Final Report

Study Title:	**ISO Agarose Overlay Using L-929 Mouse Fibroblast Cells (GLP)**
Sponsor:	**Klarity Medical Products, LLC** **1987 Coffman Road** **Newark, OH 43055** **US**
Sponsor Account Number:	**4003497**
Performing Laboratory:	WuXi AppTec 2540 Executive Drive St. Paul, MN 55120 US
Folder Number:	D00009282
Test Code:	140150.1
Report Number:	15792
Sample Number:	D00009282001
Test Article Name:	Klarity AccuCushion R550-M
Test Article Lot#:	50506Z

QUALITY ASSURANCE UNIT SUMMARY

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. This study has been performed under Good Laboratory Practice regulations (21 CFR part 58) and in accordance to standard operating procedures and a standard protocol. The Quality Assurance Unit maintains a copy of the study protocol and standard operating procedures and has inspected this study on the date(s) listed below. Each inspection was performed to assure the quality and integrity of the study.

Phase Inspected:	Date	Study Director	Management
Reading	06/17/15	06/17/15	06/20/15
Final Report	06/20/15	06/20/15	06/20/15

The findings of these inspections have been reported to management and the Study Director.

Quality Assurance Auditor: Michelle Abel Date: 06/23/15
Michelle Abel

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice (GLP) regulations set forth in 21 CFR Part 58.

The studies not performed by or under the direction of WuXi AppTec, are exempt from this Good Laboratory Practice Statement and include characterization and stability of the test compound(s)/test article.

Study Director: Date: 6/23/15
Jacob Hartman

Professional personnel involved in study:

Teri Tanquist, BS	Vice President of Operations
Roxanne Miller, AA, CVT	Sr. Director of Operations
Sarah Steinmetz, BA	Director of Study Operations
Jean Kringstad, BS	Director of *In Vitro* Operations
Jacob Hartman, BS	Associate Study Director

PURPOSE

The purpose of this assay was to evaluate *in vitro* toxicity of the test article to mammalian cells when leachable extracts were allowed to diffuse through an agarose barrier and contact cultured cells. L-929 mouse fibroblast cells were employed for this assay by a method compliant with the requirements specified in ISO 10993-5:2009.

TEST FACILITY: WuXi AppTec
2540 Executive Drive
St. Paul, MN 55120

DATE SAMPLES RECEIVED: 06/08/15
INITIATION DATE: 06/10/15
COMPLETION DATE: 06/23/15

TEST ARTICLE IDENTIFICATION

Test Article Name:	Klarity AccuCushion R550-M
Lot #:	50506Z
Sterilization Method:	Non-Sterile
Physical State:	Insoluble
Expiration Date:	5/6/18
Storage Conditions:	Room Temperature
Intended Use/Application:	Patient stabilization for Radiation therapy treatments
Physical Description:	According to the Sponsor, the test article consisted of moldable pillow with nylon fabric surface.

CHARACTERIZATION

The Sponsor was responsible for all test article characterization data as specified in the GLP regulations. The identity, strength, stability, purity, and chemical composition of the test article were solely the responsibility of the Sponsor. The Sponsor was responsible for supplying to the testing laboratory results of these determinations and any others that may have directly impacted the testing performed by the testing laboratory, prior to initiation of testing.

Furthermore, it was the responsibility of the Sponsor to ensure that the test article submitted for testing was representative of the final product that was subjected to materials characterization. Any special requirements for handling or storage were arranged in advance of receipt and the test article was received in good condition.

SAMPLE STORAGE

Upon receipt by the Sample Receiving Department, the test samples were placed in a designated, controlled access storage area ensuring proper temperature conditions. Test and control article storage areas are designed to preclude the possibility of mix-ups, contamination, deterioration or damage. The samples remained in the storage area until retrieved by a technician for sample preparation and/or testing.

SAFETY

Appropriate routine safety procedures were followed in handling the test article, unless more cautious procedures were specified by the Sponsor. All applicable WuXi AppTec safety policies and procedures were observed during the performance of the test.

EXPERIMENTAL DESIGN

Experimental Summary

The agarose overlay assay evaluated qualitatively the *in vitro* toxicity of solids, liquids or powders to L-929 mouse fibroblast cells when leachable extracts were allowed to diffuse through an agarose barrier and contact cultured cells.

