



Akkreditiert durch  
Zentralstelle der Länder  
für Gesundheitsschutz  
bei Arzneimitteln  
und Medizinprodukten  
ZLG-P-974.98.05



mdt medical device testing GmbH

Grenzenstraße 13  
88416 Ochsenhausen  
Germany  
Phone +49 – (0)73 52 – 91 14 0  
Fax+49 – (0)73 52 – 91 14 70

## FINAL REPORT

Project #	09z171
Study Title	Cytotoxicity Growth Inhibition Test in L929 Mouse Fibroblasts (Elution Test) of a Coated Blank
Test item	Coated blank: "Acktar Model-1"
Reference item	Not applicable
Authors	Dr. Beate Schaller (Study Director) Jan Peeters (Director Testing Services)
Test facility	mdt medical device testing GmbH Cytological laboratory Beim Braunland 1 88416 Ochsenhausen Germany
Study dates:	
Arrival of test item	03-December-2009
Begin of testing	10-December-2009
End of testing	14-December-2009
Final report date	14-December-2009

According to DIN EN ISO/IEC 17025, mdt medical device testing GmbH does not assume liability for potential misleading presentations of study methods and results of this report, if the report is reproduced in extracts.

## Project Staff Signatures

Ochsenhausen, .....

Dr. Beate Schaller  
Study Director  
mdt medical device testing GmbH

Ochsenhausen, .....

Jan Peeters  
Director Testing Services  
mdt medical device testing GmbH

## Quality Assurance

This study was performed in compliance with a quality management system according to DIN EN ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories), which has been established at mdt medical device testing GmbH and has been accredited by the German accreditation body "Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten" (ZLG) under the register number ZLG-P-974.98.05.

With the exception that standard test procedures do not require a separate study protocol and no archiving of study items is requested, this quality management system includes the quality requirements prescribed by the international guidelines for Good Laboratory Practice (GLP).

## Archiving

All correspondence, an original copy of the protocol, an original copy of the test report, and a documentation of all raw data generated during the conduct of the study (i.e., documentation forms as well as any other notes of raw data, printouts of instruments and computers) are stored under the project number 09z171 in the archives of the mdt medical device testing GmbH.

If the sponsor requests the study file at the end of the archiving period (at present 15 years), mdt medical device testing GmbH needs to be instructed in writing by the sponsor. Otherwise all documents and materials may be destroyed by 'mdt' at the end of the archiving period.

## Distribution and Number of Reports

mdt medical device testing GmbH:	1 (Original)
Sponsor:	1 (Original)

## Table of Contents

Page

Project Staff Signatures .....	2
Quality Assurance.....	3
Archiving.....	3
Distribution and Number of Reports.....	3
Table of Contents .....	4
1. Summary.....	5
2. Introduction .....	6
3. Materials and Methods .....	7
3.1. Chemicals and Materials .....	7
3.2. Instruments .....	7
3.3. Test Items .....	8
4. Test Methods.....	9
4.1. Preparation of Extracts .....	9
4.2. Cell System and Study Conduct .....	9
4.3. Controls.....	10
5. Test Results .....	11
6. Conclusion.....	12
7. References.....	13

## 1. Summary

The aim of this study was to find out if cytotoxic substances are extracted from the study materials with cell culture medium containing 10 % fetal calf serum.

The test was performed according to DIN EN ISO 10993-5 [1] as growth inhibition test with L929 mouse fibroblasts.

The quantitative cell growth inhibition test with L929 mouse fibroblasts showed the following results:

None of the extract-concentrations of the coated blank "Acktar Model-1" showed any cytotoxic reaction.

Conclusion:

Due to the high sensitivity of the mouse fibroblast growth inhibition test, it is assumed that a mean growth inhibition of up to 30 % does not indicate a significant risk of cytotoxicity.

Based upon the observed results and under the test-conditions chosen, the coated blank "Acktar Model-1" was considered to have no cytotoxic effects in all extracts in the growth inhibition test with L929 mouse fibroblasts.

## 2. Introduction

For medical devices the biocompatibility has to be evaluated regarding the harmonized standard DIN EN ISO 10993-1, Biological evaluation of medical devices - Part 1: Evaluation and testing [2] in which the biological test methods are listed.

The growth inhibition test is designed to ascertain the presence of extractable cytotoxic substances. The growth rate of mammalian cells is significantly decreased in the presence of toxic substances. Usually, the growth rate is determined by comparing the cell number or the protein content of cells at different time intervals. In the present quantitative cytotoxicity assay, proliferating Mouse L929 fibroblast cultures were exposed to dilution series of material extracts and the growth rates are determined in comparison to positive (e.g. culture medium with 10 % Dimethylsulfoxide) and negative controls (non-extracted culture medium).

