



**National Institute of Public Health
Centre for Laboratory Testing**



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Laboratory No.1206, accredited by Czech Accreditation Institute
according to EN ISO/IEC 17025:2017

Test Report No.3/20/261-262

Customer: RESPILON Group s. r.o.

Address: Cejl 12, 602 00 Brno, Czech Republic

Reference No.: CTZB 187-14067/2020

Test Material

Identification:

TM 3/20/261: RespiPro VK - RPVK-M/L/S-000

TM 3/20/262: RespiPro White - RPW-M/L/S-000

Laboratory Tests

SOP 1/3 Tests for in vitro cytotoxicity (EN ISO 10993–5: 2009 Biological evaluation of medical devices – Part 5, Articles 1, 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.3, 8.5, 9, 10, Annex A)

Sample reception date: 8.12.2020

Date of study: 11.1. - 15.1.2021

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Authorized by Technical Manager: Hana Bendová, M.Sc., Ph.D.

The tests were performed at the address of the laboratory. The test results refer only to the sample as submitted by the sponsor and to the objectives of the study. This test report does not substitute for any other document or certification of the product. Without written approval of the testing laboratory this report should not be reproduced in other form than as a whole.

TEST REPORT CYTOTOXICITY IN VITRO

Testing facility: Laboratories of Toxicology (Centre for Laboratory Testing, National Institute of Public Health, Šrobárova 49/48, 100 00 Prague 10, Czech Republic).

The test was carried out in compliance with: SOP 1/3 Tests for in vitro cytotoxicity. (EN ISO 10993-5: Biological evaluation of medical devices – Part 5, Articles 1, 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.3, 8.5, 9, 10, Annex A).

Aim of the study: Assessment of the in vitro cytotoxicity of the test materials.

MATERIALS AND METHODS

TEST MATERIAL (TM):

TM 3/20/261: RespiPro VK - RPVK-M/L/S-000

TM 3/20/262: RespiPro White - RPW-M/L/S-000

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602 00 Brno

Czech Republic

CELL LINE

Balb/c 3T3 mouse fibroblasts (3T3-L1), European Collection of Cell Cultures, Great Britain, ECACC No.86052701.

CULTURE MEDIUM

DMEM (Dulbecco's Minimum Essential Medium, LONZA, Lot 734457) supplemented with antibiotics (PNC 100 IU/ml, STM 100 µg/ml, LONZA, Lot 6MB 057) and 10% of inactivated calf serum (GIBCO, Lot 1794524), pH 7.2, freshly prepared, stored no longer than 1 week.



CONTROLS

Positive control (PC) – material which provides a reproducible cytotoxic response: Dodecylsulphate sodium salt SDS (SIGMA), final concentration 1, 10, 20 µg/ml DMEM without serum.

Negative control (NC) – material which does not produce a cytotoxic response: Hydron (poly[(2-hydroxyethyl) methacrylate], Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague).

Reagent control (RC) – extraction vehicle without test material subjected to extraction conditions and test procedures: culture medium without serum.

Cell control (C) - culture medium without serum.

• TEST PROCEDURE (test on extract)

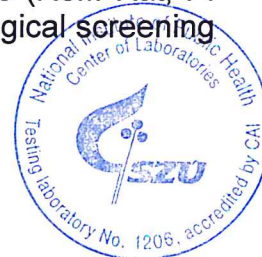
The cells were seeded into individual wells of 96-well microtitre tissue culture plates. The prepared cell suspension of 1×10^5 cells/ml DMEM was inoculated in the volume of 0.1 ml (1×10^4 cells) per well. After 24 h preincubation (37°C, 7.5% CO₂) the medium was removed and replaced by 0.2 ml of liquid extracts of the test material and controls. The extracts were diluted in culture medium without serum and all the test samples and controls were run in quadruplicates. At the end of the 24 h treatment (37°C, 7.5% CO₂) the medium was removed and the cells were stained by Neutral Red dye according to INVITTOX Protocol No. 46 (0.2 ml Neutral Red solution per well, 3 h incubation, Neutral Red desorb solution – ethanol/acetic acid). The Neutral Red uptake was measured fluorimetrically.

PREPARATION OF LIQUID EXTRACTS

The test materials were extracted according to ISO 10993-12 in the ratio of 3 cm² of the material per 1 ml of extraction vehicle (DMEM without serum) in chemically inert closed containers by using aseptic techniques. Extraction time: 24 h, extraction temperature: 37°C. The extracts were used immediately after preparation. The concentrated (100%) extracts were further diluted in DMEM without serum.

• EVALUATION OF CYTOTOXICITY

The degree of cytotoxicity (i.e. the decrease of cell viability) was quantitatively determined as the Neutral Red uptake measured by fluorescence-luminiscence reader FLX800TBI (BioTek). The evaluation of cytotoxicity by means of cold light fluorimetry is based on the incorporation of a vital dye (Neutral Red) into living cells (Neutral Red Uptake) and detection of fluorescence in the system of excitation (530 nm) and emission (590 nm) filters. The results (Fluorescence Units, FSU) obtained for wells treated with the test material were compared to untreated control wells (culture medium, 100% viability) and converted to a percentage value (Ref.: Rat, P. et al. (1994). New in vitro fluorimetric microtitration assays for toxicological screening of drugs. Cell Biology and Toxicology, 10, 329-337).



Calculation:

The mean FSU value of eight blank wells (containing only Neutral Red desorb solution) was subtracted from the mean FSU value of four treated wells (treated with the test material, positive control or culture medium).

The viability of cells was calculated as

$$\text{viability (\%)} = \frac{\text{mean FSU of test wells} - \text{mean FSU of blanks}}{\text{mean FSU of cell control wells} - \text{mean FSU of blanks}}$$

Cytotoxicity scale:

viability 70% and higher..... no cytotoxicity
 viability higher or equal to 50% and lower than 70%..... mild cytotoxicity
 viability higher or equal to 30% and lower than 50%..... moderate cytotoxicity
 viability lower than 30%..... severe cytotoxicity

RESULTS

- CYTOTOXICITY - FLUORIMETRIC EVALUATION**

Test No. 1

Test material	Fluorescence (FSU)	Viability
extract dilution	mean value	% of cell control
TM 3/20/261		
10%	3813,5	102,0
25%	3914,0	104,6
50%	4076,8	109,0
100%	3987,5	106,6
TM 3/20/262		
10%	3702,3	99,0
25%	3770,5	100,8
C	3740,3	100,0
50%	3482,0	95,9
100%	3622,3	97,0
C	3630,8	100,0
PC– SDS		
1 µg/ml	3287,0	87,9
10 µg/ml	236,0	6,3
20 µg/ml	271,5	7,3



NC	3874,8	103,6
RC	3625,8	96,9
C	3740,3	100,0

Test No. 2

Test material extract dilution	Fluorescence (FSU) mean value	Viability % of cell control
TM 3/20/261		
10%	3344,3	104,9
25%	3401,0	106,7
50%	3446,3	108,1
100%	3365,8	105,6
TM 3/20/262		
10%	3332,8	104,5
25%	3168,0	99,4
C	3187,8	100,0
50%	3215,0	101,4
100%	3270,0	103,1
C	3171,5	100,0
PC – SDS		
1 µg/ml	2933,3	92,0
10 µg/ml	1218,5	38,2
20 µg/ml	289,3	9,1
NC	3400,0	106,7
RC	3111,5	97,6
C	3187,8	100,0

Test carried out by: K. Kejlová, M.Sc., Ph.D., J. Losová

Principal investigator: K. Kejlová, M.Sc., Ph.D.

-----end of report-----

