

Original



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TESTING
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Testing Center of Radiological Medical Research Institute, Soochow University Test Report

Report Number: SDFY-2008-2806

Sample Name: Jacket-Material

Testing Item: Biocompatibility Test

Sample Supplier: Envisen

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Cytotoxicity Test

Summary

The test article, Jacket-Material, were evaluated for cytotoxicity test in accordance with the ISO 10993-5,1999: Tests for in vitro cytotoxicity ASTM F813-07 and USP(87): Biological Reactivity Test, In Vitro-Elution test. The testing sample solution was mixed with growing-well L-929 cell, and then incubated for 24h and 48h at 37°C in 5% CO₂. Intracytoplasmic granulethe and cell lysis were observed. The result showed that the Reactivity Grade of biank control was 0 grade, the Reactivity Grade of negative control was 1 grade; the Reactivity Grade of positive control was 4 grade, The cytotoxicity ratio were 87.8% (Reactivity Grade 1 grade) and 92.3% (Reactivity Grade 1 grade) respectively by calculation on the basis of cell concentrations of different groups. This meant that the test was valid and the sample article had no toxicity to L-929 cell.

Date completed: Oct 24, 2008

Edited by: *Sun Xiao Jun*

Checked by: *Lin Tengyu*

Introduction

The test article, Jacket-Material, were evaluated for cytotoxicity in accordance with ISO10993-5,1999: Tests for in vitro cytotoxicity ASTM F813-07 and USP (87) : Biological Reactivity Test, In Vitro-Elution test. The purpose of this study was to determine the potential cytotoxicity of the testing article to L-929 cell.

Test system and test system Management

L-929 mammalian fibroblast cell will be grown in RPMI 1640 supplemented with 10% serum, Cells will be seeded into the 96-well cell culture plates, and incubated at 37°C in a humidified incubator with 5% CO₂ to obtain confluent monolayers of cells prior to use. Aseptic procedures will be used in the handling of cell culture.

Personnel: Associates involved were appropriately qualified and trained.

Justification for selection of the test system

Mammalian cell culture monolayer, L-929 mouse fibroblast cells (American Type Culture Collection CCLI (NCTC clone 929), will be used. In vitro mammalian cell culture study has been used to historically evaluate the cytotoxicity of biomaterial of medical device.

Materials

1. Test Sample:

Sample Name: Jacket-Material

Materials:

Part No	10627
Reference No	660.0123.05
Material / Part Description	Jacket-Material

Size: Not supplied

Lot No: Not supplied

Receiving Date: Oct 18, 2008

2. Equipment: Autoclaves(Shanghai Medical and Technologic Equipment Company), CO₂ Incubator (Thermo, U.S.A), Inverted microscope(Olympus, Japan), Super clean working desk(Suzhou Purification Equipment CO, LTD), Refrigerator(Thermo, U.S.A), culture plate(Corning Incor), PowerWave XS Microplate Reader(Bio Tek, U.S.A), etc.

3. Reagents: Phenol (Lot No: 20070401), MTT (Lot No: 13241337), Calf Serum (Lot No:080119), Trypsinase (Lot No: 1983B39), RPMI 1640 (Lot No:1320625), Penicilline (Lot No: Y0709307), Streptomycin sulfate (Lot No: 071107), DMSO (Lot No: 20070712), etc.

4. Cell Strain: Recommended cell lines are American Type Culture Collection CCLI (NCTC clone 929).

5. Control Preparation:

Negative control: High density polyethylene +RPMI 1640 medium, with addition 10% calf serum.

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Add: No.199 Ren'ai Road, Suzhou Industrial Park, China

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(37°C 24h)

Positive control: RPMI 1640 medium, with addition 10% calf serum and 0.5 % phenol (37°C 24h) .