On the day of testing, equal amounts of 2X E-MEM + 10% FBS (pH of 7.2 – 7.4) and molten 2% agarose were combined to achieve a final concentration of 1.0% agarose in 1X E-MEM + 5% FBS. The maintenance media was carefully removed from each monolayer and replaced with 3.5 mL of the E-MEM / agarose mixture. Once the agar solidified, the controls and test articles were placed on the hardened surface for testing. Each treatment was performed in triplicate. One cell control plate was run concurrently.

The plates were incubated at 37 ± 1 °C in 5 ± 1% CO_2 for 24-25 hours. After the incubation period, the test article was outlined on the culture dish with indelible ink and removed. The plates were then flooded with 0.01% neutral red stain. The plates were returned to the incubator (37 ± 1 °C, 5 ± 1% CO_2) for 1 hour ± 10 minutes. The stain was removed and the monolayers were microscopically evaluated.

Justification for Selection of the Test System
This *in vitro* cytotoxicity assay is designed to screen the biological reactivity of mammalian cell cultures following contact by diffusion of leachable, cytotoxic chemicals in materials or formulations. The L-929 cell line has a history of use in assays of this type. Neutral red stain is used as a vital stain to aid in toxicity estimation in the treated L-929 cells.

PROTOCOL AMENDMENTS/DEVIATIONS
There were no amendments or deviations that occurred during the course of this study.

IDENTIFICATION OF THE TEST SYSTEM
Cultures of L-929 cells (mouse fibroblast) were obtained from American Type Culture Collection (ATCC # CCL-1). Cell lines which are mycoplasma-free are purchased from the vendor and are kept frozen in the lab until use. To maintain the sensitivity, they are only subcultured for up to 15 passages and then discarded.

Cultures were grown and used as monolayers in disposable tissue culture labware at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% CO_2 in air at WuXi AppTec. The media used for growth of cells was Eagle's minimal essential medium (E-MEM) supplemented with 5% (v/v) fetal bovine serum (FBS). The medium was also supplemented with the following: 2 mM L-glutamine, 10 mM HEPES, 0.01 mg/mL vancomycin, 0.01 mg/mL gentamicin, 1% 1000 u/mL penicillin, and 1% 2.50 ug/mL amphotericin B (Fungizone). WuXi AppTec has a long-standing history using antibiotics in culture medium. The use of antibiotics in culture media has not shown adverse effects when used for this assay; this is exhibited by the varying degrees of toxicity seen in the assay controls.

TEST ARTICLE PREPARATION
The test article was cut into three approximately 1 cm x 1 cm pieces for triplicate assay plates. The test article was placed directly onto the agarose surface for testing. Due to its absorbent nature, the test article was saturated with 2X E-MEM + 10% FBS prior to the test article placement onto the agarose surface. The plastic packaging was removed before testing; included nylon fabric that will come into contact with patient's skin during use for testing, per Sponsor's request.

Table 1: Control / Cell Line Record

Control Identification	Class	Lot #	Supplied By	Expiration
Latex	Positive Control	WXAT103112	Fisher	10/31/15
High Density Polyethylene (HDPE)	Negative Control	C-111	Hatano	08/2018
L-929	Cell Line	L061215	ATCC	NA
2X E-MEM + 10% FBS	Medium	052115A1	WXAT	06/18/15
2% Agarose	Agarose	TDSI1335	Balco	11/13/15

NA = Not Applicable

EXPERIMENTAL PROCEDURE
On Day 1 of the test, equal amounts of 2X E-MEM + 10% FBS (pH 7.29) and molten 2% agarose were combined to achieve a final concentration of 1% agarose in 1X E-MEM + 5% FBS. The maintenance media was carefully removed from each monolayer and replaced with 3.5 mL of the E-MEM/agarose mixture. The cultures were held at room temperature until the agarose solidified.

The test article was gently placed on the agarose surface of three culture dishes. The positive and negative controls were set up in triplicate and assayed in parallel with the test article and cell control. All cultures were held at 37 ± 1 °C in 5 ± 1% CO_2 for 24-25 hours.

At the completion of the incubation period, the perimeter of each test article and control articles were outlined in indelible ink and then removed. All cultures were flooded with 3.0 mL of 0.01% neutral red stain and returned to the incubator at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% CO_2 in air for 1 hour ± 10 minutes. The stain was removed from the agarose surface and cultures were evaluated macroscopically and microscopically.

