Test Report

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TEST FACILITY

STC (Dongguan)

68 Fumin Nan Road, Dalang,

Dongguan, Guangdong,
China. (Zip code 523770)

SPONSOR

Evan Chen
STILO OPTICAL TECHNOLOGY
Yonglong industry park, Fumin Ind. Zone,
Tanglivillage, Fenggang Town,
Dongguan City, Guangdong Province,
China.

CONFIDENTIAL

STUDY TITLE

MTT Cytotoxicity Test of FRAME using ISO 10993-5:2009 Test Methods MTT Cytotoxicity Test, Minimal Essential Medium with 10% Fetal Bovine Serum Extract

TEST ARTICLE NAME

FRAME

TEST ARTICLE IDENTIFICATION

CP-MD-1197

CSD NO.:CL20190605262

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Summary

The test article, FRAME, was evaluated for potential cytotoxic effects. This study was conducted following the guidelines of ISO 10993-5, Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (2009). A single preparation of the test articles was extracted in single strength Minimum Essential Medium at 37°C for 24 hours. A negative control, reagent control, and positive control were similarly extracted. Following extraction, the test article and positive extract were diluted to obtain solutions of approximate concentrations of 100%, 50%, 25% and 12.5%. Triplicate monolayers of L-929 mouse fibroblast cells were dosed with the full strength and diluted extracts and incubated at 37°C (humidified) in the presence of 5% CO_2 for 24 hours. Following incubation, the culture medium was replaced with a 1 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution and incubated for an additional 2 hours. Following this step, the MTT solution was replaced with isopropanol. The percent viability for the test article was determined from the reagent control. A decrease in the number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm.

The MEM extract of test showed no cytotoxic potential to L-929 mouse fibroblast cells. The test article extract met the requirements of the test since the viability was more than 70%.

Authorized Signatory Approval: Tang

Jonathan Tang

1. Introduction

1.1 Purpose

The purpose of this study was to determine the potential of a test article to cause cytotoxicity.

1.2 Testing Guidelines

This study was based on the requirements of the ISO 10993-5, Biological evaluation of medical device – Part 5: Tests for in vitro cytotoxicity (2009).

1.3 Dates

Test Article Received:	2019.06.28
Cells Dosed:	2019.07.10
Observations Concluded:	2019.07.12

2. Identification of Test and Control Articles

The test article provided by the sponsor was identified and handled as described below:

Table 1: Test Article

Name:	FRAME
Size:	N.A
CAS Code:	N.A
Model:	TRUEGRASSES
Lot:	N.A
Initial State:	Not Sterilized
Strength, Purity and Composition:	N.A
Color:	N.A
Physical Description of the Test Article:	Solid
Manufacture date:	N.A
Expiration Date:	N.A

Table 2: Negative Control Article

Name:	High Density Polyethylene
Lot:	C-161
Source:	Hatano Research Institute, Food and Drug Safety Center
Component:	Material: High Density Polyethylene Film

Table 3: Positive Control Article

Name:	ZDEC
Lot:	A-161K
Source: Component:	Hatano Research Institute, Food and Drug Safety Center 0.1% ZDEC Polyurethane Film
Name:	ZDBC
Lot:	B-172K
Source: Component:	Hatano Research Institute, Food and Drug Safety Center 0.25% ZDBC Polyurethane Film

Table 4: Ancillary Materials

Growth Media:	Single strength Minimum Essential Medium supplemented with 10% fetal bovine serum, 1% antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin)
Formulation:	44.5 mL MEM+ 5 mL FBS+0.5 mL antibiotics

Table 5: Extraction Vehicle

Name:	MEM

Table 6: Ragents

Name	Brand	Lot
MEM	GiBco	2003798
MTT	MYM	YK20180215
FBS	GiBco	42Q3382K
Penicillin, Streptomycin	GiBco	2068818

3. Test System

3.1 Test System and Justification of Test System

Mammalian cell culture monolayer consisting of L-929 mouse fibroblast cells (ATCC Number: CCL-1, Lot Number: 70001022) was used. In vitro mammalian cell culture studies have been used historically to evaluate cytotoxicity of biomaterials and medical devices.

3.2 Test System Management

L-929 mouse fibroblast cells were propagated and maintained in flasks containing IX MEM at 37°C with 5% carbon dioxide (CO_2). For this study, a 96-well plate was seeded with 1 x 10^4 cells/ well and incubated at 37°C (humidified) with 5% CO_2 to obtain semi-confluent monolayers of cells prior to use. Aseptic procedures were used in the handling of the cell cultures following approved STC Standard Operating Procedures.

