NAMSA®

Final GLP Report

TEST FACILITY

NAMSA
115 Chemin de L'Islon
38670 Chasse-sur-Rhône
France
Tel : +33 4 78 07 92 34
Fax : +33 4 72 24 08 12

SPONSOR

PlayMoovin'
Sébastien Passemard
465 rue Marie Marvingt
63118 Cébazat
France

CONFIDENTIAL

STUDY TITLE

ISO MTS cytotoxicity test

TEST ARTICLE NAME AND IDENTIFICATION

Coque KidsMoovin
reference: Polyethylene MicroFlex RM 1260 ROUGE
Batch: Manufacturing date: 12/2020

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Summary

This *in vitro* study was conducted to evaluate Coque KidsMoovin for potential cytotoxic effects following the guidelines of ISO 10993-5, Biological Evaluation of Medical Devices, Part 5: Tests for *In Vitro* Cytotoxicity.

A single preparation of the test article was extracted in single strength Eagle Minimum Essential Medium (EMEM10) at 37 ± 1°C for 72 ± 2 hours. A negative control, control blank and a positive control were similarly prepared. Following extraction, triplicate monolayers of L-929 mouse fibroblast cells were dosed with the full strength extracts (100%) and incubated at 37 ± 1°C (humidified) in presence of 5 ± 1% CO_2 for 24-26 hours. Following incubation, 20 µL of the MTS-PMS solution, prepared just before use, were dispensed in each well and incubated during 120-135 minutes at 37 ± 1°C (humidified) in 5 ± 1% CO_2. The percent viability for the test article was determined from the control blank. 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 brown formazan formed, as monitored by the optical density at 492 nm. If the cell viability is reduced to less than 70% of the control blank, a cytotoxic potential exists.

The full strength EMEM10 test article extract showed no cytotoxic potential to L-929 mouse fibroblast cells.

Supervisory Personnel:

Damien Briotet
Manager, Biocompatibility

Study Director Approval:

Laurence François
Study Director

Date February 26, 2021

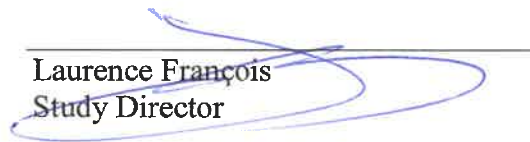
Authorization for duplication of this report, except in whole, is reserved pending NAMSA's written approval.

GLP Compliance

This non clinical study was conducted in accordance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98)17, and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58.
There were no deviations from the protocol, standard operating procedures or the Good Laboratory Practice regulations that were judged to have any significant impact on the validity or interpretation of the data.

All laboratory data have been accurately recorded and verified, as indicated by the signature below:

Study Director:



Laurence François
Study Director

Date

1. Introduction

1.1. Purpose

The purpose of this GLP study was to evaluate the potential cytotoxic effect of a test article extract using an *in vitro* mammalian cell culture.

1.2. Testing Guidelines

This study was conducted according to the ISO 10993-5, Biological evaluation of medical devices, Part 5 (2009): Tests for *In Vitro* cytotoxicity.

1.3. Dates

Test Article Received:	February 01, 2021
Signature of the Protocol:	February 04, 2021
Treatment Started:	February 11, 2021
Observations Concluded:	February 12, 2021

2. Materials

2.1. Test Article

The test article provided by the Sponsor was identified and handled as described below and according to the Protocol No.: 289945:

Table 1: Test Article

Name*	Coque KidsMoovin
Identification*	Reference: Polyethylene MicroFlex RM 1260 ROUGE, batch: Manufacturing date: 12/2020
Intended Clinical Use	Not communicated
Physical Description	Not communicated Weight: 45 g
Surface	100 cm^2 (1 side)
Sterile	No
Sterilization Process	Not applicable
Expiration Date	Not communicated
Storage Conditions	Room temperature (+ 15°C / + 25°C)
Composition	The test article is composed of the following materials: PEHD
Purity	Purity is not applicable because no active ingredients are used.
Stability Before Opening Package	Stability testing is in progress and Sponsor affirms that the test article is stable for the duration of intended testing.
Stability After Opening Package	10 YEARS
Homogeneity	Homogeneity is not applicable (because no sampling is performed before testing or the test article is a solid).
Strength	Strength is not applicable because no active ingredients are used to formulate a concentration.
Disposal	Any remaining test article will be destroyed 1 month after the end of the study.
Quantity of Test Article Used	1 unit

* : No label on the sample; identification was however confirmed by the Sponsor.

