TECHNICAL REPORT

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Biological evaluation of medical devices —

Part 55: Interlaboratory study on cytotoxicity

Évaluation biologique des dispositifs médicaux —

Partie 55: Étude interlaboratoire sur la cytotoxicité

Reference number
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Foreword

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This document was prepared by Technical Committee ISO/TC 194, *Biological and clinical evaluation of medical devices*.

A list of all parts in the ISO 10993 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The first edition of ISO 10993-5, published in 1992, allowed several different ways to assess cytotoxicity of medical devices and gave an imprecise description of how to perform the tests. Qualitative assays were accepted and only a small amount of guidance was given for the interpretation of the results. Not surprisingly, the first interlaboratory study in 2000 resulted in quite low reproducibility of results. Therefore, detailed protocols were included into the standard and in another study the practicability of the protocols and reference materials were evaluated. The results of this second interlaboratory study mainly influenced the revision of ISO 10993-5, which was published in 2009.

This document provides the historical report of the second interlaboratory study, conducted in 2006.

Biological evaluation of medical devices —

Part 55: Interlaboratory study on cytotoxicity

1 Scope

This document describes the results of an international interlaboratory study conducted in 2006 to evaluate the performance of two different test protocols in terms of the cytotoxic effects in the biological evaluation of medical devices. The results of these tests were used for the revision of ISO 10993-5.[2] Furthermore, the results of these tests were used to estimate the accuracy of these test systems with living cells to define a threshold what is considered a cytotoxic effect.

NOTE The determination of cytotoxic effects has a high relevance in the biological evaluation of medical devices; according to ISO 10993-1[1], it is one of the very few tests which are proposed to be performed for every kind of device.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 Participants

Twelve laboratories participated in this study[1)]. Eleven reports on a neutral red uptake (NRU) assay and ten reports on a colony formation assay (CFA) were received. Four participants were commercial test laboratories, four participants were internal test laboratories of medical device manufacturers and four laboratories were in research institutes.

The laboratories were located in six different countries: one each in Austria, France and the Netherlands, and three each in Germany, Japan and the United States.

5 Materials and sample preparation

The following materials were used for the study:

a) reference material-C [RM-C; Hatano Research Institute (HRI)]: high density polyethylene sheet;

b) RM-A (HRI): segmented polyurethane film containing 0,1 % zinc diethyldithiocarbamate (ZDEC);

1) The participating laboratories were: Deutsche Institute für Textil- und Faserforschung, Germany; Hatano Research Institute, Food and Drug Safety Center, Japan; Medical University Vienna, Austria; National Institute of Health Sciences, Japan; Envigo CRS GmbH, Germany; Terumo Corporation R&D, Japan; BD Technologies, United States; NAMSA, United States; Gambro BCT, United States and three other laboratories.

c) RM-B (HRI): segmented polyurethane film containing 0,25 % zinc dibutyldithiocarbamate (ZDBC).

RM-C (HRI), RM-A (HRI) and RM-B (HRI) have been widely used as reference materials for cytotoxicity tests of medical devices. The Food and Drug Safety Center of HRI has certified these materials. HRI agreed to provide them for the interlaboratory study. Test samples were cut (2 mm × 15 mm) and sterilized with ethylene oxide (EO) and were distributed from HRI to the participants. Extraction was then performed in the participating laboratories according to the protocols.

6 Test procedures

Two test protocols were chosen by the working group developing the tests for cytotoxicity in vitro: NRU and CFA. The NRU assay protocol is based on the protocol, which was used in a validation study of Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).[4] The CFA protocol is based on the cytotoxicity test of the Japanese guidelines for basic biological tests of medical materials and devices.[5] These original protocols were modified to meet the requirements of this specific study (see Annexes A and B). The protocols were sent to the participants together with the test materials.

7 Results

7.1 Neutral red uptake

7.1.1 General

Eleven laboratories participated in this study. All test samples were extracted once as described in Annex A. Each concentration of the dilution series was tested in six replicates. The mean values were used to calculate the concentration producing 50 % inhibition of cell viability (IC_{50}) values.

7.1.2 Sodium lauryl sulfate as positive control

The laboratories used different internal reference materials as positive controls. It was therefore decided that all participants use the same common chemical substance as positive control and sodium lauryl sulfate (SLS, CAS Registry Number® 151-21-3[2)]) was selected for this purpose. Table 1 summarizes the results.

Table 1 — IC_{50}-values of SLS in the NRU assay

Laboratory	IC_{50} µg/ml
1	34,0
2	83,0
3	62,4
4	77,0
5	85,9
6	75,6
7	67,8
8	47,8
9	49,8
10	62,1
11	22,0

2) CAS Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

The variation of the IC_{50} was from 22,0 µg/ml to 85,9 µg/ml, the mean IC_{50} was (60,7 ± 20,4) µg/ml.