Blank control: RPMI 1640 medium, with addition 10% calf serum. (37°C 24h)

6. Storage Conditions: Room temperature

Test method

1. Cell Strain: Recommended cell lines are American Type Culture Collection CCLI (NCTC clone 929).

2. Preparation of sample Extracts

The sterilized sample was put into a culture flask., and serum-free culture medium is then added according to the ratio of 0.2g:1ml ,finally, the flask is sealed,and the sample is extracted at 37°C for 24h to obtain the sample extract.

3. Cell culture

L929 cells were cultured in RPMI 1640 medium, supplemented with 10% calf serum at 37°C in a humidified atmosphere of 5% CO₂. L929 cells were digested by using 0.25% trypsin and the single-cell suspension formed 8.5×10^4 cells/mL suspended cells were cultured in 96-well plates with 100μl per well. After the cells grew into monolayer, discard the original culture medium, and then add 100μl the sample extraction ,positive control solution and negative control solution to each well respectively.

4. Cell morphological observation and evaluation of cytotoxicity

After 24h and 48h incubation respectively, take out a 96-well plate for cell morphological observation first ,and then add 10μl MTT (5mg/mL) to each well. The cells were further cultured for 4 hours when all liquid in each well was tipped out and 100 μl DMSO was added to dissolve the precipitate. Sample group were tested by dual-wavelength spectrophotometric measurement with the measurement wavelength at 570 nm and reference wavelength at 630 nm. The blank control, negative control and positive control which obtained by same way were tested as the method above.

The cell cytotoxicity ratio of the negative control group, positive control group and sample group are all determined by the following formula:

The cell cytotoxicity ratio = $\frac{[\bar{A}_{570} - \bar{A}_{630}] \text{ of sample group (or } [\bar{A}_{570} - \bar{A}_{630}] \text{ of positive control group)}}{[\bar{A}_{570} - \bar{A}_{630}] \text{ of blank control group}} \times 100\%$

The morphology evaluation:the reactivity will be graded as following Table 1.

Table 1. Reactivity Grades for Elution Test

Grade	Reactivity	Conditions of all Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

According to USP , test articles scoring "0", "1", or "2" will be considered NON-TOXIC. Test articles scoring "3" or "4" will be considered TOXIC. The positive control sample must have a score of "3" or "4" and the negative control sample must have a score of "0" or "1" for a valid test

Results

Results in this experiment are showed Table 2 and Table 3.

Table 2: Descriptive statistics of the cell vitality
24h

	N	Mean	Cell vitality(%)	Maximum	Minimum	Std Deviation
Blank control	5	1.379	100	1.419	1.325	0.036
Negative control	5	1.276	92.5	1.294	1.248	0.019
Positive control	5	0.057	4.1	0.062	0.053	0.003
Sample group	5	1.210	87.8	1.239	1.190	0.018

48h

	N	Mean	Cell vitality(%)	Maximum	Minimum	Std Deviation
Blank control	5	2.047	100	2.104	2.011	0.039
Negative control	5	1.903	92.9	1.950	1.859	0.033
Positive control	5	0.056	2.7	0.058	0.055	0.001
Sample group	5	1.889	92.3	1.958	1.816	0.058

Table 3: the result of the morphology evaluation

Incubate the cultures for 24h			
Group	Conditions of all Cultures	Reactivity	Grade
Blank control	Discrete intracytoplasmic granules; no cell lysis	None	0
Negative control	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present	Slight	1
Positive control	Nearly complete destruction of the cell layers	Severe	4
sample group	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present	Slight	1
Incubate the cultures for 48h			
Group	Conditions of all Cultures	Reactivity	Grade
Blank control	Discrete intracytoplasmic granules; no cell lysis	None	0
Negative control	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present	Slight	1
Positive control	Nearly complete destruction of the cell layers	Severe	4
sample group	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present	Slight	1

The testing sample solution is mixed with growing-well L-929 cell, and then incubated for 24h and 48h. Observe the morphology and measure the cytotoxicity ratio by MTT method. The cytotoxicity ratio are 87.8% and 92.3% respectively .

Conclusion

In the experiment, the Grade was 4 in positive control and 1 in negative control. So the test is regarded to be valid.