Test Article Sampling: The sampling was performed under the responsibility of the Sponsor. The test article was tested as indicated in Section 4.

2.2. Controls

The following articles were supplied by NAMSA to serve as controls.

Table 2: Negative Control

Name	High density polyethylene sheet
Stability Testing	Marketed product; stability characterized by its labeling
Strength, Purity, Composition or Other Characteristics	SRM C: Strength: Extraction rate: 6 cm^2/mL (RM/Medium); Purity: Hatano Research Institute, Food and Drug Safety Center Grade; Composition: High Density Polyethylene

Table 3: Control Blank/Extraction Vehicle

Name	Single strength Eagle Minimum Essential Medium (EMEM1X) supplemented with 10% foetal bovine serum (v/v), 1% (v/v) L-glutamine (> 2mM) and antibiotics (2% (v/v) Penicillin (100 units/mL) – Streptomycin (> 100 µg/mL) and 1% (v/v) Amphotericin B (2.5-3 µg/mL))
Stability Testing	Stable for the duration of the study
Strength, Purity, Composition or Other Characteristics	Strength: Not Applicable, no active components in the formulation; Purity: Not Applicable, multi-component article, Composition: 86% EMEM1X, 10% fetal bovine serum, antibiotics (2% (v/v) penicillin (100 units/mL)-streptomycin (> 100 µg/mL)) and 1% (v/v) amphotericin B (2.5-3 µg/mL), and 1% (v/v) L glutamine (> 2 mM)

Table 4: Positive Control

Name	SRM-A, segmented polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC) (Hatano Research Institute, Food and Drug Safety Center)
Stability Testing	Marketed products, stability characterized by labeling
Strength, Purity, Composition or Other Characteristics	SRM-A: Strength: 0.1% ZDEC; Extraction rate: 6 cm^2/mL; Composition: Polyurethane Film containing Zinc Diethyldithiocarbamate

2.3. Additional Reagents

Calcium and Magnesium free Dulbecco's Phosphate Buffer Saline (CMF-DPBS) 10X: Sigma, reference D1408 (see section 7)
Sterile water for technical use: Laboratoire Aguettant, reference 600499
Eagle Minimum Essential Medium 1X (EMEM1X): Sigma, reference M2279
Foetal Bovine Serum: Sigma, reference F7524
L-Glutamine: Sigma, reference G7513
Penicillin-streptomycin: Sigma, reference P4458
Amphotericin B: Sigma, reference A2942
Trypsin-EDTA solution: Sigma, reference T3924
Cell Titer 96® AQueous Non-Radioactive Cell proliferation Assay, kit including MTS and PMS: Promega, reference G5430

These reagents were provided by NAMSA.

3. Test System

3.1. Test System and Justification of Test System

Mammalian cell culture monolayer consisting of L-929 mouse fibroblast cells (ECACC, L929, subclone of parental strain L, reference 85011425) was used. *In vitro* mammalian cell culture studies have been used historically to evaluate cytotoxicity of biomaterials and medical device.

3.2. Test System Management

Aliquot of L-929 mouse fibroblast cells was stored in nitrogen liquid. Then one aliquot was thawed at 37 ± 1°C in a water bath for about one minute. This aliquot was used for 20 passages. The cells were propagated and maintained in vented flasks with EMEM10 at 37 ± 1°C (humidified) with 5 ± 1% carbon dioxide (CO_2). The cells were harvested with trypsin-EDTA solution and counted. For this study, wells of a 96-well plate were seeded with 1 x 10^4 cells/well and incubated at 37 ± 1°C (humidified) with 5 ± 1% CO_2 during 24 ± 2 hours to obtain semi-confluent monolayers of cells prior to use. Aseptic procedures were used in the handling of the cell cultures following approved NAMSA Standard Operating Procedures.

