DUADERM BARRIER CREAM OF PRODUCT
IN VITRO CYTOTOXICITY TEST REPORT
(EN ISO 10993-5:2009)

Report Number: 2025.05.14E

CONTENTS

STUDY VERIFICATION AND SIGNATURE

This study was performed on the mutually agreed working test product. The work has been completed in accordance with ISO standards. This report proves the truth and accuracy of the results obtained.

Dr. Oğuz ÖZTÜRK
Lab. Manager
Date

15.05.2025

1.TEST PRODUCT INFORMATION

Requesting Company Name	Akra Group Kozmetik ve İlaç San. Tic. A.Ş.
Requesting Company Address	Yakuplu Mh. 228. Sk. No:18 İç Kapı: 3 Beylikdüzü/İstanbul
Test Product Name (TM)	**Duaderm Barrier Cream**
Lot Number	LOT 1235-01
Reference Number	-
Production Date	
Expiration Date	01/2028
Colour	
Physical Condition	
Product Sterility	-
Storage Condition	
Product Acceptance Date	01.03.2025
Experiment Start-End Date	01.05.2025 / 05.05.2025
Report Date	15.05.2025
Test Result	According to the results of the MTT method; A viability rate of 90,71% was determined in the test product at a concentration of 100 µg/ml. According to the EN ISO 10993-5:2009 standard, the test product has no cytotoxic potential.

2.EXPERIMENT METHOD DESCRIPTION

MTT method [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]: In this method, MTT is reduced to formazan, while the color formed is measured colorimetrically. The amount of formazan formed gives the number of viable cells. MTT is a substance that is actively absorbed into cells and reduced to colored, water-insoluble formazan by a mitochondria-induced reaction. The MTT reduction of the cells are taken as a measure of cell viability and the dye intensity obtained from the MTT analysis correlates with the number of viable cells. The L-929 mouse fibroblast cell line (NCTC clone 929: CCL 1, American Type Culture Collection [ATCC] USA) was used for the cytotoxicity test study.

2.1 Extraction Protocol

Extraction of negative, positive groups and test material (see Item 3.0) was performed in accordance with EN ISO 10993-12:2021 standards. **Test Product**, which was the tested material, was used in the extraction. Negative, positive and test material extractions were performed under the same conditions and temperatures.

MEM (Eagle minimum essential medium) was used for the extraction for the sample prepared from the test product based on the ratios specified in the EN ISO 10993-12:2021 standard article 10.3.3 Table 1. The extract was prepared by keeping the Test Product in a shaking incubator at 200 rpm for 22±2 hours and 37±1 ºC, and was defined as 100% extract. Percentages of other extracts were established by dilution of MEM +10% FCS. At the end of extraction, the extract was clear, no discoloration or no particles were observed. Therefore, there is no need for additional processes such as filtration, centrifugation, pH. The extract was used within 25 minutes and was considered stable during this time. No color change was observed (In-laboratory test method).

Negative Control (NC)
High Density Polyethylene Material (HDPE) was extracted as 3 cm ^{2}/ml with MEM + 10% FCS.

Positive Control (PC)
Serial dilutions of DMSO (Dimethyl sulfoxide) (10-30 v/v) % were made with MEM + 10% FCS.

Test Product (TP) Concentrations
Test material extract was diluted 100 -30-10 -3-v/v (%) with MEM+ 10% FCS

Blank (Blank Sample)
It is an extraction tool that does not contain the test sample subjected to the same conditions that it was subjected to during extraction of the test sample (MEM+ %10 FCS).

