

BIOSERVICE

SCIENTIFIC
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In vitro Cytotoxicity Assay:

Cell Growth Analysis via BCA-Staining with an Extract of

**PA 2200 Reused powder (50% virgin + 50% recycled powder from
EOSINT P System)**

Report

Version: Final

Date: 08 December 2009

BSL BIOSERVICE Study No.: 094861

Sponsor:

EOS GmbH Electro Optical Systems

Robert-Stirling-Ring 1

82152 Krailling

Germany

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- The test results relate only to the items tested. -

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Amtsgericht München, HRB 109 770

Erfüllung und Gerichtsstand München

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1. Copy of the GLP-Certificate



**BAYERISCHES LANDESAMT
FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT,
LANDESINSTITUT FÜR ARBEITSSCHUTZ UND PRODUKTSICHERHEIT
Pfarrstraße 3 · 80538 München · Telefon (089) 21 84-0**

**GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)**

Eine GLP-Inspektion zur Überwachung
der Einhaltung der GLP-Grundsätze
gemäß Chemikaliengesetz bzw. Richt-
linie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP
according to Chemikaliengesetz and
Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility

☐ Prüfstandort/Test site

**BSL Bioservice Scientific Laboratories GmbH
Bohringstrasse 6 - 8
82152 Planegg**

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

**Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5 3/ OECD guidance)**

2 Prüfungen auf toxikologische Eigenschaften

3 Prüfungen auf mutagene Eigenschaften

9 Sonstige Prüfungen:

a) Mikrobiologische Sicherheitsprüfungen

b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection

(Tag Monat Jahr/day month year)

16./17.09.2008

Die/Der genannte Prüfeinrichtung/Prüfstandort
befindet sich im nationalen GLP-Überwachungs-
verfahren und wird regelmäßig auf Einhaltung der
GLP-Grundsätze überwacht

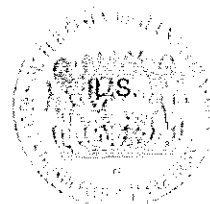
The above mentioned test facility/test site is
included in the national GLP Compliance
Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird
hiermit bestätigt, dass in dieser Prüfeinrichtung/
diesem Prüfstandort die oben genannten Prüf-
ungen unter Einhaltung der GLP-Grundsätze
durchgeführt werden können

Based on the inspection report it can be confirmed,
that this test facility/test site is able to conduct the
aforementioned studies in compliance with the
Principles of GLP.

München, 06.04.2009

Ritter
Leitender Gewerbedirektor



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3. Preface

3.1. Abbreviations

ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BGBI.	Bundesgesetzblatt
DIN	Deutsches Institut für Normung e. V.
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EDTA	Ethylene Diamine Tetraacetic Acid
EN	Europäische Norm
EWG	Europäische Wirtschaftsgemeinschaft
FCS	Fetal Calf Serum
G.I.	Growth Inhibition
GLP	Good Laboratory Practice
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
L929	strain L, clone 929
NCTC	National Collection of Type Cultures
OECD	Organization for Economic Co-operation and Development
QAU	Quality Assurance Unit
SOP	Standard Operating Procedure

3.2. General

Sponsor:	EOS GmbH Electro Optical Systems Robert-Stirling-Ring 1 82152 Krailling Germany
Study Monitor:	Ms. Monika Gessler Substitute Mr. Peter Keller
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	094861
Test Item:	PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)
Title:	<i>In vitro</i> Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

3.3. Project Staff

Study Director:	Dipl.-Biol. Dagmar Lehmeier
Deputy Study Director:	Dipl.-Ing. (FH) Jana Vogel
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

3.4. Schedule

Arrival of the Test Item:	25 November 2009
Date of Final Study Plan:	30 November 2009
Start of Experiment:	03 December 2009
End of Experiment:	07 December 2009
Date of Final Report:	08 December 2009

4. Project Staff Signatures

Study Director

Dipl.-Biol. Dagmar Lehmeier

.....
Dagmar Lehmeier

Date: 08 Dec 2009

Management

.....
W. Riedel

Print name: Dr. Wolfram Riedel

Date: 11 Dec 2009

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended and promulgated on 20 June 2002 (BGB1. I Nr. 40 S. 2090), revised 31 October 2006 (BGB1. I Nr. 50 S. 2407).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organization for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility is periodically inspected by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed Quality Assurance Statement, listing all performed audits, is included in the report.