TEST EVALUATION
Plates were stained with a neutral red solution and scored macroscopically and microscopically. Results were scored as cytotoxic or non-cytotoxic based upon observations of cell lysis and definitive cytotoxic effects at the sample sites. When scoring lysis and neutral red uptake, each sample was rated relative to the amount of lysis and neutral red uptake displayed by the negative control (the negative control was considered '0'). Sites were given numerical values to indicate the following lysis/cell death index as derived from ISO guidelines.

Zone index is a measure of area affected by the test article based upon visual observation of neutral red uptake.

Table 2: Scoring

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen.
1	Slight	Some malformed or degenerated cells under specimen.
2	Mild	Zone limited to area under specimen.
3	Moderate	Zone extending specimen size up to 1.0 cm.
4	Severe	Zone extending farther than 1.0 cm. beyond specimen.

Negative Response
According to ISO guidelines, test articles scoring '0', '1', or '2' were considered '**non-cytotoxic'.**

Positive Response
The positive control displayed a moderate to severe cytotoxic reaction, displaying a score of '3' or '4'. Test articles scoring '3' or '4' were considered '**cytotoxic**'.

VALIDITY CRITERIA
Final evaluation of the validity of the assay and test article results was based upon the criteria listed below and scientific judgment.

The positive control sample should have a score of '3' or '4' and the negative control sample should have a score equal to '0' for a valid test.

STATISTICAL METHODS: None used.

METHOD FOR CONTROL OF BIAS: Not applicable.

DATA ANALYSIS: Not applicable.

RECORD RETENTION
An exact copy of the original final report and all raw data pertinent to this study will be stored by WuXi AppTec. It was the responsibility of the Sponsor to retain a sample of the test article.

COMPLIANCE

This study was performed in compliance with the following International Standards:

ISO 10993-5:2009 Biological Evaluation of Medical Devices, Part 5: Tests for *In Vitro* Cytotoxicity.

ISO 10993-12:2012 Biological Evaluation of Medical Devices, Part 12: Sample Preparation and Reference Materials.

TEST ARTICLE DISPOSITION

Unused test samples remain in the storage area until all testing is completed. Once completed, the remaining samples were discarded or returned as requested by the Sponsor.

RESULTS

Results were scored as cytotoxic or non-cytotoxic based upon observations of cell lysis and definitive cytotoxic effects at the sample sites. Sites were given numerical values to indicate the degree of cytotoxic effect (lysis or cell death).

Table 3: Test Results

Test Article	Cytotoxic Score		
	Plate 1	Plate 2	Plate 3
Test Article	0	0	0
Positive Control	3	3	3
Negative Control	0	0	0
Cell Control	0		

ANALYSIS AND CONCLUSION

The positive control score was '3' and the negative control score was '0' indicating a valid test. The test article was scored at '0' and is considered **non-cytotoxic** under the conditions of this test.

REFERENCES

U.S. Pharmacopeia, Section 87, current revision.

WuXi AppTec Reference Library Contents, Form ALS-4650-1

WuXi AppTec SOP: MED-8700, Agarose Overlay Cytotoxicity Test

Copy of Original

Test Request Form and Protocol

药明康德 WuXi AppTec

Biocompatibility
TEST REQUEST FORM

Complete this form and include it with your test article shipment. Ship to:
WuXi AppTec • 2540 Executive Drive • St. Paul, MN 55120
(1) 651.675.2000 TOLL FREE 888.794.0077

FOR WUXI APPTEC USE ONLY
D9282-1
P.O. NUMBER KP520424
QUOTE NUMBER (QUO-#####-######-#) QUO - 15997 - SIL8W6 - 0

CLIENT INFORMATION

ACCOUNT NUMBER (Required): 4003497

COMPANY NAME • STREET ADDRESS • CITY / STATE / ZIP • COUNTRY	CONTACT NAME	
Klarity Medical Products, LLC 1987 Coffman Road Newark Ohio 43055	Peter Larson BBJ 6-8-15	
	PHONE 740-788-8107 ext 111	EMAIL peter@klaritymedica.com (2)

REQUESTED TESTING

TEST CODE	TEST NAME
140150.1	ISO Agarose Overlay Using L-929 Mouse Fibroblast Cells (GLP)

Protocol Version #: 18 • Effective Date: 12/17/2013

TEST CODE	TEST NAME
900899.1	Buehler Dermal Sensitization - Repeated Patch (GLP)

Protocol Version #: 17 • Effective Date: 05/29/2013

TEST CODE	TEST NAME
910699.1	ISO Primary Skin Irritation (GLP) – 4 hours (1)