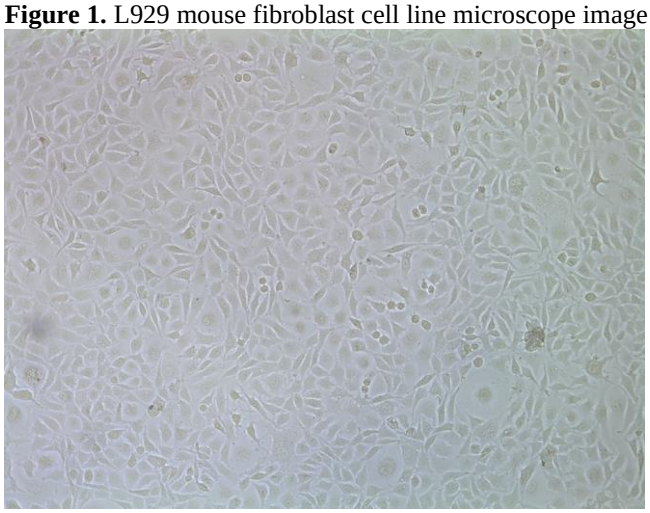
NOTE:

- The sample/ml calculation was made according to the standard surface areas and liquid extract volumes included in EN ISO 10993-12:2021 standard method 10.3.3 Table 1 considering HDPE, irregularly shaped solid devices (non-absorbent molded product), and Item 3.0 Test Material sample/ml calculation was made considering the thickness <0.5 mm group (film, sheet, pipe wall).

2.2 Cell Culture Conditions

The L-929 mouse fibroblast cell line (NCTC clone 929: CCL 1, American Type Culture Collection [ATCC]) was used for the cytotoxicity test study. No mycoplasma was observed in the stock cultures **(See. Figure 1)**. With 10% FCS (Fetal calf serum) and 2% L-glutamine It was reproduced in supplemented MEM (Eagle minimum essential medium) medium and incubated in an oven with 5% CO_2 for 24 hours at 37°C and 90% humidity.It was determined to be clean by MEM sterility test.MEM pH value was measured between 7.2 and 7.4. A mixture of 0.25% trypsin and 0.03% EDTA was used for the trypsinase of the cells according to the instructions for use of the L-929 mouse fibroblast cell line.The cells were suspended in the culture medium and 100 µl of $1x10^4$ cells per well was transferred to the 96-well plates.

Figure 1. L929 mouse fibroblast cell line microscope image



2.3 Dosing Concentrations

After 24 hours of cell culture application, the culture medium was removed and 100 µl of test material, positive and negative control extracts were added. All doses were administered at least 5 times. The culture medium was removed after the plates were examined at the end of 24 hours in a 5% CO_2 oven at 37 ^{O}C and 90 % humidity. After microscopic examination of the plate, the culture medium was removed from the wells. 50 µl of MTT solution was added to one of his test wells. Plates were incubated at 37^{O}C for 2 hours. Then, MTT solution was removed from wells and 100 µl isopropanol was added to each well. Absorbent measurements were made with a microplate reader containing a 570 nm filter and evaluation was made.

3. EQUIPMENT

Autoclave (Hanshin 60 VD)
CO_2 Incubator (Memmert INCO 108 MED)
Invert microscope (Zeiss primovert)
Electronic Balance (Shimadzu ATC224)
Clean bench (Nucleon)
Microplate reader (Thermo Scientific Multiscan Spectrum)
Water bath (Brand: JSR, Model: Jswb-11t, Serial no: 140612-04)
Cell counter or hemocytometer

4. REAGENTS

MEM (Eagle minimum essential medium) (Ref: 01-340-1B, Lot: 2013239)
MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (SIGMA, Lot No: MK.BS4732V)
Trypsin EDTA Solution (GiBco, Parti No: 1786548)
Penicillin, Streptomycin sulfate (GiBco, Parti No: 1665735) MEM (GiBco, Parti No: 8116016)
FCS (Foetal calf serum) **(**NTC, Lot No: NTC-HK007)
L-glutamine (Brand: Biological industries, Ref: 03-020-1C, Lot:2028523)

5. EXPERIMENT ACCEPTANCE CRITERIA

A-Under microscopic evaluation, cultures exposed to the test product with the negative control did not show any cytotoxic response (grade 0). Cells with a positive control showed severe cytotoxicity (grade 4). Therefore, the experiment was considered valid.