The test method is part of the BSL BIOSERVICE accreditation scope according to guideline 90/385/EWG, 93/42/EWG and DIN EN ISO/IEC 17025 for testing of medical devices.

5.2. Guidelines

This study followed the procedures indicated by internal BSL BIOSERVICE SOPs (1) and the following internationally accepted guidelines and recommendations:

Biological evaluation of medical devices:

ISO 10993-1: 2009, "Evaluation and testing within a risk management process" (2)

ISO 10993-5: 2009, "Tests for *in vitro* cytotoxicity" (3)

ISO 10993-12: 2007, "Sample preparation and reference materials" (4)

5.3. Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to GLP-regulations:

A copy of the final report, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the Sponsor concerning the study.

If test item is left, a sample will be stored according to the period fixed by the GLP Regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the Sponsor's prior consent.

Unless otherwise agreed upon, remaining test item will be discarded three months after release of the report.

6. Statement of Compliance

BSL BIOSERVICE

Study No.: 094861

Test Item: PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

Title: *In vitro* Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

Study Director: Dipl.-Biol. Dagmar Lehmeier

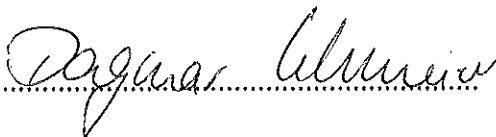
This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended and promulgated on 20 June 2002 (BGB1. I Nr. 40 S. 2090), revised 31 October 2006 (BGB1. I Nr. 50 S. 2407).

"OECD Principles of Good Laboratory Practice", as revised in 1997, Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dipl.-Biol. Dagmar Lehmeier

.....

Date: 14 Dec 2003

7. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.: 094861

Test Item: PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

Title: *In vitro* Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

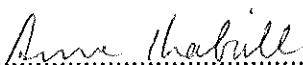
Study Director: Dipl.-Biol. Dagmar Lehmeier

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

Audit	Dates of QAU Inspections	Dates of Reports to the Study Director and Management
<i>Study Plan</i>	30 November 2009	30 November 2009
<i>Experimental Phase (Method Audit)</i>	14 September 2009	14 September 2009
<i>Report</i>	10 December 2009	10 December 2009

This report reflects the raw data.

Member of the
Quality Assurance Unit:

.....
Print name: Dipl.oec.troph (FH)
Anne Krabiell
Date: 11 Dec 2009.....

8. Summary

In the present study the cytotoxic effects of PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System) were analysed. Hereby, the test item was extracted under agitation for 24 ± 2 h with cell culture medium and the extract was incubated with L929 cells for 68 - 72 h. The protein content of the individual cultures was then analysed as a measure for cytotoxicity and compared to those of the controls.

In this study under the given conditions no leachable materials were released in cytotoxic concentrations from the test item.

9. Introduction

Cytotoxicity tests represent one of the easiest methods for the analysis of detrimental effects of substances. Cell culture techniques allow a rapid yet sensitive diagnosis of the biological reactivity of leachable or diffusable components of materials (5, 8).

The BCA test predicts cytotoxic or necrotic effects of medical devices or materials with good correlation to animal experiments and high sensitivity (6, 7).

The test item is analysed for its leachable cytotoxic contents in the BCA test. Cytotoxic effects lead to a reduction of the proliferation rate of the cells. This leads to a reduction in the protein content of the cell culture as compared to the control cultures and is detected colourimetrically after a 68 - 72 h incubation period via the BCA test (9, 10).