Protocol Version #: 18 • Effective Date: 07/15/2013

☐ *Check here if more space is needed to list requested testing. An additional page – with space for continued test listings – will be added.*

Form ALS 8000-1.15 WUXG177003695 • Page 1 of 4 Effective Date: 04.01.15

(1) Per sponsor JN 6/10/15

(2) cl. peter@klaritymedical.com JJ 6/11/15

EXACT COPY
INITIAL/DATE JN 6/10/15
PAGES 4

TEST ARTICLE INFORMATION	QUANTITY OF TEST ARTICLES SUBMITTED:	CLICK HERE for Sample Requirements Guide

TEST ARTICLE NAME (to be described on the final report)
Klarity AccuCushion R550-M

LOT NUMBER: 5US06Z ☐ Various (LIST ATTACHED) ☐ N.A. *Limit of 25 characters for lot number. To provide unique identifiers or other information that needs to be specified in test article information for the final report, enter in "Test Article Name" space above.*	EXPIRATION DATE: 5/6/18

PHYSICAL DESCRIPTION (as to be described on the final report)
Moldable pillow with nylon fabric surface

INTENDED USE / APPLICATION
Patient stabilization for Radiation therapy treatments

PHYSICAL STATE	SAFETY PRECAUTIONS	CONTROLLED STORAGE CONDITIONS
Insoluble	None/Unknown	Room Temperature

STERILITY *Select one of the three options shown.*	☐ Test article submitted sterile. Indicate sterilization method: Not Applicable	☐ Test article is not sterile. WuXi AppTec to expose to: Not Applicable *Additional fees apply.*	☑ Test article is not sterile. *To be tested non-sterile.*

For GLP studies (required by US FDA)
TEST ARTICLE CHARACTERIZATION

(1) ☒ Sponsor affirms test article has been characterized or that characterization testing is planned.

Sponsor is solely responsible for all test article characterization data as required in Good Laboratory Practices (GLP) regulations (21CFR58) – identity, strength, stability, purity, and chemical composition. Sponsor is also responsible for ensuring that test article here submitted is representative of the final product that will be subjected to materials characterization.

TEST ARTICLE DISPOSITION *(NOTE: Additional fee may apply for returns.)*	*Courier and account # for shipping: (Required for returns)*

Form ALS-BCOD-1.15 WuXi-3177003925 • Page 2 of 4 Effective Date: 04.01.15

(1) for sponsor SN 6/10/15

TEST ARTICLE HANDLING & PREPARATON

Preparation Requirements / Instructions ☐ N.A.	☐ Do NOT cut test article.	Is test article absorbent?
NOTE: Entire article will be tested unless information is provided here as to materials/components to be included/excluded for testing.	Unless box is checked, test article may be cut into sizes needed for testing. NOTE: Hemocompatibility testing typically requires the test article to be cut.	Unknown
remove plastic packaging (bag) before testing. Surface to be tested is the nylon fabric which will come into contact with patients skin during use.		

EXTRACTION PARAMETERS ☐ N.A.

If requested tests require extraction, this entire section must be completed by Sponsor. For assistance, contact your Project Manager.

NS = Normal Saline SO = Sesame Oil AS = Alcohol Saline PEG = Polyethylene Glycol PBS = Phosphate Buffered Saline DMSO = Dimethyl Sulfoxide

TYPE OF EXTRACT	EXTRACTION RATIO	EXTRACTION CONDITIONS
Cytotoxicity: Culture Media Sensitization • Irritation • Acute Systemic Toxicity • Mouse Micronucleus: Not Applicable LLNA • Ames • Mouse Lymphoma • Chromosomal Aberration: Not Applicable *Select "NS & PEG" for materials incompatible with DMSO. Select "NS & DMSO or NS & PEG" if DMSO compatibility unknown. WAI to determine extract.* Subacute/Subchronic Toxicity: Not Applicable	*Per ISO 10993-12, a surface area ratio should be used whenever possible rather than a weight ratio. The "surface area" includes the combined areas of all sides of the test article and excludes indeterminate surface irregularities.*	Cytotoxicity: 37°C / 24 hrs All other extraction testing: *Conditions recommended for most devices: 50°C / 72 hrs* Not Applicable *Extraction conditions are based on an exaggeration of product use. For insoluble materials, use highest temperature possible without causing degradation of material.*

FOR HEMOCOMPATIBILITY TESTS *(Incl. Hemolysis, PTT, PAI. Counts, IVT Hemo, PT and Complement Activation)* ☐ N.A.