The cell cultures used are regularly checked for mycoplasma contamination.

4. Method

4.1. Test Article Preparation

The test article provided by the Sponsor was handled as described below:

The entire test article was included in the preparation.
The test article was not cut into pieces before extraction.

The test article was subjected to the extraction conditions as described below:

Table 5: Extraction

Vehicle	Extraction Ratio	Article Amount	Volume of Vehicle	Extraction Conditions
EMEM 10	3 cm^2/ml - thickness >= 0.5 mm	200 cm2 (2 sides)	66.7 mL	37°C +/- 1°C for 72 hours +/- 2 hours

The extract was continuously agitated during extraction.

The extract was not centrifuged, decanted, filtered, or otherwise manipulated prior to testing. Following extraction, the extract was immediately used for testing.
It was shaken immediately before use to ensure homogeneity of the extract. The color of the culture medium was observed to determine any change in pH.

4.2. Controls Preparation

4.2.1. Negative Control

The negative control was prepared based on a ratio of 6 cm^2/mL. A single preparation of the material was made and extracted at 37 ± 1°C for 72 ± 2 hours. The negative control was tested at 100%.

4.2.2. Control Blank

The extraction vehicle was prepared in the same way and at the same time as the test article extract but without the test article and served as control blank.

4.2.3. Positive Control

The positive control was prepared based on a ratio of 6 cm^2/mL. A single preparation of the material was made and extracted using the same conditions as described for the test article. The positive control was used at 100%.

4.3. 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 control blank. The growth medium in triplicate cultures was replaced with 100 µL of the test article extract (100%). Similarly, triplicate cultures were replaced with 100 µL of negative and positive controls extracts. Each well was labelled indicating its content and replicate number. Culture Medium (CM) without cells was also placed in triplicate in Column 1 of the 96-well plates. The plates were incubated at 37 ± 1°C (humidified) in 5 ± 1% CO_2 for 24-26 hours.

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

Then, 20 µL of the MTS-PMS solution, prepared just before use, were dispensed in each well and incubated at 37 ± 1°C (humidified) in 5 ± 1% CO_2 for 120-135 minutes. Following incubation, the optical density (OD) was measured at 492 nm (Microplate reader: Tecan, Sunrise, Magellan Standard Version 6.6 software).

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

5. Evaluation

This MTS Cytotoxicity Study is a colorimetric cytotoxicity test using the Celltiter® AQ_{ueous} Non-Radioactive Cell proliferation Assay kit, that quantitatively measures cell viability and proliferation following exposure to the test extract.
This kit is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent: phenazine methosulfate (PMS). MTS (light yellow) is bioreduced by cells into a formazan product (brown) that is soluble in tissue culture medium.

The absorbance of the formazan (brown) at 492 nm can be measured directly from the 96-well assay plates. According to the supplier instructions, 100 µL of PMS solution was added to 2 mL of MTS solution. This mixture was prepared immediately before addition to the plates containing the cells.
The yellow-to-brown 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 brown formazan formed, as monitored by the optical density at 492 nm. The percent viability is compared to the control blank by using the following formula:

$$\text{Percent Viability} = \frac{100 \times ODc_{492e}}{ODc_{492cb}}$$

ODc (corrected OD) = $OD_{\text{test article/ negative control/ positive control/ control blank}} - OD_{CM}$

ODc_{492e} is the mean value of the measured optical density of the test article or negative control or positive control extract.

ODc_{492cb} is the mean value of the measured optical density of the control blank.

For the test to be valid, the average control blank OD must be > 0.3. The average of the left control blanks (column 2) and average of right control blanks (column 11) must not differ from the average of all the control blanks by more than 15%. The percent viability of the negative control extract must be ≥ 70% of the control blank extract. The percent viability of the positive control extract must be < 70% of the control blank extract (indicating a potential cytotoxic response).
In order to verify the interference of the extract with the coloration method, the extract without cell was controlled.

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 control blank extract, a cytotoxic potential exists.

No statistical analysis was conducted.