In Annex A, an IC_{50}-value between 70 µg/ml and 116 µg/ml was requested as acceptance criterion. This was an error in ISO 10993-5:2009 and will be removed in the next edition. Historical IC_{50}-values are typical for a specific laboratory but cannot be compared between laboratories.

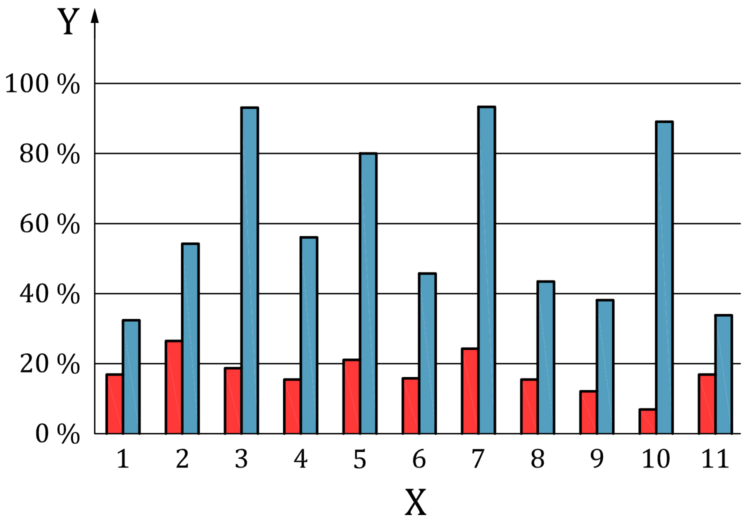
7.1.3 Test samples

The three different test samples RM-A, RM-B and RM-C were extracted as described in Annex A and the extracts were diluted as defined. The cell viabilities at different extract concentrations were determined as described in Annex A. The IC_{50} was determined from the concentration-response. This was done by using validated software, which is available in public, see Reference [6]. The results of the eleven participants are summarized in Table 2 and illustrated in Figure 1. Initially, the testing was conducted using the following concentrations of the RM-A extract: 0,25 %, 0,5 %, 1,0 %, 2,0 %, 3,0 % and 4,0 %. Unexpectedly, IC_{50}-values were higher than expected from the colony formation assay, because the neutral red assay is less sensitive probably due to the shorter exposure time. Therefore, the laboratories repeated the test with the following concentrations of the RM-A extract: 5 %, 10 %, 20 %, 30 %, 40 % and 50 %.

Table 2 — IC_{50}-values of sample extracts in the NRU assay

Laboratory	IC_{50} %		
	RM-A	**RM-B**	**RM-C**
1	16,5	32,0	—
2	26,4	54,0	—
3	18,5	93,2	—
4	15,3	56,6	—
5	20,6	79,6	—
6	15,6	45,6	—
7	24,3	93,3	—
8	15,1	43,3	—
9	11,7	38,2	—
10	6,7	89,4	—
11	16,7	33,6	—

Results for RM-A varied from 6,7 % to 26,4 %, mean IC_{50} was (17,0 ± 5,5) %. Results for RM-B varied from 32,0 % to 93,3 %, the mean IC_{50} was (59,9 ± 24,4) %.



Key

X laboratory number

Y IC_{50}

reference material A

reference material B

Figure 1 — Comparison of IC_{50}-values of sample extracts in the NRU assay

7.2 Colony formation assay

7.2.1 General

The test samples RM-A, RM-B and RM-C were those materials, which were already recommended to be used as reference materials in the Japanese Guidelines for the colony formation assay. For the study, only these materials were used by the laboratories, to assess the differences between individual laboratories. The test samples were extracted as described in Annex B and the extracts were diluted as proposed. The cell viabilities at different extract concentrations were determined by counting the colonies formed [plating efficiency (PE)]. IC_{50} was calculated from the dose-response curve as the dose with 50 % PE which was calculated from the line which passed through a dose with higher PE and a dose with lower PE than 50 %.

Ten of the twelve participants in the NRU study also participated in the CFA and communicated their results. All test samples were extracted once as described in Annex B. Each concentration of the dilution series was tested in triplicate. The mean values were used to calculate IC_{50}-values.

The plating efficiency of the controls in the different laboratories is listed in Table 3.

