			2	1.50E+01	3.00E+01			
	N/A	B	1	5.20E+01	5.70E+01	5.73E+01	181.75%	Yes
			2	6.20E+01	5.80E+01			
	Hand Sanitizer	A	1	3.10E+01	4.30E+01	6.10E+01	193.65%	Yes
			2	1.05E+02	6.50E+01			

Table 6: Neutralization Verification against *K. pneumoniae* ATCC 13883.

Test Microorganism	Test Substance	Test	Replicate	Neutralization Validation plate counts (CFU)		Average CFU	Percent Recovery Neutralization	Neutralization Verified
K. pneumoniae ATCC 13883	N/A	C	1	4.80E+01	5.70E+01	5.03E+01	N/A	
			2	5.30E+01	4.30E+01			
	N/A	B	1	5.60E+01	5.10E+01	5.08E+01	101.00%	Yes
			2	5.30E+01	4.30E+01			
	Hand Sanitizer	A	1	4.20E+01	5.20E+01	4.65E+01	92.54%	Yes
			2	5.20E+01	4.00E+01			

Table 7: Neutralization Verification against *C. albicans* ATCC 10231.

Test Microorganism	Test Substance	Test	Replicate	Neutralization Validation plate counts (CFU)		Average CFU	Percent Recovery Neutralization	Neutralization Verified
C. albicans ATCC 10231	N/A	C	1	1.42E+02	1.60E+02	1.48E+02	N/A	
			2	1.39E+02	1.52E+02			
	N/A	B	1	1.76E+02	1.75E+02	1.70E+02	114.50%	Yes
			2	1.49E+02	1.79E+02			
	Hand Sanitizer	A	1	1.88E+02	1.69E+02	1.76E+02	118.55%	Yes
			2	1.80E+02	1.66E+02			

Table 8: Sterility and Purity Controls.

Control	Result
S. aureus ATCC 6538 Purity	Pure Growth
K. pneumoniae ATCC 13883 Purity	Pure Growth
C. albicans ATCC 10231 Purity	Pure Growth
Phosphate Buffered Saline (PBS) Dilution Tube Sterility	No Growth
Dey-Engley Neutralizing Broth (D/E Broth) Sterility	No Growth
Phosphate Buffered Saline (PBS) Culture Diluent Sterility	No Growth
Tryptic Soy Agar Sterility	No Growth

REFERENCES

ASTM E2315. Standard Guide for Assessment of Activity Using a Time-Kill Procedure. West Conshohocken, PA. American Society for Testing and Materials.

Microchem Laboratory's SOP General Laboratory Safety, Organization and Personnel 008, current revision.

Microchem Laboratory's SOP Dilution, Plating, Counting, and Calculations, Testing Facility Operation 017, current revision.

Microchem Laboratory's SOP Test and Control Article/Substance Preparation, Test, Control and Reference Substances 003, current revision.

STUDY RECORD AND TEST SUBSTANCE RETENTION

The original (or certified copy) of the study report, protocol, and corresponding raw data will be held in the archives of Microchem Laboratory indefinitely. For studies not meeting the performance criteria for submission or for studies that have been canceled prior to the generation of valid data, the original (or certified copy) of the final study report, protocol, and corresponding raw data will be held in the archives of Microchem Laboratory for a minimum of two years following the study completion date at which time they may be removed from the archive or transferred to the Sponsors archive at their expense.

If requested by the Study Sponsor (or Sponsor Representative), the study file may be transferred to the Study Sponsor's archive at the Study Sponsor's expense prior to the time frames listed.

All test facility records including, but not limited to, standard operating procedures, quality assurance inspection records, temperature and equipment records including maintenance, inspection and calibration, and employee training records will be maintained at Microchem Laboratory indefinitely.

The test substance (or test control, test article, test device, as applicable) may be returned to the Study Sponsor at the Study Sponsor's request and expense following study completion unless otherwise requested to be returned earlier. If the Study Sponsor does not request return of the sample, it will be disposed >90 days following the study completion. Arrangements may be made for extended storage as necessary, at the Sponsor's request and expense.