☐ Test blood-contacting portions ONLY (per ISO 10993-4) *NOTE: Entire article will be tested unless box is checked.* Specify blood-contacting components/materials to be tested:	PTT (Partial Thromboplastin Time): RATIO: 4cm² / 1mL Platelet & Leukocyte Count and IVT Hemocompatibility: RATIO: 12cm² / 1mL Complement Activation and PT (Prothrombin Time): RATIO: Not Applicable	ASTM Hemolysis TYPE OF EXTRACT: PBS RATIO: Not Applicable CONDITIONS Direct Contact: 37°C / 3 hrs Extract Method: Not Applicable

FOR MHLW (Japan) TESTS ☐ N.A.

Extraction parameters for MHLW tests are the same as for ISO/ASTM except for the specific tests described here.

MHLW Genotoxicity and Sensitization	MHLW Cytotoxicity	MHLW Hemolysis
These tests are performed using exhaustive extraction method in methanol/acetone with terminal evaporation.	RATIO: Not Applicable	CONDITIONS: Not Applicable

☐ Check here if comparison/control article is being submitted. *(If checked, you will see an additional section that must be completed.)*

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COMMENTS

May be disposed of with normal household waste.

Sponsor signature is required before testing will be initiated.
Services requested in this form will be governed in accordance with WuXi AppTec's "Standard Terms and Conditions." To the extent WuXi AppTec's Standard Terms and Conditions are in conflict with an applicable agreement (Agreement) between Customer listed in this form and WuXi AppTec, such Agreement will govern.

Print / Save as PDF

To save an electronic copy of this completed form, select Adobe PDF Writer or Microsoft XPS Document Writer in your print dialog.

Always print a hard copy to ship with your samples.

TESTING AUTHORIZATION & PROTOCOL APPROVAL

SIGNATURE	PRINT NAME	DATE
2015.06.02 09:40:08 -05'00'	Peter M. Lasson	6/1/15

For any test being conducted GLP:
By signing above, you acknowledge that you have reviewed the most current version of the protocol(s) listed on this form and your signature constitutes approval of the protocol(s). If you would like to review any or all of the protocols, click here to email WuXi AppTec and indicate the protocol(s) you want to review.

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(For Laboratory Use Only)

WuXi AppTec Study # D9282-1

药明康德 WuXi AppTec

PROTOCOL TITLE:	**ISO Agarose Overlay Using L-929 Mouse Fibroblast Cells**
TEST CODE:	140150
PERFORMING LABORATORY:	WuXi AppTec 2540 Executive Drive St. Paul, MN 55120
EFFECTIVE DATE:	17 December 2013
GLP PROTOCOL:	140150-18

Quality Assurance has reviewed this protocol for compliance with applicable regulatory requirements and internal procedures.

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1.0 PURPOSE

The purpose of this assay is to evaluate the *in vitro* toxicity of the test article to mammalian cells when leachable extracts are allowed to diffuse through an agarose barrier and contact cultured cells. L-929 mouse fibroblast cells will be employed for this assay by a method compliant with the requirements specified in (ISO 10993-5:2009).

2.0 TEST FACILITY: WuXi AppTec
2540 Executive Drive
St. Paul, MN 55120

3.0 SCHEDULING AND DISCLAIMER

3.1 Test protocol initiation is generally within 10 working days after receipt of the test article, a signed protocol/Client Approval form, and a signed test request form. The Client Approval form and the test request form serve as addenda to this protocol. Written notification of the proposed initiation and completion dates will be provided at the time the test article and signed protocol are received by the laboratory. The estimated testing time is 2 - 3 days. Verbal results will be available from the Study Director upon completion of the study with the written report to follow approximately 10 working days after completion of the study.

3.2 Testing is performed in strict adherence to WuXi AppTec standard operating procedures (SOPs) which have been constructed to cover all aspects of the work including, but not limited to, receipt, identification, log-in and tracking of test article(s). Additionally, each test is assigned a unique Project Number. This number is used for identification during the course of the test.

3.3 The Sponsor is responsible for any rejection of the final report by the regulatory agency concerning report format, pagination, etc. To prevent rejection, the Sponsor should carefully review the WuXi AppTec final report and notify WuXi AppTec of any perceived deficiencies in these areas before submission of the report to the regulatory agency. WuXi AppTec will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

3.4 Neither the name of WuXI AppTec nor any of its employees are to be used in advertising or other promotion without written consent from WuXi AppTec.

