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TS EN ISO/IEC 17025
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ANALYSIS REPORT

(Industrial Services)

Report no : 18073060-125.05- 4241 / **18944**

Barcode no : 2230000 1265

Report date : **21.02.2023**

Requested by : ARKAN GRUP SAĞLIK ÜRÜNLERİ A.Ş

Address : VELİMEŞE MAH. HACI ŞEREMET CAD. NO:14 ERGENE / TEKİRDAĞ

Subject : IRRITATION (*), SENSITIZATION(*) AND CYTOTOXICITY(*) TESTS OF "DERMAPED ADULT DIAPER", ACCORDING TO BIOCOMPATIBILITY TESTS

The results in this report are valid only for the analyzed samples.

Approved by

Doç. Dr. Fatıma YÜCEL
Assigned for Industrial Service of Life Sciences Vice Presidency

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The test and/or measurement results, the uncertainties (if applicable) with confidence probability and test methods are given on the following pages which are part of this report.

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Address : VELİMEŞE MAH. HACI ŞEREMET CAD. NO:14 ERGENE / TEKİRDAĞ

Sample	: Single cell type	**Barcode no**	: 22S00001265
Number of samples	: 30 pieces	**Expiry date**	: 04.12.2026
Sample handling	: by Cargo	**Institute sample register no:**	**23-4476**
Condition of sample at reception: Supplied in closed original packaging in conditions declared to be sterile.		**Reception date and time**	: 27.12.2022
		Date of the analysis	:02.01.2023 – 20.01.2023

Information on retention samples:
( ) Sample returned to the customer (X) Retention sample available ( ) Retention sample is not taken

1-Samples

Upon the application no 14620 of "ARKAN GRUP SAĞLIK ÜRÜNLERİ A.Ş" dated 27/12/2022; analyses were conducted to make Irritation (*), Sensitisation (*) and Cytotoxicity (*) tests on 30 pieces samples.

Table 1. The product subjected to tests.

Sample	Specification	Pcs
DERMAPED ADULT DIAPER LOT: 221204 PRODUCED DATE: 04.12.2022 EXP DATE: 04.12.2026		30 Pieces

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2- Animal Intracutaneous (Intradermal) Reactivity Test (*)

Animal intracutaneous (intradermal) reactivity test was carried out taking into account the following standards; ""ISO 10993-23:2021 Biological evaluation of medical device: Tests for Irritation and Skin Sensitization", "ISO 10993-2:2006 Animal welfare requirements" and "ISO 10993-12:2012 Sample preparation and reference materials".

2.1. Animals, Housing and Feeding Conditions

As recommended in the standart protocol, 8-12 week-old 3 healthy young adult albino female rabbits were used in this test. The weight variation of animals does not exceed ± 20 % of the mean weight. Animals were randomly selected and individually housed in conventional cages 5 days before the test. The temperature and the relative humidity were adjusted as 22 ±3°C and 40-70 % RH. The artificial lighting sequence was adjusted as 12 h light, 12 h dark. Standardized commercial laboratorydiet was used with an unlimited supply of drinking water. Animals were caged individually.

2.2. Sample Preparation

According to the "ISO 10993-12:2012 Biological evaluation of medical devices: sample preparation", physical, chemical and biological properties of the test sample were considered. For the extraction, 3 cm_2/ml surface area/volume ratio and the incubation at 37°C for 72 hours was applied. PBS (phosphate buffer saline) and corn oil were used as polar and non-polar solvents, respectively.

2.3. Test Procedure

The backs of the animals (a sufficient distance on both sides of the spine) were shaved to obtain enough application area. The test and control samples were intracutaneously injected as shown in Figure 1. 0,2 ml of the test and control samples were applied at five injection points in every side of each rabbit. As shown in the Figure 1; the tested product was applied into the application sites 2 and 4. The negative and pozitive controls were applied into the application sites 3 and 5, respectively. The positions of injection points were marked with permanent ink. The appearance of each application site was observed and recorded immediately after injection and at 24h, 48h and 72h after injection. Observations were scored as described in Table 2.