4. Method

4.1 Test and Control Article Preparation

The test articles were measured and calculated. The preparations of the test article and the negative control were subjected to the extraction conditions as described below. The extracts were continuously agitated during extraction. The MEM extraction method was conducted in the presence of serum to optimize extraction of both polar and non-polar components.

Table 7: Extraction

Article	Extraction Ratio	Article Amount	Volume of Vehicle	Extraction Condition
Test Article	0.2 g:1 mL	5.2g	26mL	37±1°C for 24±2 hours
Negative Control	3 cm^2:1 mL	18 cm^2	6 mL	37±1°C for 24±2 hours
Positive Control (ZDEC)	6cm^2:1 mL	36 cm^2	6 mL	37±1°C for 24±2 hours
Positive Control (ZDBC)	6 cm^2:1 mL	36 cm^2	6 mL	37±1°C for 24±2 hours
Reagent Control	Not Applicable	Not Applicable	10mL	37±1°C for 24±2 hours

The following table contains a description of the test and control article extracts before and after extraction.

Table 8: Condition of Extracts

Vehicle	Time Observed	Extract	Condition of Extracts		
			Color	Clarity	Particulates
MEM	Before	Test Article	Pink	Clear	None
		Negative Control	Pink	Clear	None
		Positive Control (ZDEC)	Pink	Clear	None
		Positive Control (ZDBC)	Pink	Clear	None
		Reagent Control	Pink	Clear	None
	After	Test Article	Pink	Clear	None
		Negative Control	Pink	Clear	None
		Positive Control (ZDEC)	Pink	Clear	None
		Positive Control (ZDBC)	Pink	Clear	None
		Reagent Control	Pink	Clear	None

There appeared to be no visible changes to the test article during the extraction process. The extracts were tested immediately following extraction. The extracts were not centrifuged, filtered, or otherwise altered prior to dosing.

4.2 Test Procedure

Culture wells were selected which contained a semi-confluent cell monolayer. The growth medium contained in columns 2 and 11 of the 96-well plate was replaced with 100 µL of the reagent control. The growth medium in triplicate cultures was replaced with 100µL of the test extract at the following approximate dilutions: 100% (full strength), 50%, 25% and 12.5%. Similarly, triplicate cultures were replaced with 100 µL of the negative control and positive control extract at the following

approximate dilution: 100% (full strength) 50%, 25% and 12.5%. The wells were incubated at 37°C (humidified) in 5% CO_2 for 24 hours.

Following incubation, the cultures were examined under a phase contrast microscope to identify any systemic cell seeding errors, growth characteristics and changes in cell morphology. No determination of cytotoxicity was made from this examination.

The culture medium was replaced with 50 µL of a 1 mg/mL MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution and incubated at 37°C (humidified) in 5%CO_2 for 2hours.

The MTT solution was replaced with 100 µL of isopropanol. The optical density was measured at 570 nm (reference 650nm).

All times and temperatures reported here in are approximate and are within ranges established by the external standards described in the References section of this report and/or STC standard operating procedures.

5. Evaluation and Statistical Analysis

The MTT Cytotoxicity Study is a colorimetric cytotoxicity test that quantitatively measures cell viability and proliferation following exposure to the test extract or solution. Tetrazolium salts are used to examine cell proliferation. Metabolically active cells reduce yellow-colored tetrazolium MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble purple-colored formazan crystals, due to the action of NADPH-oxidoreductase enzymes. The yellow-to-purple color change can be quantified by spectrophotometric analysis. Absorbance values that are lower than the control cells indicate a reduction in cell viability, whereas a higher absorbance indicates an increase in cell viability.

A decrease in the number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. The percent viability is compared to the reagent control by using the following formula:

$$\text{Percent Viability} = \frac{100 \times OD570_e}{OD570_{rc}}$$

$OD570_e$ is the blank corrected mean value of the measured optical density of the test or control article extract.
$OD570_{rc}$ is the blank corrected mean value of the measured optical density of the reagent control.

For the test to be valid, the average reagent control OD must be ≥0.2. The average of the left reagent controls (column2) and average of right reagent controls (column11) must not differ by more than 15%. Examination by phase contrast microscope obviates the need for the average percent difference requirement if no systemic cell seeding errors were observed. The percent viability of the negative control extract must be ≥70% of the reagent control. The percent viability of a minimum of one dilution from each of the positive control extracts must be <70% of the reagent control (indicating a potential cytotoxic response). For test article extracts with potential cytotoxic responses (percent viability <70% of the reagent control), the 50% test article extract should have at least a similar or higher percent viability as the 100% test extract solution. Any discrepancies should be reviewed by scientific personnel.