The Grade of sample article was 1. This means the testing sample had no toxicity to L-929 cell.

Delayed Contact Sensitization Study(A Maximization Method) In the Guinea Pig

Summary

A guinea pig maximization test (ISO 10993-10:2002/Amd.1:2006) of sample Jacket-Material was conducted to evaluate the potential for delayed dermal contact sensitization. The method of Magnusson and Kligman (1970) was adapted for alcohol in a 0.9% sodium chloride USP solution (AS) test article extract.

The AS extract of the test article was intradermally injected and occlusively patched to ten guinea pigs in an attempt to induce sensitization. Following a recovery period, the original ten test and five previously untreated control animals received a challenge patch of the test article extract and the control vehicle. In addition the test article was applied to the same animals. All sites were scored at 24h and 48h after patch removal.

Under the conditions of this study, the AS test article extract and the test article showed no signification evidence of causing delayed dermal contact sensitization in the guinea pig.

Date completed: Nov 22, 2008

Edited by: *Liangfu*

Checked by: *Lin Yanbin*

Introduction

A guinea pig maximization test of the material identified below was conducted to evaluate the potential to cause delayed dermal contact sensitization. The test article was received on Oct 18, 2008. The method of Magnusson and Kligman, as reported in Allergic Contact Dermatitis in the Guinea Pig, 1970, was employed with adaptations for a test article extract. The susceptibility of the Hartley guinea pig strain to a known sensitizing agent, 1-chloro-2,4-dinitrobenzene (DNCB), has been substantiated at TCRSU with this method under lab number SDFY--2008-2151 completed on Apr 11, 2008.

Materials

The sample provided by the sponsor was identified and handled as follows:

Test Article: Jacket-Material

Materials:

Part No	10627
Reference No	660.0123.05
Material / Part Description	Jacket-Material

Size: Not supplied

Lot No: Not supplied

Equipment: Incubator

Storage Conditions: Room temperature

Vehicle: alcohol (100%) in saline 1:20 solution (AS)

Preparation:

For each phase of this test, a ratio of 0.2g: 1ml (test article to volume of vehicle) was used for the test extract. The test article was extracted in AS at $70 \pm 2^\circ\text{C}$ for 24 ± 2 hours. For the challenge phase, the vehicle (without the test article) was similarly prepared to serve as the control. In addition, the test article (as received) was cut into $2 \times 2\text{cm}$ sections at the challenge phase.

Condition of Extracts:

	TEST	CONTROL
Induction I	clear with test article particulates*	Not applicable
Induction II	clear with test article particulates	Not applicable
Challenge	clear with test article particulates	Clear

*Filtered with a $0.8 \mu\text{m}$ filter disc to yield a clear particulate free extract

Additional Materials:

Freund's Complete Adjuvant (FCA) was used at induction I, and a 10% (w/w) sodium lauryl sulfate (SLS) suspension in petrolatum was used for induction II. These materials were provided by the test facility.

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Add: No.199 Ren'ai Road, Suzhou Industrial Park, China

Tel: 0512-65880038

Fax: 0512-65880038

PC: 215123

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Method

Test System:

Species: Albino Guinea pig

Source: Provided by Animal Center, TCRSU <Permit Code: SCXK (SU) 2002-0008>

Acclimation Period: Minimum 5 days

Number of Animals: 15

Justification of Test System:

The albino guinea pig has been used historically for sensitization studies (Magnusson and Kligman, 1970). The guinea pig is believed to be the most sensitive animal model for this type of study. The susceptibility of the guinea pig to a known sensitizing agent, 1-chloro-2,4-dinitrobenzene (DNCB) has been substantiated at TCRSU with this method.

Animal Management:

Husbandry: Refer to ISO 10993-10-2002 Annex C: Animal and husbandry.

Food: All-nutrient animal food provided by Suzhou (Twin-lion) Experimental Animal Food Science & Technology Service Co. Ltd.