6. Results

6.1. Condition of Extracts

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

Table 6: Condition of Extracts

Vehicle	Extract	Condition of Extracts
EMEM 10	Test Article	The condition of the extract before extraction was clear and pink with no visible particulates present. The condition of the extract after extraction was clear and pink with plastic-like particulates present. After extraction, no physical changes of the test article were observed.
	Control Blank	The condition of the control blank before and after extraction was clear and pink with no visible particulates present.

6.2. Results

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

Table 7: Individual Test Data

Material	Percent Viability of Control Articles	System Suitability
Negative Control (100%)	92.1%	Met criteria
Positive Control (100%)	3.6%	Met criteria

Material	Percent Viability of Test Article	Cytotoxic Potential
Test Article (100%)	93.6%	No Cytotoxic Potential

7. Deviations

Due to a reagent rupture, for cell culture, Calcium and Magnesium free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) 10X (reference D1408) was diluted in sterile water in order to obtain a 1X solution instead of Calcium and Magnesium free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) 1X (reference D8537). As the final 1X solution used had the same characteristics as the reference D8537, this deviation was not considered to have affected the quality of the results.
No other incident that could have affected the quality of the raw data obtained was observed.

8. Conclusion

The full strength EMEM10 test article extract showed no cytotoxic potential to L-929 mouse fibroblast cells.

Results and conclusions apply only to the test article tested. No further evaluation of these results has been made by NAMSA. Any extrapolation of these data to other articles is the Sponsor's responsibility.

9. Quality Assurance

Inspections were conducted at adequate intervals to assure the integrity of the study, and the final report was reviewed for conformance with the OECD Good Laboratory Practice, ENV/MC/CHEM (98) 17 and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58 subparts B 35 (b) and J 185 (a). A statement of Quality Assurance activities is provided with the final report.

10. Records

All study file data including raw data, protocols, reports and ancillary documents submitted by the Sponsor at the time the order was placed will be archived at NAMSA for 5 years from the date of mailing of the final report.

After the 5 year period, these items will be subject to destruction unless specific and written instructions to return them to the Sponsor will be provided to NAMSA. Client confidentiality will be protected at all times.

The test article (and control if any: see section « materials ») will not be retained at NAMSA. As this study is a short term study, the retention of the test article of each batch for analytical purposes is not required.

11. References

OECD series on Good Laboratory Practice and compliance monitoring, number 1, ENV/MC/CHEM (98) 17, on January 26, 1998 for non clinical safety testing on pharmaceuticals, pesticides, food, and feed additives, cosmetic products, veterinary drug products and similar products and industrials chemicals adopted by Council decision on November 26, 1997, Directive C (97) 186 final.

21 CFR 58 (current version) Food and drugs - United States FDA Department of health and human services. Good Laboratory Practice for non clinical laboratory studies.

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).

Statement of Quality Assurance Activities

Phase Inspected	Date Inspected	Date Reported to Study Director	Date Reported to Management
Protocol	February 01, 2021	February 01, 2021	February 02, 2021
Cells Seeding [(1)]	December 15, 2020	December 22, 2020	December 22, 2020
Raw Data and Final Report	February 23, 2021	February 23, 2021	February 26, 2021

(1) Taking into account the routine nature of short-term studies, each study is not individually audited, except when there is a protocol change. The different experimental phases of these studies are audited every four months; this allows Quality Assurance adequate control of such studies. A report of these audits is provided to the Study Director and the Test Facility Manager.

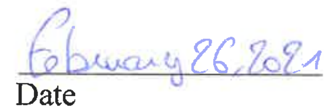
NAMSA Quality Assurance program includes process and facility inspections at regular intervals.

Based on a review of this study, it has been concluded that this report accurately describes the methods and standard operating procedures, and the reported results accurately reflects the raw data of the study.

The study was reviewed in accordance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98) 17 and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58.