The lower the percent viability value, the higher the cytotoxic potential of the test article. If viability is reduced to <70% of the reagent control extract, a cytotoxic potential exists.

6. Results

All system suitability criteria were met, indicating a valid test assay.

Table 9: Individual Test Data

Material	Percent Viability of Control Article	System Suitability
Positive Control (ZDEC, 100%, full strength)	1.40%	Met Criteria
Positive Control (ZDEC, 50%)	3.13%	
Positive Control (ZDEC, 25%)	87.97%	
Positive Control (ZDEC, 12.5%)	95.15%	
Positive Control (ZDBC, 100%, full strength)	1.38%	Met Criteria
Positive Control (ZDBC, 50%)	96.58%	
Positive Control (ZDBC, 25%)	100.00%	
Positive Control (ZDBC, 12.5%)	91.72%	
Negative Control (100%, full strength)	100.00%	Met Criteria

Material	Percent Viability of Test Article	Cytotoxic Potential
Test Article (100%, full strength)	94.87%	No Cytotoxic Potential
Test Article (50%)	96.34%	No Cytotoxic Potential
Test Article (25%)	100.00%	No Cytotoxic Potential
Test Article (12.5%)	98.25%	No Cytotoxic Potential

7. Conclusion

The MEM extract of test showed no cytotoxic potential to L-929 mouse fibroblast cells. The test article extract met the requirements of the test since the viability of test article (100%, full strength) was more than 70%.

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other articles is the sponsor's responsibility.

8. Records

All raw data pertaining to this study and a copy of the final report are retained in designated STC archive files in accordance with STC SOPs.

9. ISO Compliance

All procedures were compliance to ISO 17025.

10. References

International Organization for Standardization (ISO) 10993-1, Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process (2018).

International Organization for Standardization (ISO) 10993-5, Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (2009).

International Organization for Standardization (ISO) 10993-12, Biological evaluation of medical devices - Part 12: Sample preparation and reference materials (2012).

International Organization for Standardization (ISO) 17025, General requirements for the competence of testing and calibration laboratories (2017).

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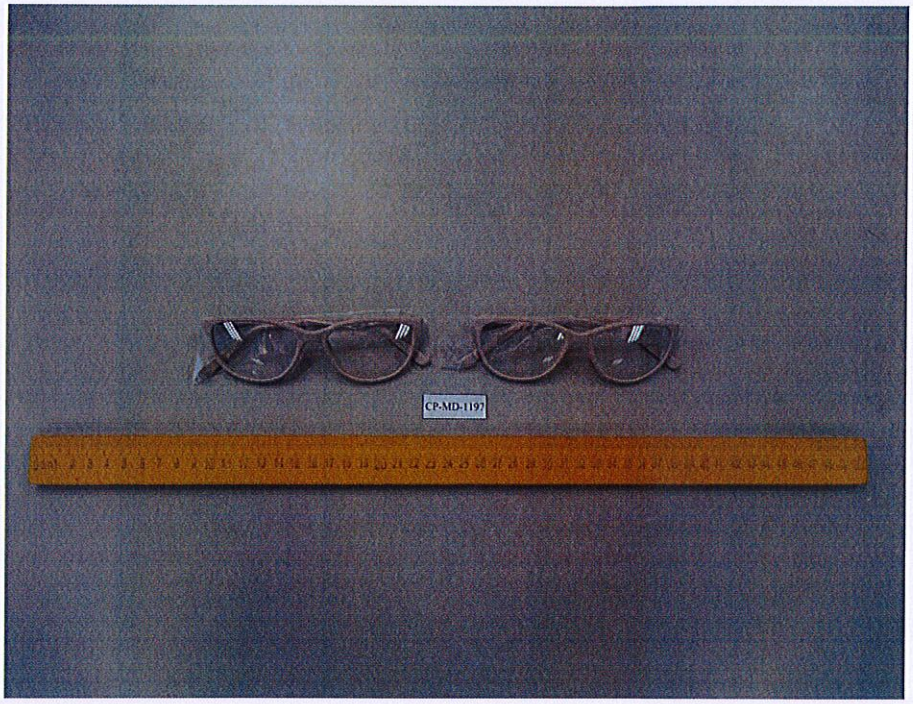
Appendix 1 – Photograph(s) of Test Articles



***** END OF TEST REPORT *****

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