Table 3 — Control PE of the CFA in the participating laboratories

Laboratory	Plating efficiency %
1	68,0
2	62,8
3	75,7
4	101,5
5	92,2
7	71,2
8	108,7
9	85,0
10	75,0

Table 3 *(continued)*

Laboratory	Plating efficiency %
12	106,3

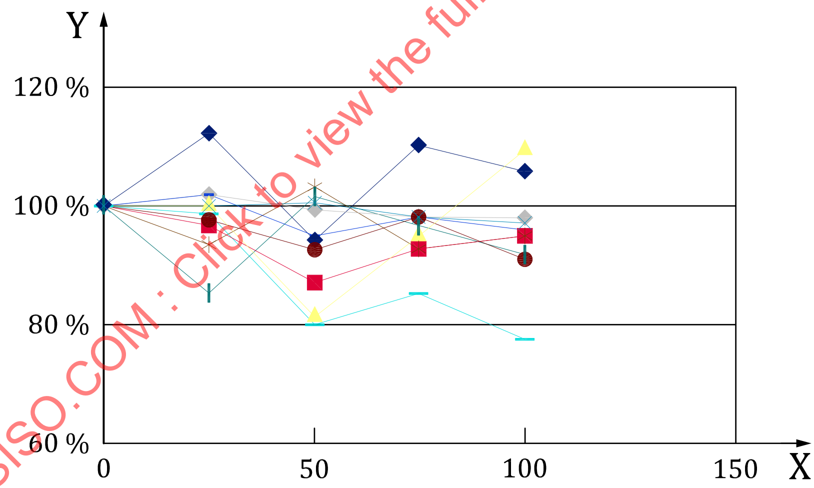
The PE in the controls varied from 62,8 % to 108,7 %, mean value was (84,6 ± 15,8) %.

7.2.2 Negative reference material

The test sample RM-C is certified not to give any positive response in the test. Nevertheless, the extract of RM-C was used in this study to detect the variation of results in this biological system. The results of the ten participants are summarized in Table 4 and illustrated in Figure 2.

Table 4 — Plating efficiencies of RM-C in the CFA

Concentration of RM-C %	Plating efficiency %									
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
25	112,0	96,8	100,0	100,2	93,3	97,8	85,3	102,4	98,7	101,9
50	94,0	87,1	81,5	100,5	103,1	92,6	101,8	94,9	79,6	99,4
75	110,0	92,9	96,0	97,5	92,9	98,2	96,6	98,5	85,3	98,4
100	106,0	94,8	110,1	97,2	95,1	91,2	92,0	96,1	77,8	98,1



Key

X extract concentration, %

Y platting efficiency of the control, %

laboratory 1
laboratory 2
laboratory 3
laboratory 4
laboratory 5
laboratory 7
laboratory 8
laboratory 9
laboratory 10
laboratory 12

Figure 2 — Plating efficiencies of RM-C in the CFA

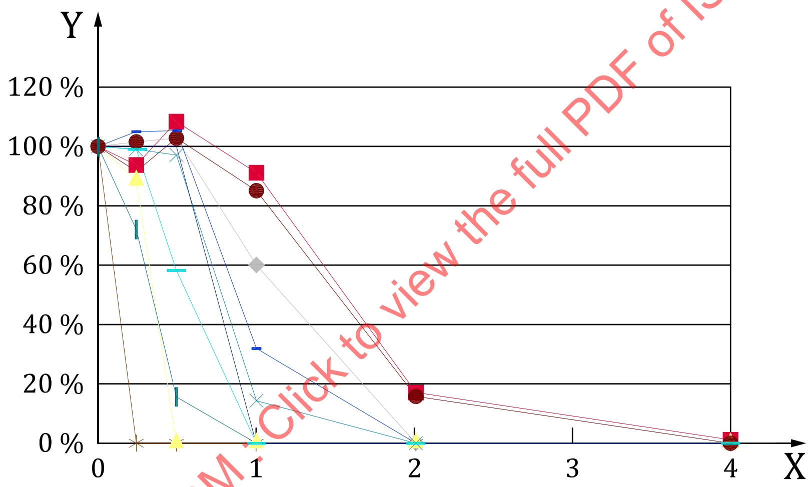
7.2.3 Positive reference materials

Extracts of the test samples RM-A and RM-B were applied in the CFA as indicated in Annex B. The results of the ten participants are summarized in Table 5 and Table 6 and illustrated in Figure 3 and Figure 4.