Quality Assurance Auditor:

J. BOURG



Date

Protocol

See attached:

- **Protocol (10 pages)**

NAMSA

GLP Protocol

TEST FACILITY

NAMSA
115 Chemin de L'Islon
38670 Chasse-sur-Rhône
France
Tel : +33 4 78 07 92 34
Fax : +33 4 72 24 08 12

SPONSOR

PlayMoovin'
Sébastien Passemard
465 rue Marie Marvingt
63118 Cébazat
France

STUDY TITLE

ISO MTS cytotoxicity test

TEST ARTICLE NAME AND IDENTIFICATION

Coque KidsMoovin,
reference: Polyethylene MicroFlex RM 1260

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Approvals

Sponsor Representative (PlayMoovin')	Sébastien Passemar
Date approved:	2/02/21
Study Director (NAMSA):	Laurence François Study Director
Date initiated:	February 04, 2021
Test Facility Management (NAMSA):	Damien Briotet
Date approved:	02 Fev 2021

1. Introduction

1.1. Purpose

The purpose of this non clinical GLP study is to evaluate the potential cytotoxic effect of a test article extract using an in vitro mammalian cell culture test.

1.2. Testing Guidelines

This study will be conducted according to the ISO 10993-5, Biological evaluation of medical devices, Part 5 (2009): Tests for *In Vitro* cytotoxicity.

1.3. GLP Compliance

This non clinical study will be conducted in accordance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98) 17 and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58.

2. Materials

2.1. Test Article

The Sponsor will submit the test article to be evaluated. The Sponsor provided detailed information about the test article to NAMSA on the "Sample submission form".

Table 1: Test Article

Article Name	Coque KidsMoovin
Article Identification	Reference: Polyethylene MicroFlex RM 1260, batch: not communicated*

*The batch(es) used will be reported in the final report.

Disposal of Test Article: Any remaining test article will be destroyed 1 month after the end of the study.

2.2. Controls

The following articles will be supplied by NAMSA to serve as controls.

Table 2: Negative Control

Name	High density polyethylene sheet
Stability Testing	Marketed product; stability characterized by its labeling
Strength, Purity, Composition or Other Characteristics	SRM C: Strength: Extraction rate: 6 cm²/mL (RM/Medium); Purity: Hatano Research Institute, Food and Drug Safety Center Grade; Composition: High Density Polyethylene

Table 3: Control Blank/Extraction Vehicle

Name	Single strength Eagle Minimum Essential Medium (EMEM1X) supplemented with 10% foetal bovine serum (v/v), 1% (v/v) L-glutamine (> 2mM) and antibiotics (2% (v/v) Penicillin (100 units/mL) – Streptomycin (> 100 µg/mL) and 1% (v/v) Amphotericin B (2.5-3 µg/mL))
Stability Testing	Stable for the duration of the study
Strength, Purity, Composition or Other Characteristics	Strength: Not Applicable, no active components in the formulation; Purity: Not Applicable, multi-component article, Composition: 86% EMEM1X, 10% fetal bovine serum, antibiotics (2% (v/v) penicillin (100 units/mL)-streptomycin (> 100 µg/mL)) and 1% (v/v) amphotericin B (2.5-3 µg/mL), and 1% (v/v) L glutamine (> 2 mM)

Table 4: Positive Control

Name	SRM-A, segmented polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC) (Hatano Research Institute, Food and Drug Safety Center)
Stability Testing	Marketed products, stability characterized by labeling
Strength, Purity, Composition or Other Characteristics	SRM-A: Strength: 0.1% ZDEC; Extraction rate: 6 cm²/mL; Composition: Polyurethane Film containing Zinc Diethyldithiocarbamate

2.3. Additional Reagents

DPBS with Ca^{2+} and Mg^{2+}: Sigma, reference D8662
DPBS without Ca^{2+} and Mg^{2+}: Sigma, reference D8537
Eagle Minimum Essential Medium 1X (EMEM1X): Sigma, reference M2279
Foetal Bovine Serum: Sigma, reference F7524
L-Glutamine: Sigma, reference G7513
Penicillin-streptomycin: Sigma, reference P4458
Amphotericin B: Sigma, reference A2942
Trypsin-EDTA solution: Sigma, reference T3924
Cell Titer 96® AQueous Non-Radioactive Cell proliferation Assay, kit including MTS and PMS: Promega, reference G5430

These reagents will be provided by NAMSA.

3. Test System

3.1. Test System and Justification

Mammalian cell culture monolayer consisting of L-929 mouse fibroblast cells (ECACC, L929, subclone of parental strain L, reference 85011425) will be used. *In vitro* mammalian cell culture studies have been used historically to evaluate cytotoxicity of biomaterials and medical devices. The cell cultures used are regularly checked for mycoplasma contamination.