2.4. Evaluation of Results

After the 72h grading, all erythma grades plus oedema grades were totalled separately for each test sample and blank for each individual animal. Each of the totals were divided to 15 (3 scoring time points X 5 injection points) to calculate the *score* of a test sample and blank. The scores for the three animals were added and divided by three to determine the *overall mean score* for each test sample and each blank.

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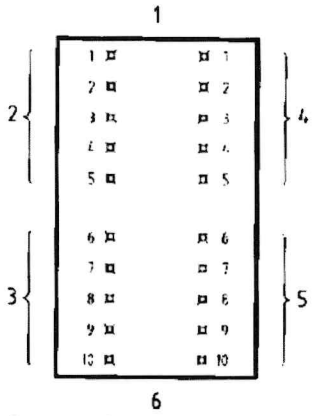

Figure 1. 1; cranial end, 2; test sample 3; negative control 4; test sample, 5; positive control, 6 caudal end.

Table 2. Grading system for intracutaneous (intradermal) reactions.

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximal possible score for irritation	8
Other adverse changes at the injection sites shall be recorded and reported.	

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2.5. Results

Observations were scored and overall mean scores were calculated as described in the section 2. 4. Results were presented in the Table 3 and 4.

Table 3. Scores of observations.

ID	Samples	Application Sites	Score
1	*Dermaped Adult Diaper (polar)*	*Left Front Site*	0
	Dermaped Adult Diaper (non-polar)	*Right Front Site*	0
	Polar Solvent Control	*Left Back Site*	0
	Non-Polar Solvent Control	*Right Back Site*	0
2	*Dermaped Adult Diaper (polar)*	*Left Front Site*	0
	Dermaped Adult Diaper (non-polar)	*Right Front Site*	0
	Polar Solvent Control	*Left Back Site*	0
	Non-Polar Solvent Control	*Right Back Site*	0
3	*Joly Adult Diaper Large (polar)*	*Left Front Site*	0
	Joly Adult Diaper Large (non-polar)	*Right Front Site*	0
	Polar Solvent Control	*Left Back Site*	0
	Non-Polar Solvent Control	*Right Back Site*	0

Table 4. Overall mean scores for test and control samples.

Samples	Application Sites	Overall Mean Score	Final Test Sample Score
Dermaped Adult Diaper (polar)	*Left Front Site*	0	0
Dermaped Adult Diaper (non-polar)	*Right Front Site*	0	0
Polar Solvent Control	*Left Back Site*	0	-
Non-Polar Solvent Control	*Right Back Site*	0	-

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2.6. Conclusion

As mentioned, after the observations at the three time points for the two criterions (Table 3), overall mean scores were obtained by averaging the scores for the test material (Table 4). In theobservations of the tested material, in any application sites and injection points serious erythema and oedema formations were not observed. According to data obtained from observations and the evaluation criterias defined in the ISO 10993-23: 2021, **the tested sample has no intracutaneous reactivity and irritation effect.**

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3- Skin Sensitization Test (*)

Sensitization test was applied according to "ISO 10993-10: 2010 Tests for irritation and delayed-type hypersensitivity" standart protocol.

Extracts were prepared by incubation at 37°C for 72 hours and extraction ratio was defined as 0.2 g/ml surface area/volume. The extraction protocol was detremined concerning of the clinical usage of the test item. PBS (Dulbecco's Phosphate Buffer Saline, Biological Industries, 02-023-1A, 500ml) and cotton oil (Zade Vital, ZV0322001, 200ml) were used for extraction as polar and apolar extraction vehicles. There was no color change or particles in the extraction solvent (pre- and post extraction). Extracts was used at the end of the extraction process without any additional processing such as; filtration, centrifugation etc. They were not stored before the administration. Sensitization tests of samples were carried out using 300 g to 500 g healthy adult nulliparous and not pregnant female guinea pigs (Cavia porcellus). As explained in the ISO 10993-10: 2010 standart protocol, experiments were carried out by injecting intradermally 0,1 ml of tested material per site. Animals are pretreated with 10 % sodium dodecyl sulfate 24 (±2) hours before the intradermal induction phase. Topical application was carried on non-injected site of the animals at 7 day after completion of the intradermal induction phase. Challenge phase was performed at 21 day after completion of the topical induction phase. Test item was applied topically to sites that were not treated during the induction stage. The experimental procedure applied on animals was shown in Figure 2.