Since there is a linear relationship between cell number and protein concentration under the conditions of this cytotoxicity assay, the number of viable cells respectively their protein concentration are indicative of the relative cytotoxicity of the tested extract concentration. If the extracts of various materials (test and reference materials) are investigated in parallel, the growth rates are indicative for the relative cytotoxicity of the extracted test and reference materials.

The test system used in this investigation is based upon the following standard:

- DIN EN ISO 10993-5, Biological evaluation of medical devices - Part 5: Tests for in vitro Cytotoxicity [1]
- DIN EN ISO 10993-12, Biological evaluation of medical devices - Part 12: Sample preparation and reference materials [3]
- USP 31, Chapter 87 - Biological reactivity tests, in vitro [4]

### 3. Materials and Methods

#### 3.1. Chemicals and Materials

- Cell culture plates: 96 well microtiterplates (Corning Lot No. 22509021)
- Cell culture dishes: 100 mm Ø (Corning Article No. 3100)
- Trypsin/EDTA: 0.05 % Trypsin-EDTA (Gibco Lot No. 670380)
- DMSO: Dimethylsulfoxide, >99.5 % (Serva Lot No. 090153)
- Ethanol: (technical grade) 99 % denatured by the addition of 1 % Butanon and other Ketons, 10 ppm Denatonium-benzoat (BfB: Bundesmonopolverwaltung für Branntwein, Offenbach)
  
- Crystal violet stain: 0.1 % in water, w/v (Crystal violet, LMK Lot No. 7406)
- Triton x100 solution: 0.2 % in water, v/v (Triton x100, LMK Lot No. 7407)
- Cell culture medium: DMEM (Dulbecco's modified Eagle Medium, LMK Lot No. 7421) containing:
  - 10 % (v/v) fetal calf serum (FCS, Gibco Article No. 10270-106)
  - 584 mg/l L-Glutamine (PAA Article No. M11-006 )
  - 50 mg/l L(+) Ascorbic acid, Na-salt (Merck Article No. F774427)
  - 140.000 U/l Penicillin (Serva 31749)
  - 140 mg/l Streptomycin (Serva 35500)
- PBS: Phosphate buffered Saline solution (PBS, LMK Lot No. 7293) containing:
  - 0.004 M  $\text{KH}_2\text{PO}_4$
  - 0.011 M  $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$
  - 0.003 M KCl
  - 0.119 M NaCl and equilibrated to pH 7.2 with NaOH
- common laboratory equipment

#### 3.2. Instruments

- Autoclave: LMK, Inv. No. 029
- CO<sub>2</sub> incubator: Heraeus BB 16, LMK, Inv. No. 1148
- Microtiter reader: For the measurement of the optical extinction a dual wavelength microtiter reader (Dynex MRX<sub>II</sub> Inv. No. 1253) was used with measuring wavelength  $\lambda = 570 \text{ nm}$  and reference wavelength  $\lambda = 405 \text{ nm}$ .

### 3.3. Test Items

09z171-1:

Name of test item:	Acktar Model-1
Product description:	Ti samples with Acktar coating
Batch No.:	97919
Cat No.:	B14-0622-001
Number of received test items:	14 pcs
Number of used test items:	4 pcs with a total surface of 10 cm <sup>2</sup>
Purity / Composition:	Titanium sheet with ceramic coating
Stability / Expiry date:	n/a
Physical state / appearance:	Solid black sheet
Storage conditions:	Room temperature
<u>Reference item:</u>	Not applicable

All data relating to the identity, purity and stability of the test materials are the responsibility of the sponsor and have not been verified by the test facility.



## 4. Test Methods

### 4.1. Preparation of Extracts

The sterile test items were subjected to medium extraction.

For that, the test items were transferred into the eluent (3 cm surface area per milliliter cell culture medium containing 10 % fetal calf serum) and incubated for  $24 \pm 2$  hours at  $37 \pm 1$  °C. The solution received after incubation was used as stock solution (100 % value) for the following dilution series with culture medium: 100 - 30 - 10 - 3 % (v/v).

### 4.2. Cell System and Study Conduct

The growth inhibition test used conforms to the guidelines and standards listed in the Chapter 7, 'References'.

L929 cells were obtained from the American Type Culture Collection, Rockville, Maryland, USA (ATCC no. CCL1, NCTC clone 929, connective tissue mouse, clone of strain L), referred to as L929 mouse fibroblasts.

A cell bank containing L929 mouse fibroblasts is kept at -196 °C. Moreover L929 cells are permanently cultivated using standard cell culture techniques (incubation at  $37 \pm 1$  °C in humidified air containing 5 % CO<sub>2</sub>). The cultivated cells are regularly controlled for cell growth and absence of mycoplasmas.

For the growth inhibition test, proliferating L929 mouse fibroblasts (passage 596) from stock cultures were used. They were trypsinized carefully, diluted with cell culture medium (DMEM) to a concentration of 40.000 cells per ml. From this suspension 100 µl were pipetted into the inner wells of 96-well microtiter plates (equivalent to  $1.3 \times 10^4$  cells/cm<sup>2</sup>). The outer wells of the plates were filled with DMEM. All plates were incubated in an incubator at  $37 \pm 1$  °C, with 5 % CO<sub>2</sub> and approximately 95 % of relative humidity.