Table 5 — Plating efficiencies of RM-A in the CFA

Concentration of RM-A %	Plating efficiency %									
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
0,00	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
0,25	100,0	93,5	90,6	99,5	0,0	92,2	71,8	105,1	100,9	101,9
0,50	101,0	108,3	0,7	96,9	0,0	102,9	15,6	105,5	58,6	102,5
1,00	0,0	91,3	0,0	14,4	0,0	85,2	0,0	32,1	0,4	61,1
2,00	0,0	17,0	0,0	1,0	0,0	16,3	0,0	0,0	0,0	0,0
4,00	0,0	1,1	0,0	0,3	0,0	0,9	0,0	0,0	0,0	0,0
8,00	0,0	0,5	0,0	0,0	0,0	0,5	0,0	0,0	0,0	0,0
	Concentration of RM-A %									
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
IC_{50}	0,75	1,56	0,36	0,78	—	1,51	0,35	0,88	0,55	1,13

The IC_{50} of RM-A concentrations ranged from 0,35 % to 1,56 %, mean IC_{50} was (0,87 ± 0,42) %.



Key

X extract concentration, %

Y platting efficiency of the control, %

laboratory 1

laboratory 2

laboratory 3

laboratory 4

laboratory 5

laboratory 7

laboratory 8

laboratory 9

laboratory 10

laboratory 12

Figure 3 — Plating efficiencies of RM-A in the CFA

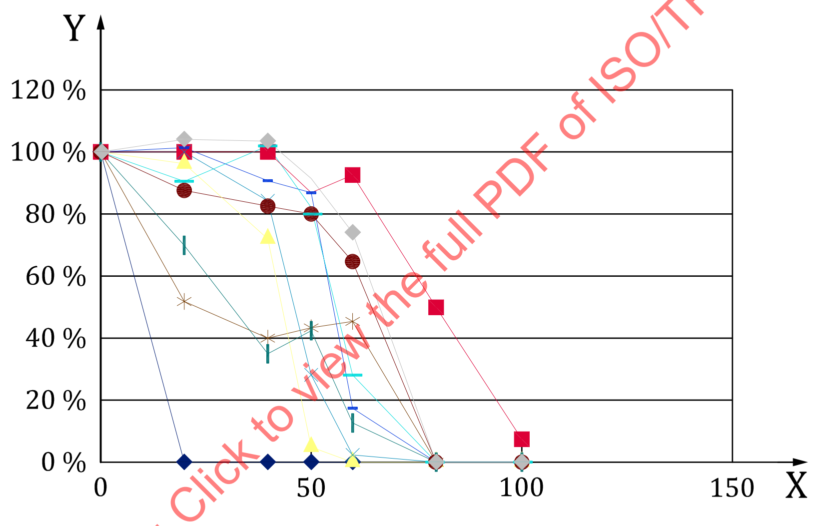
Table 6 — Plating efficiencies of RM-B in the CFA

Concentration of RM-B %	Plating efficiency %									
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
20	0,0	100,4	96,9	100,8	52,1	88,0	70,2	101,5	90,6	104,1

Table 6 *(continued)*

Concentration of RM-B %	Plating efficiency % Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
40	0,0	99,8	73,1	84,1	39,8	83,3	34,7	90,6	102,2	103,1
50	0,0	88,2	5,7	27,6	43,4	79,6	42,6	87,9	82,8	92,2
60	0,0	92,9	0,0	2,0	45,9	65,2	12,3	17,6	28,1	74,9
80	0,0	49,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,6
100	0,0	7,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
	Concentration of RM-B % Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 12
IC_{50}	—	79,95	43,43	46,04	23,41	64,66	33,96	55,39	55,94	66,70

The IC_{50} of RM-B concentrations ranged from 23,41 % to 79,95 %, mean IC_{50} was (52,2 ± 16,5) %.



Key

X extract concentration in %

Y platting efficiency in % of the control

laboratory 1

laboratory 2

laboratory 3

laboratory 4

laboratory 5

laboratory 7

laboratory 8

laboratory 9

laboratory 10

laboratory 12

Figure 4 — Plating efficiencies of RM-B in the CFA

8 Assessment of results

Two different tests were used in this study to determine cytotoxicity of three different test samples, which are recommended as reference materials in this document. The study demonstrated that both tests are suitable for this purpose. Despite a considerable variation of the results between the participating laboratories, the assessment resulted in the same conclusions for nearly all participants.

The negative reference material RM-C was non-cytotoxic in both tests and for all laboratories. The strongest variation from this result was a plating efficiency for the 100 % extract in one laboratory, which was reduced to 77,8 %. As the non-toxic effect had already been certified, all extract concentrations ought to have resulted in a 100 % plating efficiency. Therefore, it was considered that the variations found in the CFA test for RM-C reflect the measurement uncertainty of this kind of test on extracts with living cells and a threshold value was introduced in ISO 10993-5:2009; only a reduction of cell viability of more than 30 % is now considered as a cytotoxic effect.

All laboratories found a severe cytotoxicity caused by RM-A in both test systems. IC_{50} values were in a quite close range and were clearly lower in the CFA test than in the NRU test. This is expected because contact duration to the extract is one day in NRU but six days in CFA. All groups met the validation criteria for the CFA test (IC_{50} < 7 %).

