Ryerson University

Faculty of Science

Nanosafe Coatings1 Inc.

TECHNICAL REPORT:

ISO 22196:2011 Measurement of antibacterial activity on plastics and other non-porous surfaces "Modified Large droplet Inoculation Method."

Antimicrobial Activity against *Staphylococcus aureus* on ***VITRO-SKIN®***

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1.Materials Submitted for Testing:

Test Substrates: Hydrated VITRO-SKIN® (15 x 1.0 x 1.0 in.)

Treatments:
A. 3 x control - untreated
B. 3 x treated 1 – BZK (0.13%): 0 hour residual protection
C. 3 x treated 2 – BZK (0.13%) + BIO-PROTECT (0.13%): 0 hour residual protection
D. 3 x treated 1 – BZK (0.13%): 6 hour residual protection
E. 3 x treated 2 – BZK (0.13%) + BIO-PROTECT (0.13%): 6 hour residual protection

Test Organism: *Staphylococcus aureus*

VITRO-SKIN© swatches (9 x 1.0 x 1.0 in.) were hydrated by Dr. Lukasz Porosa overnight (24 hrs) using the recommended glycerol/water humidifier provided with the VITRO-SKIN starter kit prior to ISO 22196 testing.

VITRO-SKIN© is an advanced testing substrate that effectively mimics the surface properties of human skin. It has been formulated to have topography, pH, critical surface tension and ionic strength that is similar to human skin.

VITRO-SKIN© is currently used by over 155 leading companies worldwide and has been referenced in numerous scientific presentations and patents. It has been successfully applied in a broad range of in vitro methods including the measurement of SPF and UVA protection factors, evaluation of the water resistance of prototype sunscreen formulations, rapid assessment of the performance of sunless tanning formulations, evaluation of the performance of adhesive bandages, assessment of prototype and emollient spreading. Testing done on VITRO-SKIN is generally more reproducible than that performed on human skin due to the consistent topography and wetting properties across each sheet. VITRO-SKIN with N-19 topography is optimized to mimic human back skin. It is a synthetic (non-biological) product.

2.Significance and Use:

Large-droplet inoculation method

Many pathogens are able to remain viable during extended periods of desiccation on surfaces at the solid-air interface. Long-term survival of pathogens in the inanimate environment poses a significant risk for infection transmission and cross-contamination in high-risk environments such as hospital rooms or food-processing plants.

The large-droplet inoculation method was developed to simulate the deposition of bacterial species onto exposed surfaces and to determine the ability of these cells to survive desiccation. In this experiment ***VITRO-SKIN*** was sanitized with a common BZK (0.13%) antiseptic foam hand sanitizer with and without BIO-PROTECT (0.13%) to assess the residual kill of the protective cationic polymer right after product application/drying and after six hours of application (6 hour residual sanitizer protection).

3.Preparation of Bacterial Inoculum.

Challenge culture of Staphylococcus aureus – Strain (ATCC #4330) was grown in 5-10 mL10% tryptic soy broth for 24 hours on a shaking incubator at 37 C. and washed twice by centrifugation to replace the growth media with sterile water (2 x 2 mL tubes washed with 2 x 1 mL water) to give to a target range of 1.96 $x10^8$ cfu/mL.

4.Preparation of the Test Specimen:

Prior to application of hand sanitizer a sheet of ***VITRO-SKIN*** was cut into a total of twelve 1 inch by 1 inch squares (3 x control, 3 x treatment 1 x 2, 3 x treatment 2 x 2) using high-quality paper cutters or shears and hydrated for 24 hrs inside a closed, controlled-humidity plastic chamber provided with the ***VITRO-SKIN*** starter-kit. The humidity in the chamber was regulated by a solution of 85% water / 15% glycerine (350g / 52 g), placed in the bottom of the chamber. *The substrate was placed above the liquid on a shelf or tray*. This step insures reproducible hydration of the ***VITRO-SKIN*** prior to product application. Nitrile gloves were worn while handling the sheets.