Four hours after seeding the cells into one complete plate they were stained with crystal violet for the determination of the initial cell density (at  $t = 0$ ).

At the same time the wells of the other plates were filled with the dilution series of the test materials and controls. Ten replicates of every dilution step of each extract or sample were tested in different wells.

Thereafter, the plates were incubated for  $72 \pm 2$  h at  $37 \pm 1$  °C, in humidified atmosphere containing 5 % CO<sub>2</sub>.

At the end of the incubation period ( $t = 72$  h) the cells were studied under an inverted microscope at 100 x magnification for their condition. Thereafter, the biological end point was determined by staining with crystal violet:

- supernatant medium was removed
- cells washed twice with PBS
- fixed with 100 µl Ethanol, 99 % for 10 min
- Ethanol removed
- stained with 100 µl of crystal violet solution for 25 min

- washed four times with distilled water to remove remaining stain
- covered with 50 µl of Triton x100 solution
- 10 min later optical extinctions were measured at 570 nm

The measured biological values (BV), which are the optical extinctions were used to calculate the percentage of growth inhibition (GI) according to the following mathematical formula:

$$\% \text{ GI} = 100 \cdot \left( 1 - \frac{BV_{(\text{Sample, } t = 72\text{h})} - BV_{(\text{Control, } t = 0\text{h})}}{BV_{(\text{neg. control, } t = 72\text{h})} - BV_{(\text{Control, } t = 0\text{h})}} \right)$$

#### 4.3. Controls

- Negative control I: Cell culture medium (DMEM + 10 % FCS), not incubated
- Negative control II: Cell culture medium (DMEM + 10 % FCS), incubated for 24 hours under the same conditions as the extracts
- Positive control: Dilution series of Dimethylsulfoxide (10 - 3 - 1 - 0.3 % DMSO)

5. Test Results

The test results relate only to the materials supplied by the sponsor.

The quantitative cell growth inhibition test showed the following results:

None of the extract-concentrations of the coated blank "Acktar Model-1" showed any cytotoxic reaction.

The individual test results are summarized in the tables below.

Table 1: Results of growth inhibition

	Mean value growth inhibition [%] Standard deviation (SD)					
	Concentration of dilutions (v/v)					
	100 %	30 %	10 %	3%	1%	0.3 %
09z171-1 Acktar Model-1	7 ±9	2 ±6	3 ±6	2 ±6	-	-
DMEM, not incubated (Negative control I)	-2 ±6	-	-	-	-	-
DMEM, incubated (Negative control II)	5 ±6	-	-	-	-	-
DMSO (Positive control)	-	-	105 ±1	84 ±4	16 ±7	-8 ±11

All values are presented as mean values out of ten (bold letters) together with the corresponding standard deviation (SD).

Table 2: Results of microscopical evaluation

	Score values as defined below (after 72 hours of cell culture)					
	concentration of dilutions (v/v)					
	100 %	30 %	10 %	3%	1%	0.3 %
09z171-1 Acktar Model-1	0	0	0	0	-	-
DMEM, not incubated (Negative control I)	0	-	-	-	-	-
DMEM, incubated (Negative control II)	0	-	-	-	-	-
DMSO (Positive control)	-	-	4	3	0-1	0

Definition of score values (according to USP):

0 = no reactivity	Discrete intracytoplasmatic granules; no cell lysis
1 = slight reactivity	Not more than 20 % of the cells are round, loosely attached, and without intracytoplasmatic granules; occasional lysed cells are present
2 = mild reactivity	Not more than 50 % of the cells are round and devoid of intracytoplasmatic granules; no extensive cell lysis and empty areas between cells
3 = moderate reactivity	Not more than 70 % of the cell layers contain rounded cells or are lysed
4 = severe reactivity	Nearly complete destruction of the cell layers

## 6. Conclusion

Due to the high sensitivity of the mouse fibroblast growth inhibition test, it is assumed that a mean growth inhibition of up to 30 % does not indicate a significant risk of cytotoxicity.

Based upon the observed results and under the test-conditions chosen, the coated blank "Acktar Model-1" was considered to have no cytotoxic effects in all extracts in the growth inhibition test with L929 mouse fibroblasts.

## 7. References

- [1] DIN EN ISO 10993-5, 2009, Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity
- [2] DIN EN ISO 10993-1, 2003, Biological evaluation of medical devices - Part 1: Evaluation and testing
- [3] DIN EN ISO 10993-12, 2008, Biological evaluation of medical devices - Part 12: Sample preparation and reference materials
- [4] USP 31, 2008, Chapter 87 - Biological reactivity tests, in vitro