3.2. Test System Management

Aliquot of L-929 mouse fibroblast cells are stored in nitrogen liquid. Then one aliquot will be thawed at 37 ± 1°C in a water bath for about one minute. This aliquot will be used for 20 passages. The cells will be propagated and maintained in

vented flasks containing EMEM10 at 37 ± 1°C (humidified) with 5 ± 1% carbon dioxide (CO_2). The cells will be harvested with trypsin-EDTA solution and counted. For this study, wells of a 96-well plate will be seeded with 1×10^4 cells/well, labelled with passage number and date, and incubated at 37 ± 1°C (humidified) in 5 ± 1% CO_2 during 24 ± 2 hours to obtain semi-confluent monolayers of cells prior to use. Aseptic procedures will be used in the handling of the cell cultures following approved NAMSA Standard Operating Procedures.

4. Method

4.1. Test Article Preparation

The following information was completed based on the Sponsor providing the information to NAMSA.

The test article will be prepared as described below:

The entire test article will be included in the preparation.

The test article could be cut if necessary.

The extraction conditions selected by the Sponsor will be as described below:

Table 5: Extraction

Vehicle	Extraction Ratio	Extraction Conditions
EMEM 10	3 cm²/ml - thickness >= 0.5 mm*	37°C +/- 1°C for 72 hours +/- 2 hours

* If needed, the ratio could be adapted by the Study Director in order to ensure an optimal extraction of the test article.

The extract will be continuously agitated during extraction.

Following extraction, the extract will be remained at room temperature (15°C to 25°C) and will be used for testing within 24 hours of completion of extraction. It will be shaken immediately before use to ensure homogeneity of the extract.

The color of the culture medium will be observed to determine any change in pH. A color shift toward yellow will indicate an acidic pH range and a color shift toward magenta to purple will indicate an alkaline pH range. If necessary, the pH and the osmolality of the extract will be determined before use.

Physical change(s) in the test article and extract will be assessed visually (i.e., vehicle only, test article in vehicle at time zero, and test article in vehicle at post-extraction) and documented. If a problem is encountered during extraction, the Sponsor will be advised.

4.2. Controls Preparation

4.2.1. Negative Control

The negative control will be prepared based on a ratio of 6 cm^2/mL. A single preparation of the material will be made and extracted at 37 ± 1°C for 72 ± 2 hours. The negative control will be tested at 100%.

4.2.2. Control Blank

The extraction vehicle will be prepared in the same way and at the same time as the test article extract but without the test article and will serve as control blank.

4.2.3. Positive Control

The positive control will be prepared based on a ratio of 6 cm^2/mL. A single preparation of the material will be made and extracted using the same conditions as described for the test article. The positive control will be used at 100%.

4.3. Extract Observation

A qualitative macroscopic observation (naked eyes) of the test and control extracts will be done before and after the extraction process. A description of the extract color, clarity and the presence or absence of particulates will be recorded.

4.4. Test Procedure

Each culture well will be selected which contains a semi-confluent cell monolayer. The growth medium in triplicate cultures will be replaced with 100 µL of the test article extract (100%). Similarly, triplicate cultures will be replaced with 100 µL of control blank, negative and positive controls extracts. Each well will be labeled indicating its content and replicate number. To check cell seeding errors, the control blank will be placed both on the right and the left sides of the 96-well plates (Column 2 and Column 11). Culture Medium (CM) without cells will be also placed in triplicate in Column 1 of the 96-well plates. The plates will be incubated at 37 ± 1°C (humidified) in 5 ± 1% CO_2 for 24-26 hours.

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

If necessary, in order to avoid any interference of the test article extract with the reading method, each well will be rinsed with 100µL of DPBS one or several times and then 100µL of fresh culture medium will be added to cells before coloration.

Then, 20 µL of the MTS-PMS solution, prepared just before use, will be dispensed in each well and incubated at 37 ± 1°C (humidified) in 5 ± 1% CO_2 for 120-135 minutes. Following incubation, the optical density (OD) will be measured at 492 nm (Microplate reader: Tecan, Sunrise, Magellan Standard Version 6.6 software).