4.0 TEST AND CONTROL ARTICLE IDENTIFICATION

4.1 Test article information to be included in the final report will be provided solely by the Sponsor on the WuXi AppTec test request form attached to this protocol.

4.2 Controls

4.2.1 A negative control will be prepared from a material known to yield a non-cytotoxic response under the test conditions. The negative control for a liquid test article will be E-MEM + 6% FBS. The negative control for a solid test article will be high density polyethylene (HDPE).

4.2.2 A positive control will be prepared from a material known to yield a cytotoxic response under the test conditions. The positive control for a liquid test article will be 500 µM $CdCl_2$. The positive control for a solid test article will be latex.

4.2.3 The cell control will consist of one plate with a monolayer of L-929 cells covered with the agarose mixture alone.

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5.0 CHARACTERIZATION

The Sponsor is responsible for all test article characterization data as specified in the Good Laboratory Practices (GLP) regulations. The identity, strength, stability, purity, and chemical composition of the test article is solely the responsibility of the Sponsor. The Sponsor is responsible for supplying to the testing laboratory results of these determinations and any others that may directly impact the testing performed by the testing laboratory, prior to initiation of testing.

Furthermore, it is the responsibility of the Sponsor to ensure that the test article submitted for testing is representative of the final product that will be subjected to materials characterization. Any special requirements for handling or storage must be arranged in advance of receipt and the test article must be received in good condition.

6.0 SAFETY

Appropriate routine safety procedures will be followed in handling the test article, unless more cautious procedures are specified by the Sponsor. All applicable WuXi AppTec safety policies and procedures will be observed during the performance of the test.

7.0 EXPERIMENTAL DESIGN

7.1 Experimental Overview

The agarose overlay assay evaluates qualitatively the in vitro toxicity of solids, liquids or powders to L-929 mouse fibroblast cells when leachable extracts are allowed to diffuse through an agarose barrier and contact cultured cells.

On the day of testing, equal amounts of 2X E-MEM + 10% FBS (pH of 7.2 – 7.4) and molten 2% agarose will be combined to achieve a final concentration of 1.0% agarose in 1X E-MEM + 5% FBS. The maintenance media will be carefully removed from each monolayer and replaced with 3.5 mL of the E-MEM / agarose mixture. Once the agar solidifies, the controls and test articles will be placed on the hardened surface for testing. Each treatment will be performed in triplicate. One cell control plate will be run concurrently. The plates will be incubated at 37 ± 1 °C in 5 ± 1% CO_2 for 24-25 hours.

After the incubation period, the test article will be outlined on the culture dish with indelible ink. The test article will be removed and the plates will be flooded with 0.01% neutral red stain. The plates will be returned to the incubator (37 ± 1 °C, 5 ± 1% CO_2) for 1 hour ± 10 minutes. The stain will be removed and the monolayers microscopically evaluated.

7.2 Justification For Selection Of The Test System

This *in vitro* cytotoxicity assay is designed to screen the biological reactivity of mammalian cell cultures following contact by diffusion of leachable, cytotoxic chemicals in materials or formulations. The L-929 cell line has a history of use in assays of this type.

7.3 Amendments / Deviations

If it becomes necessary to make changes in the approved protocol, the revisions and reasons for changes will be documented, signed by the Study Director, dated, maintained with the protocol and reported to the Sponsor. If an event occurs which may have an effect on the validity of the study, the Sponsor will be notified as soon as is practical. If the Study Director is unable to complete the study, an alternate Study Director with full responsibility and authority regarding the study will be assigned.

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8.0 IDENTIFICATION OF THE TEST SYSTEM

Cultures of L-929 mouse fibroblast cells will be obtained from American Type Culture Collection (ATCC # CCL-1). Cell lines which are mycoplasma-free are purchased from the vendor and are kept frozen in the lab until use. To maintain the sensitivity, they are only subcultured for up to 15 passages and then discarded.