Water: Drinking water met the sanitary standard

Housing: Animals were housed in groups in stainless steel suspended cages identified by a card indicating the lab number, animal numbers, test code, sex, animal code and first treatment date.

Personnel: Associates involved were appropriately qualified and trained.

Selection: Only healthy, previously unused animals were selected.

Intradermal induction phase I :

A pair of 0.1ml intradermal injections was made for each of the following, into each animal, at the injection sites (A, B and C) as shown in Figure 1 in the clipped intrascapular region.

Site A: A 50:50 (volume ratio) stable emulsion of Freund's complete adjuvant mixed with the chosen solvent. Physiological saline (equivalent) was used for water-soluble material.

Site B: The test sample (undiluted extract); the control animals were injected with the solvent alone.

Site C: The test sample at the concentration used at site B, emulsified in a 50:50 volume ratio stable emulsion of Freund's complete adjuvant and the solvent (50%) was injected into the control animals with an emulsion of the blank liquid with adjuvant.

Topical induction phase II :

Seven days (± 1 day) after completion of the intradermal induction phase, the test sample was administered by topical application to the intrascapular region of each animal, using a patch of area approximately 8cm^2 (filter paper or absorbent gauze), so as to cover the intradermal injection sites. The concentration selected in Intradermal induction phase I for Site B was used. The maximum concentration that could be achieved in Intradermal induction phase I did not produce irritation, when the area was pretreated with 10% sodium dodecyl sulfate massaged into the skin $24\text{h} \pm 2\text{h}$ before the patch was applied. The patches were secured with an occlusive dressing. The dressings and patches were removed after $48\text{h} \pm 2\text{h}$.

The control animals were treated similarly, using the blank liquid alone.

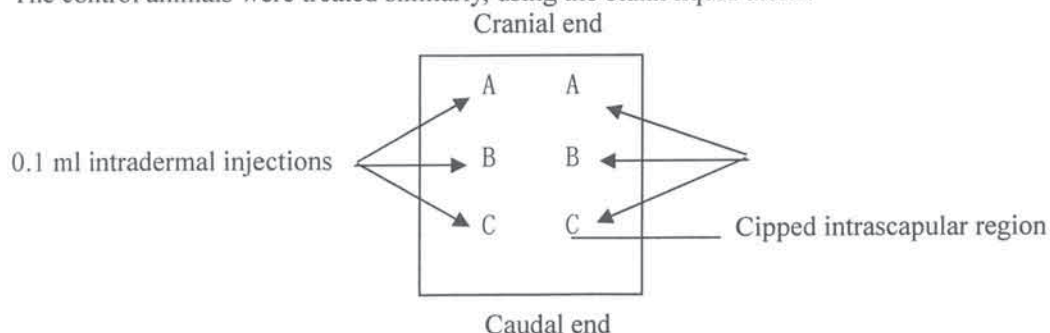


Figure 1-Location of intradermal injection sites

Challenge phase :

At 14 days (± 1 day) after completion of the topical induction phase, all test and control animals were challenged with the test sample. The test sample and a vehicle control were administered by topical application to sites that were not treated during the induction stage, such as the upper flank of each animal,

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using appropriate patches or chambers soaked in the test sample at the concentration selected in the intradermal induction phase I for site C. Dilutions of this concentration were also applied to other untreated sites in a similar manner. Occlusive dressings were used to secure areas treated. The dressings and patches were removed after 24 ± 2 h.

Observation of animal:

The appearance of the challenge skin sites of the test and control animal were observed after 24h and 48h removal of the dressings. Natural or full-spectrum was used to visualize the skin reactions.

The skin reactions for erythema and oedema were observed and graded according to the Magnusson and Kligman grading given in Table 1 for each challenge site and at each time interval. It is highly recommended that reading be done without knowledge of the treatment, in order to minimize bias in the evaluation of the results.

