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# Investigation Report

No.: B0220/07

## Cytotoxicity Investigations

- I. **Customer:** Clinical House Europe GmbH  
**Monitor:** Mr. Dipl.-Ing. Geschinski
- II. **Order Number:** A178-10/07
- III. **Address:** Am Bergbaumuseum 31  
44791 Bochum
- IV. **Object of investigation:** coated sample

Delivery Description	Sample Number	Date of Receipt
Zirkonnitrid beschichtete cp grade 4 Reintitanplättchen Ø 10 x 3 mm 15 samples, non sterile	Z0370-10/07	October 1, 2007

### Remarks:

- \* The individual results of the analysed samples apply exclusively to the objects of investigation that have been placed at our disposal.
- \* This investigation report must not be duplicated or published in parts without the written permission of the BMP GmbH laboratory.



Labor für medizinische Materialprüfung GmbH

## Investigation Report B0220/07



Accredited by  
Zentralstelle der Länder  
für Gesundheitsschutz  
bei Arzneimitteln  
und Medizinprodukten  
ZLG-P-585.00.08

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1. **Investigation period:** from October 1, 2007 to October 12, 2007

2. **Inspection method:**

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations of biological evaluation of medical devices:

- DIN EN ISO 10993-1: 2003 "Evaluation and testing"
- DIN EN ISO 10993-5: 1999 "Tests for in vitro cytotoxicity"
- DIN EN ISO 10993-8: 2001 "Selection and qualification of reference materials for biological tests"
- DIN EN ISO 10993-12: 2005 "Sample preparation and reference materials"

Using a quality management system based on DIN EN ISO 17025: 2005 in accordance with:

- the inspection plan PP 096/07 and
- the respective special instructions of the BMP GmbH laboratory.

3. **Specific instructions:** none

4. **Sampling:** by the customer

5. **Sample preparation:**

The sample was delivered in a non sterilized state.

According to the customer, the sample **Z0370-10/07** was first cleaned in an ultrasonic bath for 5 min at room temperature with Aqua dest. and then sterilized with steam at 121°C for 20 min.

According to the customer, only the coated side of the sample was brought in contact with the investigation media.

## 6. Investigation conditions and results – Cytotoxicity

### 6.1 Indirect contact

#### a) Culture and investigation conditions

**Cell line used:** L929 (BioWhittaker BE71-131F)

The murine fibroblast cell line L 929 is known for its high proliferation rate and the high reproducibility of the results.

The absence of mycoplasmas is tested regularly.

**Culture medium:** RPMI 1640 + 10% FBS  
penicillin/streptomycin solution (100 units/ml; 100 µg/ml)

**Extraction method:** Extraction 72 h at 37°C in RPMI 1640.

After extraction creation of the extraction medium by using penicillin/streptomycin solution.

surface/volume ratio: 3 cm<sup>2</sup>/ml

**Negative control:** extraction medium incubated for 72 h at 37°C without material contact

**Positive control:** dilution of the negative control extraction with 10% ethanol v/v (96%)

**Incubation time:** 24 hours

**Number of test runs:** two separate investigation runs in eightfold determination (PA 18)

**Distinguishing feature:** none

#### b) Tetrazolium salt splitting test (PA 18 XTT test)

The XTT test is suitable for determining the metabolic activity of the cell culture. In the XTT test, the mitochondrial activity of the cells is determined by colorimetric quantification of the coloured tetrazolium salt (XTT) converted by the mitochondrial dehydrogenases. The vitality of the non-proliferating cells is recorded in this way. The quantification is performed in the ELISA reader at a wavelength of 450 nm. The optical density of the negative control is standardised at 100% and compared with the relative values of the material samples and the positive control. The graph summarises the two repeated investigations in eightfold determination.

Cytotoxicity of test samples was graded following the quantitative grading system listed:

Grade	Reactivity	Conditions of Cell Cultures
0	none	less than 30 % reduction of XTT value in comparison to NC
1	slight	30 % - 50 % reduction of XTT value in comparison to NC
2	moderate	50 % - 70 % reduction of XTT value in comparison to NC
3	severe	more than 70 % reduction of XTT value in comparison to NC

NC= negative control

After an extraction time of 72 hours, the test medium was given to the cells for 24 hours (incubation time).

After a 24-hour incubation period with the investigation media of the **negative control** (non-toxic), the cells reveal no reduction of the mitochondrial activity. The mitochondrial activity corresponds to the undisturbed metabolic activity of the cell line used (fig. 1).

