STUDY TITLE
Non-GLP Suspension Time-Kill ASTM E2315

Product Identity
StellaLife® VEGA® Oral Care Peppermint Rinse

Test Microorganisms
S. mutans ATCC 25175
B. fragilis ATCC 43858

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PERSONNEL INVOLVED IN THE STUDY

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TEST ARTICLE INFORMATION

Test Substance:	StellaLife® VEGA® Oral Care Peppermint Rinse
Test Substance Manufacturer:	StellaLife
Special Handling Requirements:	None
Test Substance Dilution:	No dilution performed

TEST PARAMETERS

Contact Time(s):	30 minutes
Test Temperature:	Ambient
Testing Replicates:	Single Replicate
Control Substance (Volume):	Phosphate Buffered Saline (10.0 ml)
Test Substance Volume:	10.0 ml
Inoculum Target Concentration:	≥1 x 10^6 CFU/ml
Inoculum Volume:	0.100 ml
Serial Dilution Media (Volume):	Phosphate Buffered Saline (0.900 ml)
Neutralizer Used (Volume):	Dey-Engley Broth (D/E Broth) (9.0 ml)
Neutralization Inoculum Target Concentration:	10-100 CFU
Neutralization Dwell Time:	≥10 minutes
Volume Plated for Neutralization Plates:	1.00 ml
Growth Media:	Brain-Heart Infusion Broth (*S. mutans*), Tryptic Soy with 5% Sheep's Blood Agar (*B. fragilis*)
Plating Media:	Brain-Heart Infusion Agar (*S. mutans*), Tryptic Soy with 5% Sheep's Blood Agar (*B. fragilis*)
Incubation Conditions:	Aerobic (*S. mutans*), Anaerobic (*B. fragilis*)
Incubation Temperature:	36 ± 1°C
Incubation Duration:	18-48 hours

TEST METHOD

Preparation of Test Substance

Test substance was mixed well by shaking for at least 30 seconds. 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 monthly working stock cultures or freezer stock cultures by using a sterile inoculating loop to transfer a loop (~0.010 ml) of the target microorganism to the appropriate sterile growth media. The test cultures were incubated for an appropriate length of time and at an appropriate temperature to allow for adequate growth.

To prepare the test inoculum, the test culture tubes were re-suspended accordingly in sterile PBS to reach a target concentration of $\geq 1 \times 10^6$ CFU/ml.

Inoculation of Test Substance

The individual test substance aliquots were each inoculated with 0.100 ml, ≤5% of the volume of test substance used in testing, of the target test microorganism. A digital timer was started at the time of inoculation to ensure each aliquot met the appropriate contact time. The inoculated test substances were vortex mixed and allowed to dwell for the appropriate contact time. Following the completion of the contact time, the inoculated suspension was neutralized by transferring 1.00 ml of the suspension to 9.00 ml of sterile D/E Broth and enumerated by 1:10 serial dilutions in 0.900 ml of sterile PBS. The appropriate dilutions, targeting 25-250 CFU, were individually plated to the appropriate growth agar.

Inoculation of Control Substance for Microbial Population Control

The microbial population control, Time Zero, was taken at the start of testing for each test microorganism to determine the concentration of the inoculum. The same volume of test inoculum used in testing was added to 10.0 ml of sterile PBS. Following inoculation, the inoculated time zero suspensions were immediately neutralized by transferring 1.00 ml of the suspension to 9.00 ml of sterile D/E Broth. The neutralized suspensions were each enumerated by 1:10 serial dilutions in 0.900 ml of sterile PBS. The appropriate dilutions were individually plated to the appropriate growth agar.

Neutralization Verification

The neutralization verification tests were performed on both test microorganisms. Each test microorganism was serially diluted 1:10 in 0.900 ml of sterile PBS, targeting a final concentration of 10-100 CFU. The diluted test microorganisms were used as the neutralization verification inoculum.

TEST METHOD (cont.)

Neutralization Verification (cont.)

Neutralization Control Test A – Neutralization Effectiveness
The same volume of neutralization media used in testing, 9.00 ml D/E Broth, was inoculated with the dilute target test microorganism and vortex mixed. A volume of test substance equal to the volume harvested from the test replicates for neutralization (1.0 ml) was added to the inoculated neutralization suspension and vortex mixed. The inoculated neutralization suspensions were left to dwell at ambient temperatures for >10 minutes and then a 1.0 ml aliquot was plated in duplicate to the appropriate growth agar.