RM-B showed a much broader variation of the results. For all participants, the material was significantly cytotoxic [100 % extracts had viabilities of 0 % to 14 % for NRU (data not shown) and 0 % to 7 % for the CFA test (see Table 6)], but the cytotoxic effect was much lower than for RM-A. IC_{50} values were similar in both tests, but variation of the results was broader.

The study demonstrated that the proposed testing protocols for the colony formation assay and the neutral red uptake assay are suitable to assess cytotoxic effects of medical devices after extraction. These protocols were therefore included in ISO 10993-5:2009. The testing of the 100 % extract gives a secured finding of extractable components with a cytotoxic potential, the possible grade of cytotoxicity can be estimated by testing a concentration series.

Annex A
(informative)

Interlaboratory study protocol for the neutral red uptake cytotoxicity test

A.1 General aspects

The test protocol is based on Annex C of Reference [4].

The following protocol describes only those parts of Annex C of Reference [4], relevant for the implementation of the interlaboratory test. For the general parts (background, rationale as well as general cell culture procedures like the preparation of a stock), refer to the original document.

A.2 Experimental procedure

A.2.1 Basic procedure

The BALB/c 3T3 cells were seeded into 96-well plates and maintained in culture for 24 h (approximately one doubling period) to form a semi-confluent monolayer (see Reference [4] for more information on cell maintenance and culture procedures). Cells were then exposed to the test compound over a range of eight concentrations. After 24 h exposure, NRU was determined for each treatment concentration and compared to that determined in control cultures. For each treatment (i.e. concentration of the test chemical), the percent inhibition of growth was calculated. The IC_{50} (i.e. the concentration producing 50 % reduction of NR uptake) was calculated from the concentration-response and expressed as µg/ml.

A.2.2 Test limitations

Test limitations as they were described in Reference [4] for the neutral red assay were considered for this test.

A.2.3 Material

A.2.3.1 Cell lines

BALB/c 3T3 cells, clone 31 (e.g. ECACC # 86110401, European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK; CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA).

A.2.3.2 Technical equipment

The following technical equipment was used:

— incubator, 37 °C, humidified, 5 % CO_2/air;

NOTE 1 The original document asked for 7,5 % CO_2/air because cells are very sensitive to pH-changes but was changed to use 5 %, which is more common in most laboratories and to add HEPES (4-(2-Hydroxyethyl) Piperazine-1-Ethanesulfonic Acid) for better buffering additionally.

— laminar flow clean bench (standard: "biological hazard");

— water bath, 37 °C;

NOTE 2 Incubation of solutions is technically feasible in an incubator.

— inverse phase contrast microscope;

— laboratory burner;

— centrifuge (optionally: equipped with microtiter plate rotor);

— laboratory balance;

— 96-well plate photometer equipped with 540 nm filter;

— shaker for microtiter plates;

— cell counter or haemocytometer;

— pipetting aid;

— pipettes, 8-channel-pipettes and dilution block;

— cryotubes;

— tissue culture flasks (80 cm^2, 25 cm^2);

— 96-well tissue culture microtiter plates.

A.2.3.3 Chemicals, media and sera

The following chemicals, media and sera were used:

— Dulbecco's Modification of Eagle's Medium (DMEM) without L-glutamine;

— L-glutamine 200 mM;

— Newborn calf serum (NBCS).

ATTENTION — Foetal calf serum (FCS) was not used. FCS causes a strongly reduced optical density (OD) due to the formation of vacuoles in the cells.

Due to lot variability of NBCS, each lot was first checked for growth stimulating properties with 3T3 cells (20 h to 25 h doubling time) and then reserved in sufficient quantity.

— Trypsin/ethylenediaminetetraacetic acid (EDTA) solution.

— Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization).

— HEPES.

— PBS with Ca^{2+} and Mg^{2+}(for rinsing).

— Penicillin/streptomycin solution.

— Neutral red (NR).

— Dimethyl sulfoxide (DMSO), analytical grade.

— Ethanol (ETOH), analytical grade.

— Glacial acetic acid, analytical grade.

— Distilled H_2O or any purified water suitable for cell culture.

A.2.3.4 Preparations

All solutions (except NR stock solution, NR medium and NR desorbing solution), glassware, etc., were sterile and all procedures were carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

A.2.3.5 Media

A.2.3.5.1 General

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

a) for freezing:

- 20 % NBCS, and
- 7 % to 10 % DMSO;

b) for routine culture:

- 10 % NBCS,
- 4 mM glutamine or glutamax,
- 100 IU penicillin,
- 100 µg/ml streptomycin, and
- 20 mM HEPES;

c) for treatment with test samples:

- 5 % NBCS,
- 4 mM glutamine or glutamax,
- 100 IU penicillin,
- 100 µg/ml streptomycin, and
- 20 mM HEPES.