The BCA reagents are comprised of the water soluble and stable BCA (Bicinchoninic acid) and an alkaline Cu^{2+} solution. The amino acids cysteine, cystine, tryptophan and tyrosine, which are a constituent of every cell, bind to these reagents, i.e. these amino acids reduce Cu^{2+} to Cu^{+} , which then binds to bicinchoninic acid to form a water soluble violet dye. The intensity of the dye correlates with the cell number in the culture.

9.1. Aim of the Study

This *in vitro* method analyses the cytotoxic potential of the test item. The test is carried out using the mouse cell line L929 cultured with different concentrations of an extract of the test item. The vitality of the cells or potential cytotoxic effects of the extract are registered via the protein content of the cell culture as compared to the controls.

9.2. Justification for Selection of the Test System

L929 is a widely used and well established cell line for *in vitro* experiments since many years. It is known for its cloning efficiency and high proliferation rate.

9.3. Justification for Selection of the Test Method

This cell culture method is applicable for the cytotoxicity analysis of all medical devices and materials which are destined for implantation or come in contact with tissue or tissue fluids for a longer period.

10. Materials and Methods

10.1. Characterization of the Test Item

The test item and the information concerning the test item were provided by the Sponsor. All data related to the test item are the responsibility of the Sponsor and have not been verified by the test facility.

Name:	PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)
Batch No.:	919389
Specifications:	Recycled powder was taken from part cake of an EOSINT P system after build
Sterility:	unsterile
Storage:	at room temperature
Expiry Date:	not applicable
Nature of material:	synthetic polymer: Polyamide 12
Safety precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety

10.2. Extraction of the Test Item

The extraction was carried out in compliance with ISO 10993-5, -12. The test item was extracted under agitation for 24 ± 2 h in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at $37 \pm 1^\circ\text{C}$. The surface/volume ratio in the assay was $3 \text{ cm}^2/\text{mL}$ which corresponds to 100% extract concentration. The extract was processed by sterile filtration. The test item was tested as provided by the Sponsor.

10.3. Controls

Controls were set up in parallel to the test item cultures in order to confirm the validity of the test.

Negative control

The negative control, Polyethylene material (Art. No. 188.271, Lot E09060FX, Greiner), was extracted at a weight/volume ratio of 1 g/5 mL medium for 24 ± 2 h at $37 \pm 1^\circ\text{C}$.

Positive control

The positive control, Dimethylsulfoxide (DMSO 99.5%, Lot 8V011644, Applichem), was set up in a final concentration of 5% in DMEM 10% FCS.

Solvent control

A solvent control, consisting of extraction vehicle (DMEM 10% FCS) alone and treated in the same way as the treatment groups was included.

10.4. Cells

The test was carried out with L929 cells (ATCC No. CCL1, NCTC clone 929 (connective tissue mouse), clone of strain L (DSMZ)). For the test cells were cultured in 75 cm^2 culture flasks (Greiner) in DMEM (Invitrogen) with 10% FCS-Gold (PAA) at $37 \pm 1^\circ\text{C}$ and 5.0% CO_2 .

10.5. Dose Groups

1. Solvent control	DMEM 10% FCS
2. Negative control	Polyethylene extracted in DMEM 10% FCS
3. Positive control	DMSO (5%) in DMEM 10% FCS
4. Test Item	6 concentrations of the test extract: 13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

10.6. Experimental Procedure

The extract of the test item and the solvent control were diluted five times with DMEM 10% FCS at a ratio of 2:3. 100 µL of the different dilutions or 100 µL of the controls were given to 3 parallel cultures in a 96 well plate (Greiner).

Log phase L929 cultures were washed and trypsinized with Trypsin EDTA for approximately 3 minutes. The enzymatic reaction was stopped with DMEM 10% FCS and a single cell suspension was made at a density of 1.0×10^5 cells per mL. 50 µL of this cell suspension were pipetted to all cultures with the exception of the blanks. The highest concentration of the extract in the cell cultures corresponds to a surface/volume ratio of 3 cm²/mL. The cell culture plates were then incubated with the test extract for 68 - 72 h at $37 \pm 1^\circ\text{C}$, 5.0% CO₂ / 95% air.