After a 24-hour incubation period with the investigation media from the **positive control** (toxic), the cells reveal an almost complete inhibition of the mitochondrial activity down to approx. 0% relative to the negative control (fig. 1).

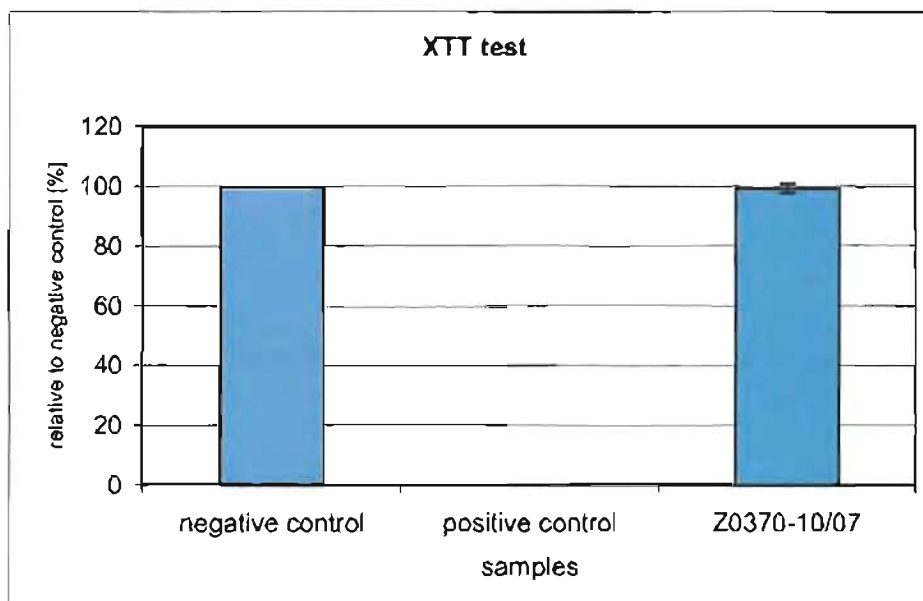


Fig. 1: Mitochondrial activity after a 24-hour incubation period with the material extracts of the sample **Z0370-10/07** (mean  $\pm$  SD; number of measurements = 16)

After a 24-hour incubation period with the investigation media of the sample **Z0370-10/07**, the cells reveal a mitochondrial activity of approx. 99% compared with the negative control (fig. 1). This measurement is above the 70% cytotoxicity threshold value and thus indicates no reduction of mitochondrial activity.

In this test system, there is no evidence of a toxic effect on the mitochondrial activity of the material extracts of the sample **Z0370-10/07**.

## 6.2 Direct contact

### a) Culture and investigation conditions

**Cell line used:** L929 (BioWhittaker BE71-131F)

The murine fibroblast cell line L 929 is known for its high proliferation rate and the high reproducibility of the results.

The absence of mycoplasmas is tested regularly.

**Culture medium:** RPMI 1640 + 10% FBS  
penicillin/streptomycin solution (100 units/ml; 100 µg/ml)

**Negative control:** commercial PTFE film

**Positive control:** commercial polyvinylchloride film

**Investigation:** two separate investigation runs (PA 17)

**Distinguishing feature:** none

### b) Membrane integrity (PA 17 vital coloration)

Testing the membrane integrity, the vitality of the cells after contact with the test sample is investigated.

The membrane integrity is determined by means of a double coloration with fluorescein diacetate (cytoplasm with intact cells; green) and ethidium bromide (DNA in cell nuclei with damaged cell membrane; red). Fluorescein diacetate easily permeates the membranes of living cells, is converted into fluorescein in the cell and can thus no longer leave vital cells. Vital cells are coloured green. Within short periods of time, ethidium bromide only penetrates cells with disturbed membrane integrity, intercalates there with the DNA of the cell and colours the cell nucleus red. Detection of vital cells is done under fluorescence microscopy with a blue filter (I3 filter, 450-490 nm), detection of damaged cells with a green filter (N2.1 filter, 515-560 nm).