Cultures are grown and used as monolayers in disposable tissue culture labware at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% CO_2 in air at WuXi AppTec. The media used for growth of cells is sterile Eagle's minimal essential medium (E-MEM) supplemented with 5% (v/v) fetal bovine serum (FBS). The medium is also supplemented with the following: 2 mM L-glutamine, 10 mM HEPES, 0.01 mg/mL vancomycin, 0.01 mg/mL gentamicin, 1% 1000 units/mL penicillin, and 1% 2.50 ug/mL amphotericin B (Fungizone). WuXi AppTec has a long-standing history using antibiotics in culture medium. The use of antibiotics in culture media has not shown adverse affects when used for this assay; this is exhibited in the varying degrees of toxicity seen in the assay controls.

9.0 TEST METHOD

9.1 Test Article Preparation

Table 1: Standard Surface Areas and Extract Liquid Volumes

EXAMPLES OF FORMS OF MATERIALS	THICKNESS	EXTRACTION RATIO (SURFACE AREA OR MASS/VOLUME) ± 10%
Film, sheet, tubing wall	<0.5 mm	6 cm^2/mL
Tubing wall, slab, small molded items	0.5 to 1.0 mm	3 cm^2/mL
Larger molded items	>1.0 mm	3 cm^2/mL
Elastomeric closures	>1.0 mm	1.25 cm^2/mL
Powder, pellets, foam, non-absorbent molded items	Irregularly shaped solid devices	0.2 g/mL
Membranes, textiles	Irregularly shaped porous devices (low-density materials)	0.1 g/mL
NOTE: While there are no standardized methods available at present for testing absorbents and hydrocolloids, a suggested protocol is as follows: - determine the volume of extraction vehicle that each 0.1 g or 1.0 cm^2 of material absorbs; - then, in performing the material extraction, add this additional volume to each 0.1 g or 1.0 cm^2 in an extraction mixture.		

The size of the article to be tested will be determined by the following ISO guidelines:

9.1.1 Liquids or extracts will be prepared by saturating three biologically inert 10 mm filter paper discs with the liquid under test. If a test article is extracted it may be extracted using standard ISO ratios, at 37 ± 1 °C for 24 ± 2 hours. Other test article sizes may be used if requested by the Sponsor.

9.1.2 When a test article is sufficiently small enough to fit into the culture dish leaving an adequate margin of cells for evaluation, the entire article will be used.

9.1.3 Large solid materials and devices will be cut in cross-section to obtain a flat surface having an area of approximately 1 cm x 1 cm to be placed in direct contact with the agarose surface.

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9.1.4 Articles of rod or tubing shaped devices will be cut into approximately 1 cm segments and placed side by side to form a 1 cm x 1 cm patch.

9.1.5 Absorbent test articles will be moistened with E-MEM media prior to application to the plate.

9.2 Control Article Preparation

9.2.1 A negative control will be prepared from a material known to yield a non-cytotoxic response under the test conditions. This control will be of a similar physical state to the test material.

a. A liquid negative control will be prepared by saturating a sterile disc with E-MEM.

b. A solid negative control will be high density polyethylene (HDPE).

9.2.2 A positive control will be prepared from a material known to yield a cytotoxic response under the test conditions. This control will be of a similar physical state to the test material.

a. A liquid positive control will be prepared by saturating a sterile disc with 500 μM $CdCl_2$.

b. A solid positive control will be sterile latex.

9.2.3 The cell control will consist of one plate with a monolayer of L-929 cells covered with the agarose mixture alone.

9.3 Experimental Procedure

L-929 cells will be plated at 7.5 x 10^6 cells per 60 mm plate at least 24 hours prior to use. Just prior to testing, the monolayers will be evaluated for the level of confluency. This will be achieved by microscopic examination of a representative portion of the total number of plates to be used in the assay. Cultures will only be used if they appear healthy and have not reached complete confluency.

The maintenance media will be removed from the plates and a 1:1 mixture of 2X E-MEM + 10% FBS (pH 7.2 – 7.4) and molten 2% agarose will be added to cultures of L-929 cells. After addition of the agar the cells will be examined to ensure they appear healthy.

After the mixture has solidified, the test article, control, or saturated filter discs will be carefully placed on the agar layers. The test article and the positive and negative controls will be plated in triplicate. One cell control plate will be prepared at this time by adding the agar solution but no control or test material.

After a 24-25 hour incubation at 37 ± 1 °C in 5 ± 1% CO_2. The test article and controls will be outlined on the culture dish with indelible ink. The test article will be removed and the plates will be flooded with 0.01% neutral red stain. The plates will be returned to the incubator (37 ± 1 °C, 5 ± 1% CO_2) for 1 hour ± 10 minutes. The stain will be removed and the monolayers microscopically evaluated and scored.