NOTE The serum concentration of treatment medium was reduced to 5 %, since serum proteins can mask the toxicity of the test substance. The serum was not totally excluded because the cell growth is markedly reduced in its absence.

Complete media was kept at 4 °C and stored for less than two weeks.

A.2.3.5.2 Neutral red stock solution 0,4 %

The neutral red stock solution (0,4 %) was composed of:

- 0,4 g NR dye;
- 100 ml H_2O.

Solution was made prior to use and stored in the dark at room temperature for up to two months. As an alternative liquid NR stock solutions were permissable. The expiry dates were noted.

A.2.3.5.3 Neutral red medium

The neutral red medium was composed of:

— 1 ml NR stock solution;

— 79 ml DMEM.

IMPORTANT — The final concentration was 0,005 %. This was considered when using another stock concentration.

The NR medium was incubated overnight at 37 °C and centrifuged at 600 × g (where is g the gravitation) for 10 min (to remove NR crystals) before it was added to the cells. Alternative procedures (e.g. millipore filtering) were permitted as long as they guaranteed that NR medium was free of crystals. Aliquots of the NR Medium were maintained at 37 °C (e.g. in a water bath or incubator) before being added to the cells and used within 30 min of preparation but also after 15 min of removal from 37 °C storage.

A.2.3.5.4 Ethanol/acetic acid solution

The ethanol/acetic acid solution was composed of:

— 1 % glacial acetic acid solution;

— 50 % ethanol;

— 49 % H_2O.

Solution was prepared immediately prior to use. It was stored for less than 1 h (the solutions quickly form an aldehyde and the resulting desorption is worse).

A.2.3.6 Preparation of sample extract

A.2.3.6.1 General

Within the study defined biomaterials were used instead of test chemicals. Samples were extracted according to ISO 10993-5. Test samples were cut (2 mm × 15 mm) and sterilized with EO, then were distributed from HRI. According to ISO 10993-12[3], 6 cm^2/ml can be used as the extraction ratio for films. As it was difficult to fill 100 of these stripes into 10 ml extraction medium, the extraction conditions were reduced to 0,1 g/ml for all tests. This ratio was used in several publications with these materials.

A.2.3.6.2 Extraction of the test samples

One gram of sample was cut into small pieces and placed in a sterile 15 ml screw-cap vial and 10 ml of medium C (see A.2.3.5) was added (the final concentration was 0,1 g/ml). The vial (with the cap loosely closed) was placed in the (37 ± 1) °C incubator for (24 ± 2) h. The extract was separated by decantation, being the designated 100 % extract. The 100 % extract was diluted with medium C to give the various percentages of diluted extract.

A.2.4 Methods

A.2.4.1 General

For routine methods, see Annex C of Reference [4].

A.2.4.2 Quality check of assay (I): Positive control (PC)

A positive control was permitted in the test.

Of the many chemicals backed by sufficient history or intralaboratory and interlaboratory repeat tests sodium lauryl sulfate (SLS, CAS # 151-21-3) is one of the most frequently tested, and was therefore recommended as a PC. It was recommended that SLS be tested in a 4-concentration scale: 0,05 mg/ml; 0,1 mg/ml; 0,15 mg/ml; 0,2 mg/ml.

The historical mean IC_{50} of SLS (see Reference [7]) is 0,093 mg/ml.

The 95 % CI is 0,070 mg/ml to 0,116 mg/ml.

IMPORTANT — A test met acceptance criteria, if the IC_{50} for SLS was within the 95 % CI.

A.2.4.3 Quality check of assay (II): Vehicle control (VC)

The absolute value of optical density (OD_{540} of NRU) obtained in the untreated vehicle control (medium C in A.2.3.5) indicated whether the 1 × 10^4 cells seeded per well had grown exponentially with normal doubling time during the two days of the assay.

IMPORTANT — A test met acceptance criteria if the mean OD540 of VCs was ≥0,3.

To check for systematic cell seeding errors, VCs were treated under extraction conditions (see A.2.3.6) were placed both at the left side (row 2) and the right side (row 11) of the 96-well plate, see Annex E in Reference [4].

IMPORTANT — A test met acceptance criteria if the left and the right mean of the VCs did not differ by more than 15 % from the mean of all VCs.

Checks for cell seeding errors were also performed by examining each plate under a phase contrast microscope to ensure that cell quantity is consistent. Microscopic evaluation obviates the need for two rows of VCs.