Cytotoxicity of test samples was graded following the qualitative grading system listed:

Grade	Reactivity	Conditions of Cell Cultures
0	none	less than 30 % of the cells reveal disturbed membrane integrity
1	slight	30 % - 50 % of the cells reveal disturbed membrane integrity
2	moderate	50 % - 70 % of the cells reveal disturbed membrane integrity
3	severe	more than 70 % of the cells reveal disturbed membrane integrity

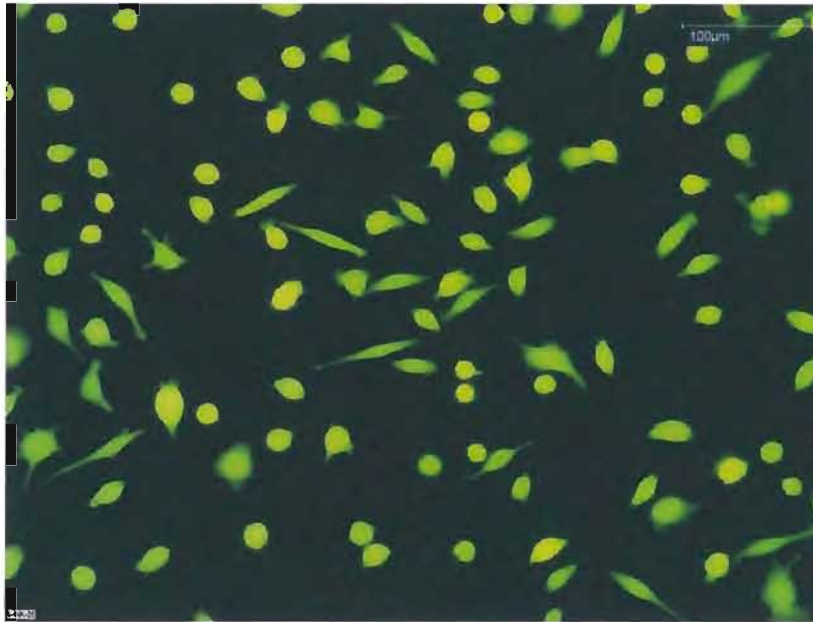
Cell attachment rate and form of cells (rounded/not rounded) were examined and expressed as differences to the negative control. These factors were examined and interpreted separately and can influence the cytotoxicity grading. In case of deviation from the grading listed above, a separate explanation is added.

After a 24-hour incubation period on the **negative control** (non-toxic), the cells do not reveal disturbed membrane integrity in each of the three investigations. After coloration, the cytoplasm appears uniformly green (blue filter, fig. 2a) and the cell nuclei are not marked with ethidium bromide (green filter, fig. 2b).

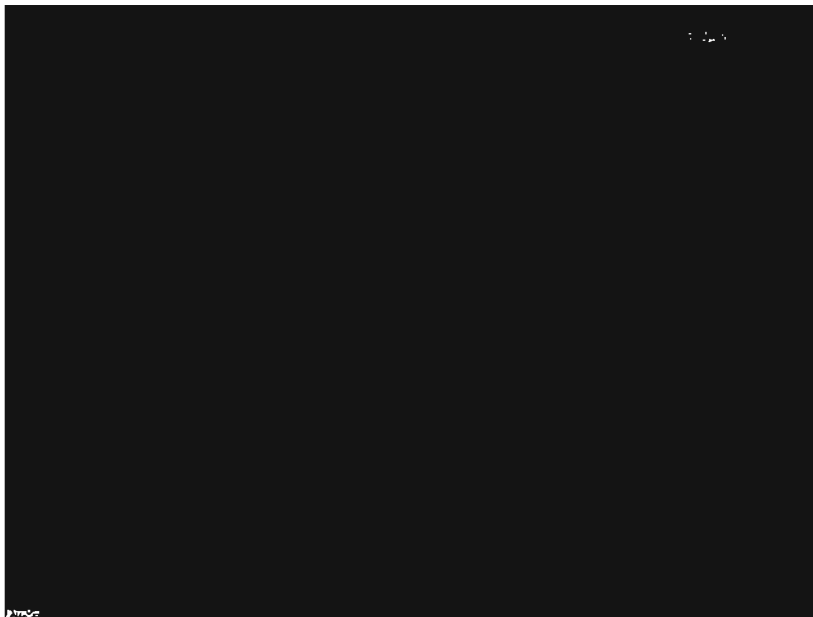
After a 24-hour incubation period on the **positive control** (toxic), the cells reveal disturbed membrane integrity in each of the three investigations. The dissemination efficiency is very strongly reduced. The cells have not absorbed any fluorescein diacetate. The cytoplasm appears to have not been coloured in the damaged cells (fig. 3a). The cell nuclei in the damaged cells fluoresce orange/red after interaction with the ethidium bromide (fig. 3b).