Evaluation of results:

Magnusson and Kligman grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 are seen in control animal. If grades of 1 or greater are noted in control animal, then the reactions of test animal which exceed the most severe reaction in control animals are presumed to be due to sensitization. If the response is equivocal, re-challenge is recommended to confirm the results from the first challenge. The outcome of the test is presented as the frequency of positive challenge results in the test and control animal.

Table 1 Magnusson and Kligman scale

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

Result

Individual results of dermal scoring for the challenge appear in Table 1. No evidence of sensitization was observed.

Clinical Observations: All animals appeared clinically normal throughout the study.

Conclusion

Under the conditions of this study, the test article extract and the test article showed no significant evidence of causing delayed dermal contact sensitization in the guinea pig.

Table 2 Guinea pig Sensitization Dermal Reactions – Challenge

Animal Number/ Group	Topical induction phase II	Hours following patch removal (h)		Weight (g)	
		24	48	Before injection	After experiment
1 Test	0	0	0	313	378
2 Test	0	0	0	304	368
3 Test	0	0	0	327	391
4 Test	0	0	0	341	405
5 Test	0	0	0	333	396
6 Test	0	0	0	321	384
7 Test	0	0	0	319	382
8 Test	0	0	0	312	375
9 Test	0	0	0	337	401
10Test	0	0	0	326	390
11control	0	0	0	320	384
12control	0	0	0	321	386
13control	0	0	0	336	398
14control	0	0	0	305	368
15control	0	0	0	318	382

Skin irritation Test

Summary

The test article, Jacket-Material was evaluated for skin irritation in accordance with the ISO 10993.10-2002/Amd.1:2006: Tests for Irritation and Sensitization. The skin responses on application sites in 1h, 24h, 48h and 72h respectively after removal the patches (about 2.5cm×2.5cm) which moistened by the extract were observed and recorded. The tissue reaction was graded for erythema and oedema according to the classification system given in Table 1. According to what was observed, the response of skin on testing side does not exceed that on the control side. The primary irritation index for the test article was calculated to be 0. The test result showed that the applied sample does not induce irritation to rabbit skin.

Date completed: Nov 7, 2008

Edited by: Liu Chun Li

Checked by: ni shisu

Approved by: Zhang Tongchang

Nov 27, 2008

Nov 27, 2008

Nov 27, 2008

Testing Center of Radiological Medical Research Institute, Soochow University (Stamp)

Introduction

The test article was evaluated for skin irritation in accordance with the guidelines of the ISO 10993.10-2002/Amd.1:2006: Tests for irritation and sensitization. This study was to determine the potential skin irritation after the patches which moistened by the extract apply to the animal back. The test article was received on Oct 18, 2008. The test started on Nov 4, 2008, and concluded on Nov 7, 2008.

Materials

Test Article: Jacket-Material

Materials:

Part No	10627
Reference No	660.0123.05
Material / Part Description	Jacket-Material

Size: Not supplied

Lot No: Not supplied

Equipment: Incubator

Storage Conditions: Room temperature

Sample and Control Preparation:

A ratio of 0.2g: 1ml (test article to volume of vehicle) was used for the test extract. The test article was extracted in 0.9% NaCl at 70°C±2°C for 24 ±2 hours. The vehicle (without the test article) was similarly prepared to serve as the control. Then apply the 0.5ml extract(s) to 2.5cm×2.5cm absorbent gauze patches.

Method

Test System:

Species: Rabbits.

Breed: New Zealand white (single strain)

Source: Provided by Animal Center, TCRSU<Permit Code: SCXK (SU)2002-0008>

Body Weight Range: Not less than 2 kg.

Age: Young adult

Acclimation Period: Minimum 5 days.

Number of Animals: Three

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Justification of Test System:

The rabbit is specified as an appropriate animal model for evaluating potential skin irritants by the current ISO testing standards. The rabbit is widely used for this purpose and relative ranking of irritant scores can be determined.

Animal Management:

Husbandry: Refer to ISO 10993-10: 2002 Annex C: Animal and husbandry

Food: All-nutrient animal food was provided by the Suzhou (Twin-lion) Experimental Animal Food Science & Technology service Co., Ltd.