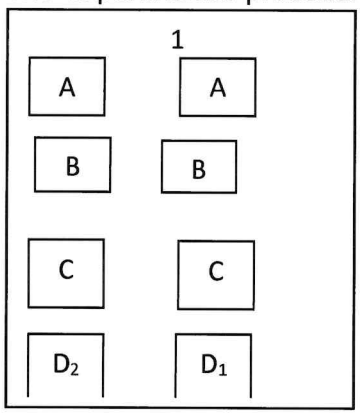

Figure 2.

1- Cranial end of animal.

A- 50:50 (volume ratio) stable emulsion of Freund's complete adjuvant (FCA) mixed with the physiological saline applied test sites.

B- Only test material applied test sites.

C- 50:50 (volume ratio) stable emulsion of the sample used at Site A mixed with test sample used at Site B applied test sites.

D- Topical induction at intrascapular region using 0,3 ml of test material.

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A pair of 0,1 ml intradermal injections into each animal (right and left sites) at the A, B, C injection sites were administrated.
At D site; the test materials were applied at day 7 left topical site (D_1), and at day 21 right topical site (D_2).

Negative control was carried out comparatively at 2 different sites at 2 different applications (Figure 3).

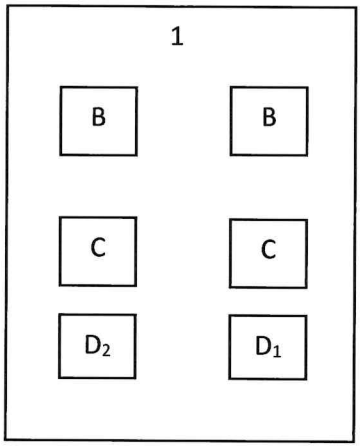

Figure 3.
1- Cranial end of the experimental animal.
B- 0,1 ml of serum physiologic.
C- (FCA) and serum physiologic mixed at 50:50 (volume ratio) was applied.
D- 0,3 ml of serum physiologic was applied on topical sites.

The fur of experimental animals were shaved to obtain enough application sites and a day after shaving, test materials were applied as shown in Figure 3 and in control animals as shown in Figure 1. All injections were carried out intradermally using 0,1 ml test samples. After application, no dressing was applied to the sites. In topical application, test material in experimental animals and 0,3 ml of serum physiologic in control animals were administered onto skin and absorbent gauze patch was applied and wrapped by an elastic bandage. The bandages were removed after 48 hours and the skin reactions were visualized. The second topical induction was performed 14 days after that and same experimental procedures were followed.

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Applied Test Materials
In the application, 10 animals for test sample and 5 animals as control were used. A total of 15 animals were used for one test material.

Test Material: Dermaped Adult Diaper

Table 5. Evaluation criteria and grading.

Reaction	Grading scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

Table 6. Observed scores for polar extract (PBS).

Groups	ID	24h	48h	Mean	Group Means	Score
Male Test	1	1	0	0,5	0.6	0.6
	2	1	1	1		
	3	1	1	1		
	4	1	0	0,5		
	5	0	0	0		
Female Test	1	1	1	1	0.6	
	2	1	1	1		
	3	1	1	1		
	4	0	0	0		
	5	0	0	0		
Negative Control	1	0	0	0	0.3	0.3
	2	0	0	0		
	3	1	0	0,5		
	4	1	0	0,5		
	5	1	0	0,5		

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Mean Scores

Table 7. Mean score values.