After a 24-hour incubation period on the sample **Z0370-10/07**, the cells reveal no changed membrane integrity in each of the three investigations compared with the negative control. The dissemination efficiency is corresponding with the negative control. After coloration, the cytoplasm appears uniformly green (blue filter, fig. 4a-c) and the cell nuclei are not marked with ethidium bromide (green filter, fig. 4d).

In this test system, there is no evidence of a toxic membrane-influencing effect from the sample **Z0370-10/07**.



**Fig. 2a:** Vital coloration of L929 cells after a 24-hour incubation period on the **negative control** (x200 magnification, blue filter)



**Fig. 2b:** Vital coloration of L929 cells after a 24-hour incubation period on the **negative control** (x200 magnification, green filter)



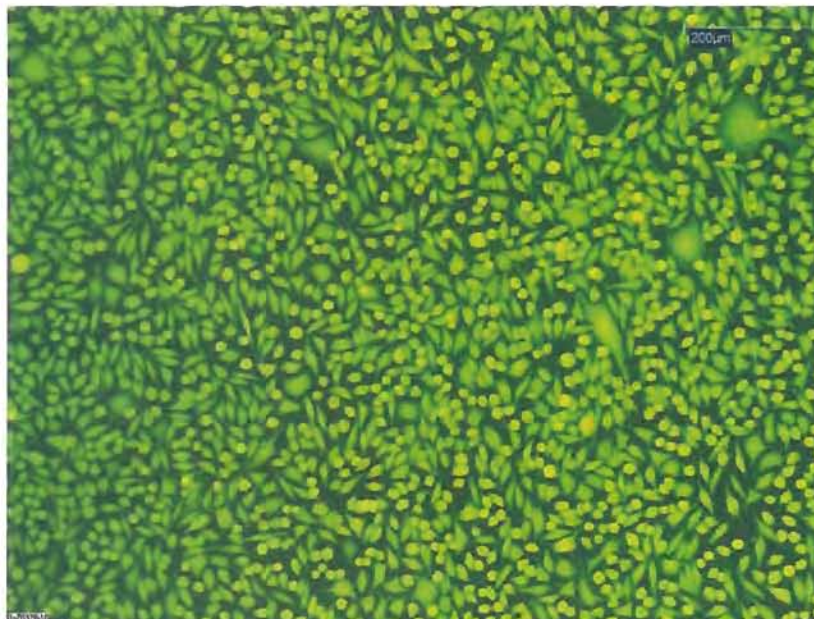
**Fig. 3a:** Vital coloration of L929 cells after a 24-hour incubation period on the **positive control** (x200 magnification, blue filter)



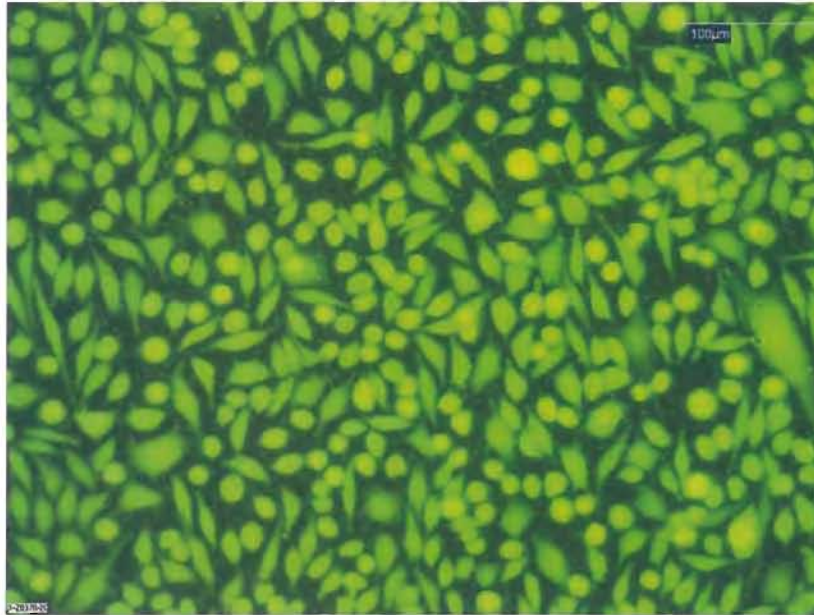
**Fig. 3b:** Vital coloration of L929 cells after a 24-hour incubation period on the **positive control** (x200 magnification, green filter)