BCA-staining

The protein contents of the individual cultures were measured colourimetrically using the BCA reagents (Uptima). The absorption at 550 nm was measured using a micro plate auto reader.

The mean absorption ($A_{550\text{nm}}$) and standard deviation of the three parallel cultures was calculated and used for assessing the percentage of growth inhibition (% G.I.) following the depicted formula:

$$\% \text{ G.I.} = 100 - 100 \times \frac{(A_{550 \text{ nm}} \text{ sample}) - (A_{550 \text{ nm}} \text{ blank})}{(A_{550 \text{ nm}} \text{ control}) - (A_{550 \text{ nm}} \text{ blank})}$$

$A_{550 \text{ nm}}$ sample:	Absorption value of the test extract
$A_{550 \text{ nm}}$ blank:	Absorption value of the blank cultures (without cells)
$A_{550 \text{ nm}}$ control:	Absorption value of the solvent control

10.7. Data Analysis

According to ISO 10993-5 (3) cytotoxic effects can be based on the protein content of the cultures, which is used as a measure for cell growth. Clear cytotoxicity is hereby defined as an effect leading to an inhibition of cell growth of more than 30% as compared to the cultures treated with solvent controls.

11. Deviations from the Study Plan

In the present study there were no deviations from the study plan.

12. Results

Test item: PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System)

	Rel. Protein content (A550) (a)						Growth inhibition in %
	1	2	3	x	±	s	
Blank	0.116	0.122	0.114	0.118	±	0.003	
Positive control (b)	0.199	0.206	0.219	0.208	±	0.008	92
Negative control (c)	1.243	1.269	1.205	1.239	±	0.026	1
Solvent control							
100% v/v	1.229	1.279	1.248	1.252	±	0.021	0
66.7% v/v	1.214	1.218	1.229	1.220	±	0.006	0
44.4% v/v	1.243	1.206	1.270	1.240	±	0.026	0
29.6% v/v	1.249	1.207	1.194	1.217	±	0.023	0
19.8% v/v	1.235	1.235	1.222	1.231	±	0.006	0
13.2% v/v	1.232	1.199	1.184	1.205	±	0.020	0
Test extract (d)							
100% v/v	1.219	1.199	1.213	1.210	±	0.008	4
66.7% v/v	1.249	1.203	1.206	1.219	±	0.021	0
44.4% v/v	1.242	1.210	1.227	1.226	±	0.013	1
29.6% v/v	1.232	1.161	1.185	1.193	±	0.029	2
19.8% v/v	1.251	1.213	1.249	1.238	±	0.017	0
13.2% v/v	1.274	1.248	1.237	1.253	±	0.016	0

(a) 3 parallel cultures, mean ± standard deviation

(b) 5% DMSO in DMEM 10% FCS

(c) PE material extracted in DMEM 10% FCS

(d) The test item was extracted under agitation in DMEM 10% FCS for 24 ± 2 h at $37 \pm 1^\circ$ C and the extract was cultured for 68 - 72 h with L929 cells at a final surface/volume ratio of 3 cm² test item / mL culture.

13. Discussion

Changes of cell proliferation due to the presence of cytotoxic substances were analysed in a cell growth inhibition test by comparing the protein content of the cell cultures treated with an extract of the test item with that of the untreated controls.

In the present study PA 2200 Reused powder (50% virgin + 50% recycled powder from EOSINT P System) was extracted under agitation for 24 ± 2 h with DMEM 10% FCS. L929 cells were then incubated for 68 - 72 h with the following end concentrations of the extract:

13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

The highest extract concentration corresponds to the ISO10993-5, -12 described surface/volume ratio of $3 \text{ cm}^2/\text{mL}$.

Growth analysis of cells cultured with the test extract showed no relevant growth inhibition of L929 cells.

The controls confirmed the validity of the study. Cell growth of the positive cultures was inhibited by 92%. The extract of the negative control did not show a relevant inhibition of cell growth (1%).

13.1. Conclusions

In this study under the given conditions no leachable substances were released in cytotoxic concentrations from the test item.

14. Distribution of the Report

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15. References

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