Sample	Mean Score	Result
DERMAPED ADULT DIAPER	0.6	0,45
Negative control	0.3	

Results

In the experiment carried out for test and control samples, the observations were graded according to the evaluation and grading criteria defined in Table 5. In the evaluation of the "Dermaped Adult Diaper" extract applied group of animals, no visible skin reaction was observed in the application sites. Sensitization score was obtained as 0.45 (Table 7). There was no discrete weight loss in the tested animals. There was also no visible change in the overall health situation of the tested animals.

According to the results of observations and the evaluation, criterions defined in the ISO 10993-10: 2010 international standard protocol; **the tested product does not have a sensitization (against material) effect.**

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4- Cytotoxicity Test (*)

The Biological Evaluation of the test of samples was performed by following Part 5: Tests for in Vitro Cytotoxicity and Part 12: Sample preparation and reference materials (ISO 10993)

Start date of analysis: 11.01.2023

End date of analysis: 13.01.2023

Definition of Samples: Sample of "DERMAPED ADULT DIAPER" was defined as in Section 1 and sample was supplied by ARKAN GRUP SAĞLIK ÜRÜNLERİ A.Ş.

Rationality of the selection of the cell line: L929 mouse cell line (NCTC clone 929) was used as the test subject. This cell line is one of the recommended ISO 10993-5: 2009 cell lines and it is suitable to represent the mammalian system under study.

Name of company and batch of medium, serum and antibiotics, when added: Minimum Essential Medium (MEM, Sigma Cat # D0547, lot # SLBH5487) was used as medium supplemented by %10 Fetal bovine serum (FBS, heat inactivated, Gibco, #10500-064) + 1% antibiotic-antimycotic solüsyon (Gibco, #15240-062), 1% Non-Essential Amino Acids, (NEAA, Gibco, #11140050), 1% GlutaMAX™ (Gibco, #35050061), 1% Sodium Pyruvate (Gibco, #11360070).

Assay Method: Extraction method

Rationale: To be able to measure the cytotoxicity from the saple as a result of soluble toxic substances.

Method: The "DERMAPED ADULT DIAPER" product was not sterile when it was received. The size of the 3 sample measured using digital calipers was cut based on measured dimensions and sterilized under UV. The extraction ratio of the sample (certain surface area or weight per extraction volume) was calculated based on surface area 3 cm^2/ml and thickness > 0.5 mm as described in the ISO 10993-12 standards. The samples were extracted with medium including serum at 120 rpm, 37°C for 24 h.

Cytotoxicity Method: L929 cells were seeded at a cell density of 10×10^3 cells/well into the 96-well plates and incubated at 37°C, %5 CO_2 for 24 h. The test item extract and controls were added (without modification or storage) to the 96-well plates and incubated at 37°C, %5 CO_2 for 24 h. After that, the medium containing 0,5 mg/ml of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma #5655) was added into each well and incubated for another 4 hours. Following, DMSO in 100 µl was added to dissolve formazan crystals into the each well and the 96-well plate was placed in a shaker for 2 hr. To determine the cell viability, the absorbance was measured at 570 nm and 650 nm reference wavelength in the microplate reader.

Measure of Cytotoxicity: MTT Cell viability assay (Colorimetric)

Rationale: Measurement of cell viability in precise and reproducible techniques.

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Negative, positive and other controls

Control 1: MEM fresh medium including serum without any treatment

Control 2: MEM extracts treated at the same conditions of the sample 5% CO2, 37oC and 120 rpm for 24 hr.

Negatif Kontrol: High Density Polyetheylene (HDPE), USP. MD (Cat No:1546809; Lot:K0M357)

Pozitif Kontrol: Low Density Polyethylene (LDPE), USP, MD (Cat No:1546809; Lot:R063J0)

RESULTS: Cell response and other observations

The effects of samples and controls on cell viability were observed morphologically in an inverted microscope. It has been determined that the morphological grading upon "DERMAPED ADULT DIAPER" exposure was observed as discrete intracytoplasmatic granules, no cell lysis, and no reduction of cell growth were observed.