B-An experiment meets acceptance criteria when the mean OD570 values of blank samples are ≥ 0.2. A 100% viability value of the test material above 70% of the value obtained from the blank sample indicates that it has no cytotoxic potential. The positive control sample should show >30% cytotoxic activity.

6.EVALUATION CALCULATION OF CYTOTOXICITY EFFECT

% Viability value is determined according to the formula below.

$$\textbf{\% Viability} = \frac{\textbf{100 x OD570}^{\textbf{TM}}}{\textbf{OD570}^{\textbf{b}}}$$

OD_{570TM} = It is the mean value of the measured optical density of the 100 % extracts of the test sample

$OD570_{NK}$= It is the mean value of the measured optical density of the blanks.

Table 1: Qualitative morphological observations of cells (cell culture status)

Groups	Reaction	Before Inoculation	Before extract treatment	24 hours after extract treatment
Negative Control	None (grade 0)	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, no cell lysis.
Test Product	None (grade 0)	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, no cell lysis.
Positive Control	Severity (grade 4)	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmic granules, There is cell lysis.

Table 2: Absorbance values of Negative Control (NC), Blank sample (B), Test Material (TM) and Positive Control (PC) groups of test material observed in polar solvent .

Repeat Number (n)	NC	B	*TM100 %*	*TM30%*	*TM10%*	*TM 3%*	PC 30%	PC 10%
1	0,655	0,587	0,554	0,504	0,604	0,525	0,300	0,174
2	0,524	0,606	0,449	0,636	0,625	0,506	0,225	0,168
3	0,544	0,559	0,537	0,549	0,517	0,578	0,110	0,271
4	0,533	0,502	0,582	0,427	0,442	0,586	0,091	0,174
5	0,507	0,589	0,457	0,511	0,517	0,583	0,081	0,252

Table 3. The results of the quantitative measurement of cytotoxic effects of the test material with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

	Optical Density (OD) Mean Value 570 nm ± Standard Deviation (SD)				Vitality %			
	Dilution Concentrations (v/v) %				Dilution Concentrations (v/v)			
	100	*30*	*10*	*3*	*100*	*30*	*10*	*3*
Test Product	0,516±0,053	0,525±0,068	0,541±0,066	0,556±0,033	90,71	92,40	95,15	97,71

Blank	0,569±0,037				100,00			
Positive Control		0,161±0,086	0,208±0,044			28,39	36,55	
Negative Control	0,553±0,053				97,19			

7. CONCLUSION

In this study, which was carried out in line with the EN ISO 10993-5:2009 standard MTT method directives, the percent vitality value of the test product;

It was determined as 90,71% for 100% extract. According to these test results, the test product does not have cytotoxic potential.

8. RECORD STORAGE

All raw data of this study and a copy of the final report are kept in our archive for 5 years.

9. CONFIDENTIALITY AGREEMENT

Statements of confidentiality were as agreed upon prior to study initiation.

10. DEVIATION STATEMENT

There were no deviations from the approved study protocol which were judged to have any impact on the validity of the data.

REFERENCES

1.Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, EN ISO 10993-1:2018.

2. Biological Evaluation of Medical Devices - Part 5, Tests for in vitro Cytotoxicity, EN ISO 10993-5:2009.

3. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, EN ISO 10993-12:2021.

4. OECD Principles of Good Laboratory Practice. OECD Environmental Health and Safety Publications, Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1. ENV/MC/CHEM (98)17. 5. General Requirements for the Competence of Testing and Calibration Laboratories, ISO/IEC 17025:2017(E).

6. Use of International Standard ISO 10993-1, “Biological Evaluation of Medical Devices, ISO 10993 - Part 1. Evaluation and Testing Within a Risk Management Process. Guidance for Industry and Food and Drug Administration Staff. September 4, 2020.

APPROVAL