Water: Provided by Sanitary Standard for drinking water.

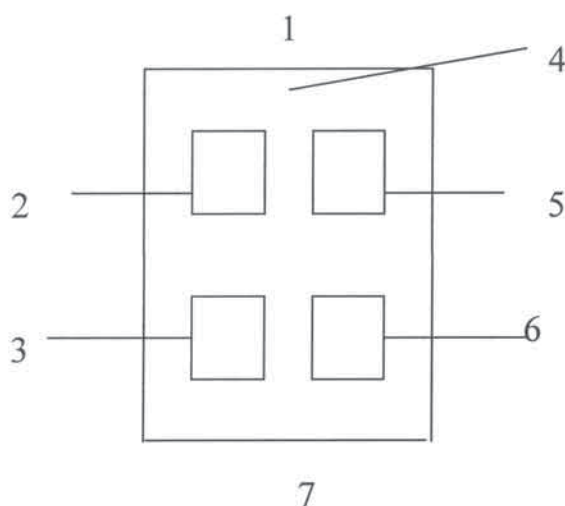
Housing: Animals were individually housed in stainless steel suspended cages identified by a card indicating the lab number, animal number, test code, sex, and date dosed.

Personnel: Associates involved were appropriately qualified and trained.

Selection: Only healthy, previously unused, animals free from irritation or other dermatological lesions that could interfere with the test were selected.

Experimental Procedure

Use only animals with healthy intact skin. Fur is generally clipped within 24h to 4h of testing on the backs of the animals a sufficient distance on both sides of the spine for application and observation of all test sites(approximately 10cm×15cm)



- 1- Cranial end 2- Test site 3- Control site 4- Clipped dorsal region
5- Control site 6- Test site 7- Caudal end

Figure1-Location of skin application sites

Apply the appropriate extract(s) to 2.5cm×2.5cm absorbent gauze patches. Apply one patch on each side of the animal as shown in Figure 1. Similarly, Apply a control patch of gauze moistened with the extract vehicle as indicated in Figure 1. Then wrap the application sites with a bandage(semi-occlusive or occlusive) for a minimum of 4h. At the end of the contact time, remove the dressing.

Describe and score the skin reaction for erythema and oedema according to the scoring system given in Table 1 and Table 2 for each application site at each time interval. Record the appearance of each application site at 1h, 24h, 48h and 72h following removal of the patches.

Result

According to what observed, the response of skin on testing side does not exceed that on the control side. Thus, it is identified as grade 0. See table 2.

Conclusion

The test result shows that of the applied sample does not induce irritation to skin.

Record Storage

All raw data pertaining to this study and a copy of the final report are to be retained in designated SDFY archive files.

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Add: No.199 Ren'ai Road, Suzhou Industrial Park, China

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Table.1 Classification System for Skin Reaction

Erythema and Eschar Formation:	Numerical Grading
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
Edema Formation:	
No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1mm)	3
Severe edema (raised more than 1mm and extending beyond exposure area)	4
Total possible score for irritation	8
Irritation Response Categories in the Rabbit	
Response Category	Mean score
Negligible	0 to 0.4
Slight	0.5 to 1.9
Moderate	2 to 4.9
Severe	5 to 8

NOTE: Other adverse changes at the skin sites were recorded and are reported

Table 2. Dermal Observations

Rabbit No	Group		Interval (hours)			
			1	24	48	72
1	Test	Erythema	0	0	0	0
		Oedema	0	0	0	0
	Control	Erythema	0	0	0	0
		Oedema	0	0	0	0
2	Test	Erythema	0	0	0	0
		Oedema	0	0	0	0
	Control	Erythema	0	0	0	0
		Oedema	0	0	0	0
3	Test	Erythema	0	0	0	0
		Oedema	0	0	0	0
	Control	Erythema	0	0	0	0
		Oedema	0	0	0	0