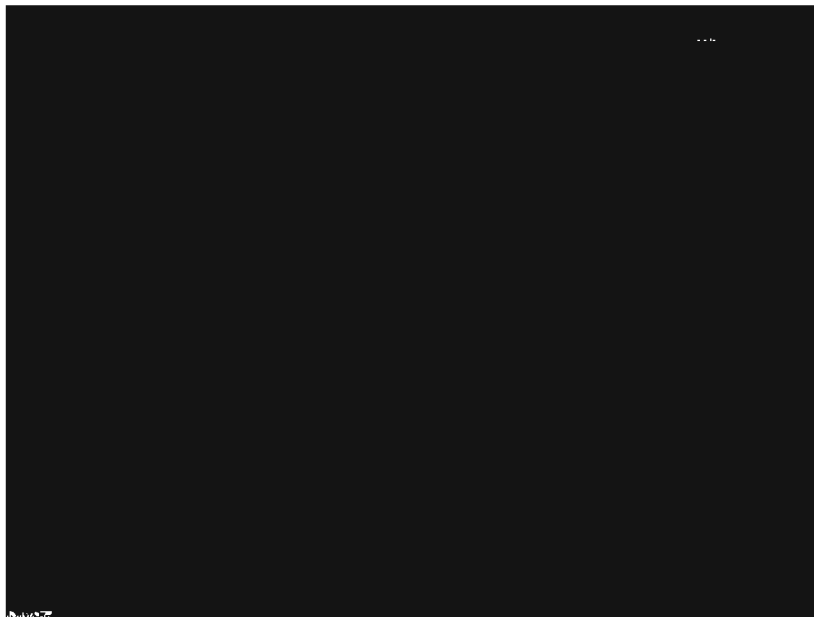
**Fig. 4a:** Vital coloration of L929 cells after a 24-hour incubation period on the sample **Z0370-10/07** (x40 magnification, blue filter)



**Fig. 4b:** Vital coloration of L929 cells after a 24-hour incubation period on the sample **Z0370-10/07** (x100 magnification, blue filter)



**Fig. 4c:** Vital coloration of L929 cells after a 24-hour incubation period on the sample **Z0370-10/07** (x200 magnification, blue filter)



**Fig. 4d:** Vital coloration of L929 cells after a 24-hour incubation period on the sample **Z0370-10/07** (x200 magnification, green filter)

## 7. Summary of the investigation results

In these investigations, the sample **Z0370-10/07** should be tested on cytotoxicity. The investigations have been carried out indirectly and directly.

For the indirect investigations, an extraction of the sample **Z0370-10/07** in cell culture medium was carried out for 72 hours at 37°C. L929 cells were incubated with the extraction medium for 24 hours to determine the influence of the extracts on mitochondrial activity.

For the direct investigations, L929 cells were incubated directly on the sample **Z0370-10/07** for 24 hours to determine the influence of the sample on membrane integrity.

### Indirect contact

- The activity of the mitochondrial dehydrogenases is not reduced after a 24-hour incubation period with the material extract of sample **Z0370-10/07** compared with the negative control.

In conclusion, the material extracts of the sample **Z0370-10/07** shows in indirect contact with L929 cells a non toxic influence measuring mitochondrial activity (XTT).

The control extracts showed the expected reactions: the **negative controls** (non-toxic) produced a reaction that could be tolerated safely; the **positive controls** (toxic) produced a toxic reaction.

### Direct contact

- There is no evidence of a membrane influencing effect of the sample **Z0370-10/07**. The disseminating efficiency of the cells corresponds to that of the negative control.

In conclusion, there is a non toxic influence after direct contact of the cells with the sample **Z0370-10/07** measuring membrane integrity.

The controls showed the expected reactions: the **negative controls** (non-toxic) produced a reaction that could be tolerated safely; the **positive controls** (toxic) produced a toxic reaction.

## 8. Judgment of the results

In these investigations, the sample **Z0370-10/07** showed no relevant cytotoxic effect according to DIN EN ISO 10993-5.

**Grade of cytotoxicity (0 to 3)**


**0 (no cytotoxicity)**

**Cytotoxicity testing (passed/failed)**

**passed**

## 9. Responsibilities


Responsible for the professional contents of this report:



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Christopher Niedhart, MD, PhD  
Technical Director  
Cytotoxicity Laboratory

Responsible for the total contents of the investigation report:



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Ute Müller, Dr.-Ing.  
Head of Laboratory  
BMP GmbH

Aachen, October 12, 2007