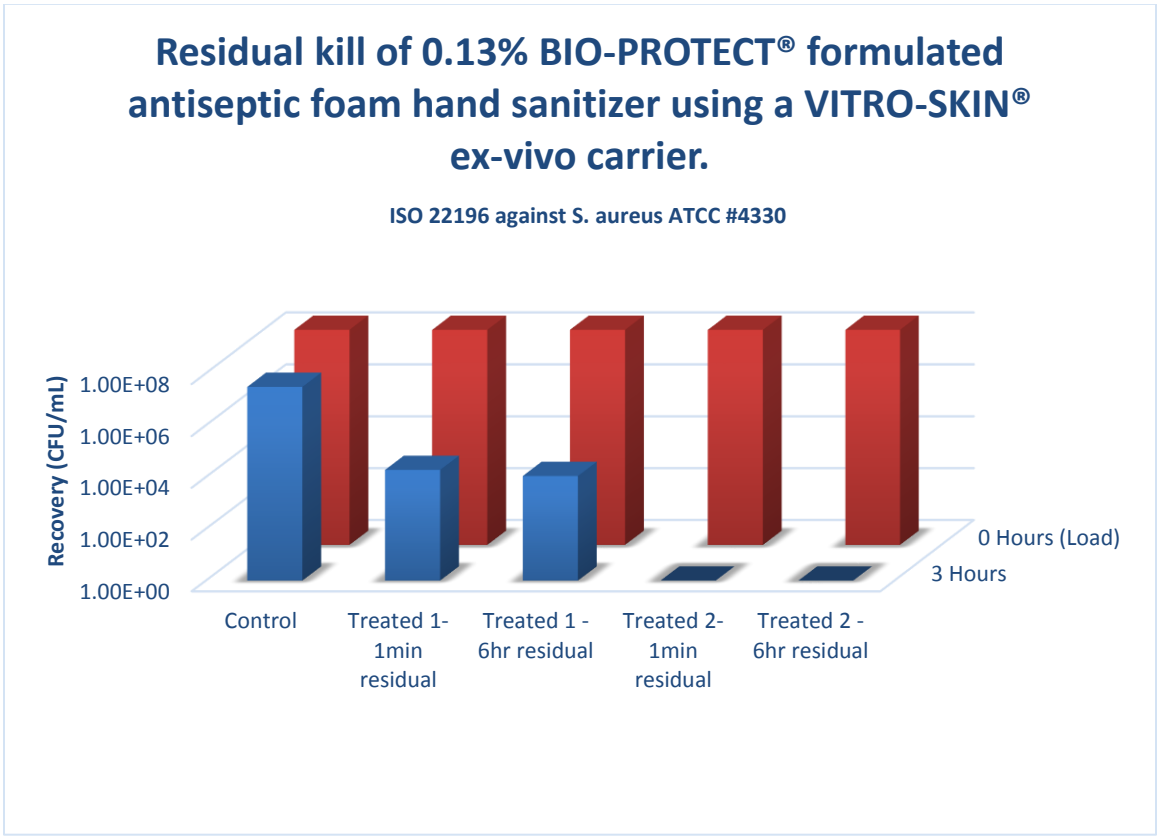
All samples were sanitized with 1 mL 70% (v/v) ethanol and rubbed with a sterile nitrile glove until dry. One pump of a BZK (0.13%) antiseptic foam hand sanitizer delivering 0.4 g of product was spread across 3 1 inch x 1 inch VITRO-SKIN squares and rubbed for 30 seconds until completely dry. The above product application was repeated with the BZK + BIO-PROTECT product. Two sample in triplicate were inoculated immediately and the remaining two samples in triplicate were inoculated after 6 hours of leave on residual.

5.Testing Procedure:

The untreated and treated VITRO-SKIN® samples in triplicate were each placed into three separate sterile petri dishes and 0.05 mL *Staphylococcus aureus* inoculum was placed on each 1.0 by 1.0 inch square for a total of 0.6 mL (12 x 0.05 mL). Samples were left inside the petri dishes and allowed to air-dry in a biological safety cabinet and incubated at 36 ± 1 °C until the droplet of bacteria was visibly dry. Drying typically occurred 3 hours after 0.05 mL inoculations, and surviving cells were enumerated immediately after drying. For enumeration, inoculated coupons were sacrificed in triplicate and placed inside separate tubes containing 5mL of a 0.9% saline collection liquid. Each coupon was agitated vigorously for 1 minute with a bench-top vortex to transfer cells from the test surface to the collection liquid. Standard agar plate counts after 48-72 hours at 35ºC were then performed on serial dilutions of the collection liquid, and colony counts of *Staphylococcus aureus* colonies (20-200) from triplicate treated surfaces were averaged and compared to colony counts from tests of untreated control surfaces carried out in parallel.

6.Evaluation of Results:

Sample ID	CFU/mL	Log Reduction	%Log Kill
Control T = 0	1.96E+08		
Control T = 3	2.98E+07	8.18E-01	84.778912
Treated 1 T = 1 min	1.89E+04	4.02E+00	99.990357
Treated 1 T = 6 hr	1.10E+04	4.25E+00	99.994388
Treated 2 T = 1 min	0.00E+00	8E+00	99.999999
Treated 2 T = 6 hr	0.00E+00	8E+00	99.999999



7.Calculation of the "Antibacterial Activity": This is the difference in the logarithm of the viable cell count found on an antimicrobial-treated product and a control product after inoculation with, and incubation of, the bacteria. The following equation were used:

(a)Log Reduction Calculation

$$\text{Log Reduction} = \log_{10}\left(\frac{A}{B}\right)$$

or,

$$\text{Log Reduction} = \log_{10}(A) - \log_{10}(B)$$

A = the average number of viable bacteria (bacteria/mL) recovered from the control test specimens..
B = the average number of viable bacteria (bacteria/mL) after 3 hr of contact time

(b) Log Reduction to Percent Reduction Calculation

$$P = (1 - 10^{-L}) x 100$$

P = % reduction
L = log reduction

(c) Relationship between log reduction and percent reduction.

1 log reduction = 10^1 times less organisms = 90% reduction = ninety
2 log reduction = 10^2 times less organisms = 99% reduction = one hundred
3 log reduction = 10^3 times less organisms = 99.9% reduction = one thousand
4 log reduction = 10^4 times less organisms = 99.99% reduction = ten hundred thousand
5 log reduction = 10^5 times less organisms = 99.999% reduction = one hundred thousand
6 log reduction = 10^6 times less organisms = 99.9999% reduction = one million
7 log reduction = 10^7 times less organisms = 99.99999% reduction = ten million
etc...

8. Antibacterial Activity

Antibacterial Activity	%Kill compared to control	Comment
<1.5	**<96.8**	**poor**
1.5 to 2.0	**96.8-99.0**	**borderline**
2.0 to 3.0	**99.0-99.9**	**good**
>3.0	**>99.9**	**excellent**

9. Conclusion

Typically, when a product performs with > 3-logs (99.9%) or greater it is deemed bacteriostatic by FDA standards. Antimicrobial hand sanitizer formulated with BIO-PROTECT exhibited excellent antimicrobial performance > log 6 (99.9999) reduction against *Staphylococcus aureus* (S.*aureus*) a common skin inhabitant in ISO large droplet inoculation method (50 uL) on VITRO-SKIN as a leave on surface sanitizing antiseptic. Long lasting residual antimicrobial activity was demonstrated up to 6 hours after product application which is expected to last for 24 hours with regular use and abuse conditions.