5. Evaluation

The MTS Cytotoxicity Study is a colorimetric cytotoxicity test using the Celltiter® AQueous Non-Radioactive Cell proliferation Assay Kit, that quantitatively measures cell viability and proliferation following exposure to the test extract.
This kit is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent: phenazine methosulfate (PMS). MTS (light yellow) is bioreduced by cells into a formazan product (brown) that is soluble in tissue culture medium. The absorbance of the formazan (brown) at 492 nm can be measured directly from the 96-wells assay plates. According to the supplier instructions, 100 µL of PMS solution will be added to 2 mL of MTS solution. This mixture will be prepared immediately before addition to the plates containing the cells.
The yellow-to-brown 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 brown formazan formed, as monitored by the optical density at 492 nm. The percent viability will be compared to the control blank by using the following formula:

$$\text{Percent Viability} = \frac{100 \times ODc_{492e}}{ODc_{492cb}}$$

ODc (corrected OD) = $OD_{\text{test article/ negative control/ positive control/ control blank}} - OD_{CM}$
ODc_{492e} is the mean value of the measured optical density of the test article or negative control or positive control extract.
ODc_{492cb} is the mean value of the measured optical density of the control blank.

For the test to be valid, the average control blank OD must be > 0.3. The average of the left control blank (column 2) and average of right control blank (column 11) must not differ from the average of all the control blank by more than 15%. The percent viability of the negative control extract must be ≥ 70% of the control blank extract. The percent viability of the positive control extract must be < 70% of the control blank extract (indicating a potential cytotoxic response).
In order to verify the interference of the extract with the coloration method, the extract without cell will be controlled.

Any discrepancies will 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 control blank extract, a cytotoxic potential exists.

No statistical analysis will be conducted.

6. Report

A final report will be supplied in English to the Sponsor and will include the following:

- Test article identification and characterization.
- Description of the methods and test system used.
- Description of all circumstances that may have affected the quality and integrity of the data.
- Description of the data and transformations, calculations or operations performed on the data.
- An analysis and statement of the conclusions drawn from analysis.

7. Quality Assurance

Inspections will be conducted at adequate intervals to assure the integrity of the study, and the final report will be reviewed for conformance with the OECD Good Laboratory Practice, ENV/MC/CHEM (98) 17 and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58 subparts B 35 (b) and J 185 (a). A statement of Quality Assurance activities will be provided with the final report.

8. Proposed Dates

Receipt date of the test article: February 01, 2021
Study initiation date: date on which the Study Director signs the Protocol
Proposed experimental starting date: February, 2021
Proposed experimental completion date: February, 2021
The study starting date (first data collected) and the study completion date (final report release) will be defined by the Study Director once this Protocol is approved and once appropriate material for the study is received. These dates will then be provided to the Sponsor (or representative of the Sponsor) before the start of the experimentation.

9. Records

All study file data including raw data, protocols, reports and ancillary documents submitted by the Sponsor at the time the order was placed will be archived at NAMSA for 5 years from the date of mailing of the final report.

After the 5 year period, these items will be subject to destruction unless specific and written instructions to return them to the Sponsor will be provided to NAMSA. Client confidentiality will be protected at all times.

The test article (and control if any: see section « materials ») will not be retained at NAMSA. As this study is a short term study, the retention of the test article of each batch for analytical purposes is not required.

10. References

OECD series on Good Laboratory Practice and compliance monitoring, number 1, ENV/MC/CHEM (98) 17, on January 26, 1998 for non clinical safety testing on pharmaceuticals, pesticides, food, and feed additives, cosmetic products, veterinary drug products and similar products and industrials chemicals adopted by Council decision on November 26, 1997, Directive C (97) 186 final.

21 CFR 58 (current version) Food and drugs - United States FDA Department of health and human services. Good Laboratory Practice for non clinical laboratory studies.

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).

11. Protocol Changes

Any necessary changes to the protocol after Sponsor approval or study initiation will be documented and approved by the study director as protocol amendments. Copies will be distributed to the Sponsor, the raw data file and the NAMSA Quality Assurance Department. Any relevant deviation during the study will be recorded and noted in the final report.