Sample	Degree	Positive and negative controls	Degree
"DERMAPED ADULT DIAPER"	0	Control 1, MEM fresh	0
		Control 2, MEM extracts	0
		Negative Control, HDPE	0
		Positive Control, LDPE	4

Table 8. Qualitative morphological grading of cytotoxicity of dilutions

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

Quantitative Assessment: Measure cell death, inhibition of cell growth, cell proliferation or colony formation. The number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye or any other measurable parameter may be quantified by objective means.

Reduction of cell viability by more than 30 % is considered a cytotoxic effect.

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According to the evaluation criteria given in Table 8, L929 cell viability upon exposure to the "DERMAPED ADULT DIAPER" extract for 24 hr was quantitatively calculated compare to the control sample. According to the ISO 10993-5: 2009 cytotoxicity test, the cell viability was found to be **95.69 ± 3.78%.**

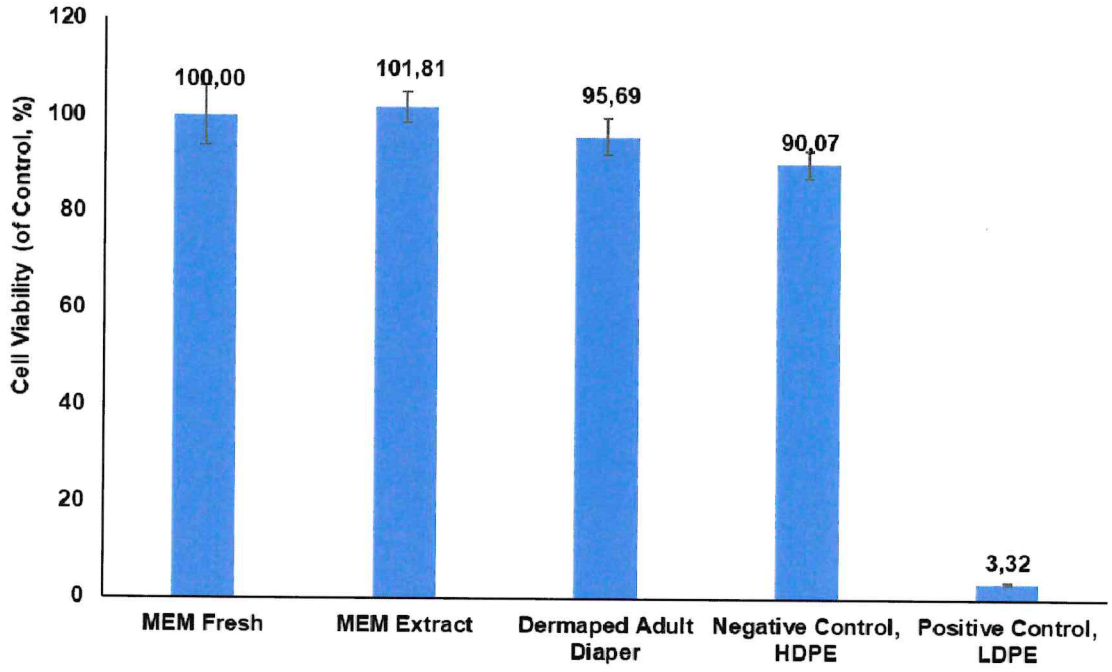

Figure 4. The percentage of cell viability of L929 cell line relative to the control upon 24 hr exposure to the "DERMAPED ADULT DIAPER" sample extracts.

The cytotoxicity of "DERMAPED ADULT DIAPER" samples was analyzed based on normalization to the 100 % cell viability of MEM fresh medium, which was incubated at the same conditions with the samples without any treatment. The results was obtained from triplicate experiments from 3 randomly selected samples.

CONCLUSION:

The results of the MTT assay was showed that "DERMAPED ADULT DIAPER" at the tested dose range using L929 cell line was **found to be non-cytotoxic.**

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