A.2.4.4 Quality check of concentration-response

The IC_{50} derived from the concentration-response was backed by at least two, or if possible, three responses between 10 % and 90 % inhibition of NRU. If this was not possible and the concentration progression factor could be reduced, the experiment was rejected and it was repeated with a smaller progression factor.

A.2.4.5 Concentrations of test samples

The following concentrations of test samples were used:

— RM-A (positive RM): 4,0 %, 3,0 %, 2,0 %, 1,0 %, 0,5 %, and 0,25 %;

— RM-B (positive RM): 100 %, 80 %, 60 %, 50 %, 40 %, and 20 %;

— RM-C (negative RM): 100 %, 75 %, 50 %, and 25 %;

— 100 % = 0,1 g/ml.

NOTE The test with RM-A were repeated with concentrations 50 %, 40 %, 30 %, 20 %, 10 % and 5 % because IC_{50} values were higher than expected.

A.2.4.6 Test procedure

A.2.4.6.1 General

See Table A.1 for a flow chart of the test procedure. A template table for documenting the relevant data generated by the BALB/c 3T3 NRU assay was given together with the samples.

A.2.4.6.2 First day after growing up the cells from frozen stock

After thawing from stock, cells were passaged two to three times before using in the test.

— A cell suspension of 1 × 10^5 cells/ml in culture medium was prepared. A multi-channel pipette dispensed 100 µl of culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (as blanks, see Annex E of Reference [4]). In the remaining wells, 100 µl of a cell suspension of 1 × 10^5 cells/ml was dispensed (1 × 10^4 cells/well). One plate per material to be tested was prepared, one plate for the PC and one plate for the negative control material if available.

— Cells were incubated for 24 h (5 % CO_2, 37 °C) so that cells formed a half-confluent monolayer. This incubation period ensured cell recovery and adherence, and progression to exponential growth phase.

— Each plate was examined under a phase contrast microscope to ensure that cell growth was relatively even across the microtiter plate. This check was performed to identify experimental errors.

A.2.4.6.3 Second day

— After 24 h incubation, culture medium was aspirated from the cells.

— Per well, 100 µl of treatment medium containing either the appropriate concentration of sample extract, or the negative control, or the PC, or nothing but VC was added.

— Cells were incubated for 24 h (5 % CO_2, 37 °C).

A.2.4.6.4 Third day

A.2.4.6.4.1 Microscopic evaluation

After 24 h treatment, each plate was examined under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Changes in morphology of the cells due to cytotoxic effects of the sample extract were recorded, but these records were not used for the calculation of the highest tolerable dose (HTD) or any other quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells indicated experimental error and were cause for rejection of the assay.

A.2.4.6.4.2 Measurement of NRU

This method was based upon Reference [8]. The uptake of NR into the lysosomes/endosomes and vacuoles of living cells was used as a quantitative indication of cell number and viability.

— Cells were washed with 150 µl pre-warmed PBS. The washing solution was removed by gentle tapping. The 100 µl NR medium was added and incubated at 37 °C in a humidified atmosphere of 5 % $C0_2$ for 3 h.

— After incubation, the NR medium was removed and cells were washed with 150 µl of PBS.

— PBS was decanted and blotted totally (optionally: the reversed plate was centrifuged).

— Exactly 150 µl of NR desorbing solution (ETOH/acetic acid) was added to all wells, including blanks.

— The microtiter plate was shaken rapidly on a microtiter plate shaker for 10 min until NR was extracted from the cells and formed a homogeneous solution.

— The absorption of the resulting coloured solution was measured at 540 nm in a microtiter plate reader, using the blanks as a reference. Raw data was saved in a file format (e.g. ASCII, TXT, XLS) appropriate for further analysis of the concentration-response and calculation of IC_{50}.

Table A.1 — 3T3 NRU cytotoxicity test: Flow chart

Time h	Procedure which was followed
00:00	Seed 96-well plates: 1 × 10^4 cells / 100 µl DMEM culture medium / well Incubate (37 °C / 5 % CO_2 / 22 h to 24 h) ↓
23:00	Remove culture medium ↓
24:00	Treat with eight concentrations of test sample extract in treatment medium (100 µl) (untreated vehicle control = treatment medium) Incubate (37 °C / 5 % CO_2 / 24 h) ↓
48:00	Microscopic evaluation of morphological alterations Remove treatment medium Wash once with 150 µl of PBS Add 100 µl of NR medium Incubate (37 °C / 5 % CO_2 / 3 h) ↓
51:00	Discard NR medium Wash once with 150 µl of PBS Add 150 µl NR desorbing solution (ETOH/acetic acid solution) ↓
51:40	Shake plate for 10 min
51:50	Detect NR absorption at 540 nm (i.e. cell viability)

A.2.5 Data analysis

A calculation of cell viability expressed as NRU was made for each concentration of the test sample extract by using the mean NRU of the six replicate values per test concentration. This value was compared with the mean NRU of all VC values (provided VCs met the VC acceptance criteria). Relative cell viability was then expressed as percent of untreated VC. If achievable, the eight concentrations of each compound tested spanned the range of no effect up to total inhibition of cell viability.