Neutralization Control Test B – Neutralizer Toxicity
The same volume of neutralization media used in testing, 9.00 ml D/E Broth, was inoculated with the dilute target test microorganism and vortex mixed. A volume of sterile PBS equal to the volume harvested from the test replicates for neutralization (1.0 ml) was added to the inoculated neutralization suspension and vortex mixed. The inoculated neutralization suspensions were left to dwell at ambient temperatures for >10 minutes and then a 1.0 ml aliquot was plated in duplicate to the appropriate growth agar.

Neutralization Control Test C – Test Organism Viability
9.00 ml of sterile PBS, equal to the volume of neutralization media used in testing, was inoculated with the target test microorganism and vortex mixed. A volume of sterile PBS equal to the volume harvested from the test replicates for neutralization (1.0 ml) was added to the inoculated suspension and vortex mixed. The inoculated neutralization suspensions were left to dwell at ambient temperatures for >10 minutes and then a 1.0 ml aliquot was 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 at an appropriate temperature for an incubation time which 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, Time Zero control, must demonstrate a starting concentration of at least $1x10^6$ CFU/ml.
- The test microorganism concentration obtained from each neutralization verification test must demonstrate a test microorganism count of 10-100 CFU.
- The average CFU from neutralization test A or test B must each be ≥50% of the average CFU of test C for neutralization to be considered effective.
- The media sterility controls demonstrate no growth.
- The test microorganism purity control plate(s) demonstrate(s) 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

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

Log_{10} Reduction
$LR = Log_{10}(A) – Log_{10}(B)$
Where:
LR= the 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
$PR = 100 * (1-10^{-LR})$
Where:
PR = Percent Reduction versus microbial population control
LR = the mean Log_{10} of surviving microbial population

Percent Recovery Neutralization
% Recovery = (X/Y) * 100
Where:
X = the values associated with the neutralizer test A or B
Y = the values associated with the neutralizer test C

RESULTS

Table 1: Percent Reduction and Log_{10} Reduction at 30 Minutes Compared to Time Zero

Test Microorganism	Test Substance	Contact Time	CFU/mL	Percent Reduction Compared to Time Zero Concentration	Log Reduction Compared to Time Zero Concentration
S. mutans ATCC 25175	N/A	Time Zero	4.40E+06	N/A	
	StellaLife VEGA Oral Care Peppermint Rinse	30 minutes	<5.00E+01*	>99.9989%	>4.94
B. fragilis ATCC 43858	N/A	Time Zero	5.60E+07	N/A	
	StellaLife VEGA Oral Care Peppermint Rinse	30 minutes	<5.00E+01*	>99.99991%	>6.05
*Lower limit of detection. A value of 5.00E+01 was used for calculations.					

Table 2: Neutralization Verification against *S. mutans* ATCC 25175

Test Microorganism	Test Substance	Test	Neutralization Validation Plate Counts (CFU)		Average CFU	Percent Neutralization Verified	Neutralization Verified
S. mutans ATCC 25175	Control Substance	Test C	50	108	79.0	N/A	
		Test B	92	126	109.0	137.97%	Yes
	StellaLife VEGA Oral Care Peppermint Rinse	Test A	140	166	153.0	193.67%	Yes

Table 3: Neutralization Verification against *B. fragilis* ATCC 43858

Test Microorganism	Test Substance	Test	Neutralization Validation Plate Counts (CFU)		Average CFU	Percent Neutralization Verified	Neutralization Verified
B. fragilis ATCC 43858	Control Substance	Test C	135	126	130.5	N/A	
		Test B	128	151	139.5	106.90%	Yes
	StellaLife VEGA Oral Care Peppermint Rinse	Test A	128	116	122.0	93.49%	Yes

RESULTS (cont.)

Table 4: Sterility and Purity Controls

Control	Result
S. mutans ATCC 25175 Purity	Pure Growth
B. fragilis ATCC 43858 Purity	Pure Growth
Phosphate Buffer Saline Culture Re-suspension/Plate Wash Media Sterility	No Growth/Sterile
Phosphate Buffer Saline Serial Dilution Media Sterility	No Growth/Sterile
Dey-Engley Neutralizing Broth (D/E Broth) Sterility	No Growth/Sterile
Brain Heart Infusion Agar (BHIA) Sterility	No Growth/Sterile
Tryptic Soy w/ 5% Sheep's Blood Agar (BLA) Sterility	No Growth/Sterile

The results of this study apply to the tested substances(s) only. Extrapolation of findings to related materials is the responsibility of the Sponsor.