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10.0 TEST EVALUATION

Plates will be stained with a neutral red solution and scored macroscopically and microscopically. Results will be scored as cytotoxic or non-cytotoxic based upon observations of cell lysis and definitive cytotoxic effects at the sample sites. When scoring lysis and neutral red uptake, each sample will be rated relative to the amount of lysis and neutral red uptake displayed by the negative control (the negative control will be considered '0'). Sites will be given numerical values to indicate the following lysis/cell death index as derived from ISO guidelines.

Zone index is a measure of area affected by the test article based upon visual observation of neutral red uptake.

Table 2: Scoring

GRADE	REACTIVITY	DESCRIPTION OF REACTIVITY ZONE
0	None	No detectable zone around or under specimen.
1	Slight	Some malformed or degenerated cells under specimen.
2	Mild	Zone limited to area under specimen.
3	Moderate	Zone extending specimen size up to 1.0 cm.
4	Severe	Zone extending farther than 1.0 cm. beyond specimen.

10.1 Positive Response

The positive control will display a moderate to severe cytotoxic reaction, displaying a score of '3' or '4'.

Test articles scoring '3' or '4' will be considered '**cytotoxic**'.

10.2 Negative Response

Test articles scoring '0', '1', or '2' will be considered '**non-cytotoxic**'.

10.3 Repeat Assays

A test will be repeated in part or in total if a control failure occurs.

11.0 ASSAY VALIDITY

Final evaluation of the validity of the assay and test article results will be based upon the criteria listed below and scientific judgment.

The positive control should induce a score of '3' or '4'. If the positive control does not induce this response, the assay will be repeated.

The negative control should score '0', displaying no cytotoxicity. Should these criteria not be met, the test will be repeated.

12.0 METHOD FOR CONTROL OF BIAS: Not applicable.

13.0 DATA ANALYSIS: Not applicable.

14.0 STATISTICAL METHODS: None used.

15.0 FINAL REPORT

The final report will include, but will not be limited to: the date of the study initiation and completion, the purpose as stated in the approved protocol, changes in the approved protocol, identification of the test system, a description of the methods used and conclusion as it relates to the test.

16.0 RECORD RETENTION

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16.1 Study Specific Documents

All of the original raw data developed exclusively for this study shall be retained according to WuXi AppTec's standard operating procedures for archival. These original data include, but are not limited to the following:

16.1.1 All handwritten and equipment generated raw data for control(s) and test article(s).
16.1.2 Any protocol amendments/deviation notifications.
16.1.3 Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
16.1.4 Original signed protocol.
16.1.5 Certified copy of final study report.
16.1.6 Study-specific SOP deviations made during the study.
16.1.7 QA reports for each QA inspection with comments.

16.2 Facility Specific Documents

The following records shall also be retained according to WuXi AppTec's standard operating procedures for archival. These documents include, but are not limited to, the following:

16.2.1 SOPs which pertain to the study conducted.
16.2.2 Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
16.2.3 Methods which were used or referenced in the study conducted.
16.2.4 Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
16.2.5 Current job descriptions and summary of experience and training for all personnel involved in the study.

17.0 COMPLIANCE

17.1 GLP Status

The study will be conducted under GLP compliance (FDA, 21 CFR, Part 58 - Good Laboratory Practice for Nonclinical Laboratory Studies). The study will be inspected during at least one phase and the final report will be audited by the WuXi AppTec Quality Assurance unit.

17.2 International Standards

17.2.1 ISO 10993-5: 2009. Biological Evaluation of Medical Devices, Part 5: Tests for *In Vitro* Cytotoxicity.

17.2.2 ISO 10993-12:2012 Biological Evaluation of Medical Devices, Part 12: Sample Preparation and Reference Materials.

18.0 TEST ARTICLE DISPOSITION

It is the responsibility of the Sponsor to retain a sample of the test material. All unused test material will be discarded following study completion unless otherwise requested by Sponsor.

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19.0 REFERENCES

19.1 U.S. Pharmacopeia, Section 87, current revision.

19.2 WuXi AppTec Reference Library Contents, Form ALS-4650-1

19.3 WuXi AppTec SOP: MED-8700, Agarose Overlay Cytotoxicity Test

20.0 VERSION CHANGE SUMMARY -- from Version 140150-16 to 140150-17

20.1 Updated section 9.3 to specify cells should be plated at least 24 hours before use.

20.2 Section 11.0 was updated for conciseness.

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