Where possible, the concentration of a test chemical reflecting a 50 % inhibition of cell viability (i.e. the IC_{50}) was determined from the concentration-response. This was done either by applying:

— a manual graphical fitting method: probability paper with “x = log” and “y = probit” scales was used because in most cases the concentration-response function became almost linear in the relevant range. Semi-log paper was an alternative for this technique; or

— any appropriate nonlinear regression procedure (preferably a Hill function or a logistic regression) to the concentration response data was used; before use of IC_{50} for further calculations, the quality of the fit was appropriately checked.

NOTE A Hill function is monotonous and sigmoidal in shape and represents an acceptable model for many dose response curves.

A complete set of data was requested from every participating laboratory.

A.2.6 Test report

The following items were recorded in the test report. A template for the data was given to the participants with the samples.

— name and address of testing facility;

— name of the person(s) who conducted the test as study director and project member;

— starting and finishing dates of the test;

— cell lines used in the test and sources;

— name of company and batch of medium, serum and antibiotics when added;

— extraction condition and procedure;

— all test data;

— evaluation and discussion based on the relevant data.

Annex B
(informative)

Interlaboratory study protocol for the colony formation cytotoxicity test

B.1 General aspects

The test protocol was based on part I of the Cytotoxicity Test of the Japanese Guidelines for Basic Biological Tests of Medical Materials and Devices.[5] The following description covers only those parts of part I, relevant for the implementation of this interlaboratory test.

B.2 Experimental procedure

B.2.1 Basic procedure

V79 cells were seeded into 6-well plates and maintained in culture for 24 h to start growing in a log phase. Cells were then exposed to the test compound over a range of concentrations. Cells were incubated for 6 d to make colonies large enough to count. Colonies were fixed with methanol, stained with Giemsa solution, and counted. The IC_{50} (the concentration inhibiting plating efficiency to 50 %) was calculated and expressed as % of the extract.

B.2.2 Test limitations

Test limitations were already considered for the study.

B.2.3 Material

B.2.3.1 Cell line

V79 cells (JCRB 0603, Human Science Research Resources Bank, Osaka, Japan, available from other cell banks of USA and EU) was used.

NOTE V79 cells were recommended because they make large and clear colonies.

B.2.3.2 Technical equipment

The following technical equipment was used:

— incubator: 37 °C, humidified, 5 % CO_2/air;

— laminar flow clean bench (standard: “biological hazard”);

— water bath: 37 °C;

— inverted phase contrast microscope;

— stereomicroscope;

— laboratory burner;

— centrifuge (rotation speed: 1 000 rpm);

— centrifuge tubes (15 ml);

— laboratory balance;

— cell counter or haemocytometer;

— pipetting aid;

— pipettes;

— tissue culture flasks (75 cm^2, 25 cm^2) or tissue culture dish with a diameter of 100 mm;

— 6-well tissue culture plates (diameter of 35 mm, e.g. Costar 3516) or culture dish with a diameter of 35 mm.

B.2.3.3 Chemicals, media and sera

The following chemicals, media and sera were used:

— Eagle minimum essential medium (MEM) containing Earle's balanced salt solution;

— FCS;

NOTE 1 Due to lot variability of FCS, each lot was checked for growth stimulating properties with V79 cells and then reserved in sufficient quantity.

NOTE 2 No other cell culture medium than Eagle MEM was used, because the medium composition influences the results. Cells delivered in another medium were allowed to grow for at least two passages in Eagle MEM before use in the study.

— trypsin/EDTA solution;

— PBS without Ca^{2+} and Mg^{2+} (for trypsination);

— penicillin/streptomycin solution;

— DMSO, analytical grade;

— methanol, analytical grade;

— Giemsa's solution;

— phosphate buffered solution (for dilution of Giemsa's solution), any kind, pH 6,5 to pH 7,5;

— distilled H_2O or any purified water suitable for cell culture;

— sodium bicarbonate;

— L-glutamine;

— MEM non-essential amino acids solution;

— sodium pyruvate 100 mM.

B.2.3.4 Reference materials (RMs)

B.2.3.4.1 Negative reference material

The negative RM was the material, certified not to give any positive results when tested according to the procedure in B.2.4.1, such as RM-C (HRI): high density polyethylene sheet.