Anhang 4

Prüfbericht Sensibilisierungstest (LLNA) mit C.I. Reactive Yellow 174

BIOSERVICE

SCIENTIFIC LABORATORIES GmbH

Test for Sensitization

(Local Lymph Node Assay - LLNA)

with

C.I. Reactive Yellow 174

Report

BSL BIOSERVICE Project No.: 053226D

Sponsor

Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA)

Friedrich-Henkel-Weg 1-25

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Akkreditiert durch
Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten
ZLG-P-986.96.01

Copy of the GLP Certificate

BAYERISCHES LANDESAMT
FÜR ARBEITSSCHUTZ,
ARBEITSMEDIZIN UND SICHERHEITSTECHNIK
Pfarrstraße 3 · 80538 München · Telefon (089) 21 84 0

LfAS

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

BSL Bioservice Scientific Laboratories GmbH
Behringstrasse 6
82152 Planegg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen auf toxikologische Eigenschaften
3 Prüfungen auf mutagene Eigenschaften (in vitro/in vivo)
9 Sonstige Prüfungen:
a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month year)
11./12.02.2004

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

München, 21.07.2004

i.V.
Ritter
Leitender Gewerbedirektor

L.S.

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Preface

General

Sponsor:	Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA) Friedrich-Henkel-Weg 1-25 44149 Dortmund Germany
Study Monitor:	Mrs Heidi Ott
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6 82152 Planegg Germany
BSL BIOSERVICE- Project No.:	053226D
Test Item:	C.I. Reactive Yellow 174
Title:	Test for Sensitization (Local Lymph Node Assay - LLNA) with C.I. Reactive Yellow 174

Project Staff

Study Director:	Dr. Ingrid Haist
Deputy Study Directors:	Dr. Daniela Brummer Dr. Achim Albrecht
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Quality Assurance Unit:	Dipl. Biol. Uwe Hamann Dr. Margarete Hoechst Dr. Helga Köhn

Schedule

Arrival of Test Item:	December 29, 2005
Date of Draft Project Protocol:	December 27, 2005
Date of Project Protocol:	January 23, 2006
Start of Study (preliminary test):	February 08, 2006
End of Study:	March 08, 2006
Date of Draft Report:	May 09, 2006
Date of Report:	September 07, 2006

Project Staff Signatures

Study Director: Dr. Ingrid Haist

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Date: 07.09.2006

Management:

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Date: Sept. 8, 2006

Quality Assurance

GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended on May 08, 2001. Published May 14, 2001 in Bundesgesetzblatt 2001 part I no. 21, pp. 844 – 854.

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1.
Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the project protocol, the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections and audits are included in this report. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study was audited. A Quality Assurance Statement, signed by the Quality Assurance, is included in this report.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, number 429 "Skin Sensitization: Local Lymph Node Assay" (adopted: 24th April 2002).

EPA Health Effects Test Guidelines, OPPTS 870.2600 "Skin Sensitization", EPA 712-C-03-197, March 2003.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-Regulations:

A copy of the final report, the project protocol, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the project. Default archiving period for the study documentation is 15 years.

If test item is left over a sample will be stored according to the GLP-Regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the sponsor's prior consent. Remaining test item will be returned to the sponsor as requested.

Statement of Compliance

BSL BIOSERVICE-Project No.:	053226D
Test Item:	C.I. Reactive Yellow 174
Title:	Test for Sensitization (Local Lymph Node Assay - LLNA) with C.I. Reactive Yellow 174
Study Director:	Dr. Ingrid Haist

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended on May 08, 2001. Published May 14, 2001.

"OECD Principles of Good Laboratory Practice (as revised in 1997) ", Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Ingrid Haist

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Date: 04.10.2006

This statement does not include the preliminary test.

Statement of the Quality Assurance Unit

BSL BIOSERVICE-
Project No.: 053226D

Test Item: C.I. Reactive Yellow 174

Title: Test for Sensitization
(Local Lymph Node Assay - LLNA) with
C.I. Reactive Yellow 174

Study Director: Dr. Ingrid Haist

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

Phases of QAU Inspections	*Dates of QAU Inspections*	*Dates of Reports to the Study Director and Management*
Audit Project Protocol/ Study Plan:	January 26, 2006	January 26, 2006
Experimental Phase Audit (Method Audit):	January 19, 2006	January 19, 2006
Draft Report Audit:	May 15, 2006	May 15, 2006
Report Audit:	September 27, 2006	September 27, 2006

This report reflects the raw data.

Member of the
Quality Assurance Unit: Helga Köhn

Date: Sept. 27 2006

This statement does not include the preliminary test.

Summary

Four concentrations were chosen to gain a wide spectrum for the test design:

Due to results of a solubility test and in consultation with the sponsor, the test item was assayed at concentrations of 15%, 9%, 3% and 1% (w/v). A positive control group was carried along in accordance to demonstrate appropriate performance of the assay.

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil). Stability of the test item in the vehicle was proven (details see Annex to this report).

Each mouse was treated by topical application of the prepared test item to the entire dorsal surface of each ear once daily over three consecutive days.

Five days after the first topical application all mice were injected intravenously with ^{3}H-methyl thymidine.

Directly prior to the first application and shortly before excising the lymph nodes the thickness of both ears from all animals was measured.

No different ear thickness development between test and control groups could be found.

Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed and the draining "auricular lymph nodes" were excised and weighed individually.

The lymph nodes of all test groups outweighed the lymph nodes of the negative control group.

A single cell suspension of the lymph node cells for each animal was prepared. The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations per minute (DPM). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was calculated as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test item / negative control, was calculated for each concentration.

All tested concentrations exceeded the stimulation index of 3.

The stimulation index at a concentration	of	15%	was	**7.8**
The stimulation index at a concentration	of	9%	was	**5.5**
The stimulation index at a concentration	of	3%	was	**3.3**
The stimulation index at a concentration	of	1%	was	**4.2**

All animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the study.

At the daily clinical observation the animals did not show any visible clinical symptoms.

Conclusions

The EC3 value could not be calculated as the stimulation indices of all concentrations were above 3. This finding was confirmed by the second endpoint, the weight of the lymph nodes, as all of the test groups showed increased lymph node weights compared to the control group.

Consequently, according OECD 429 the test item C.I. Reactive Yellow 174 has shown skin sensitizing properties under the given experimental conditions.

Introduction

The LLNA has been developed as an alternative method for the identification of skin sensitizing test items and measures the proliferation of lymphocytes isolated from lymph nodes (auricular lymph nodes) draining the site of exposure (dorsal aspect of the ears) in mice.

Lymphocyte proliferation is measured by determining the incorporation of ^{3}H-methyl thymidine.

This experiment being on hand was conducted in order to receive a quantitative result of the sensitizing properties of the test item. This is necessary for the Federal Institute of Occupational Safety and Health (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, BAuA) to evaluate a ranking system for dyestuffs which have shown positive results in the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman.

No validated *in vitro* method is available for assessing sensitization potency.

Materials and Methods

Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor.

Substance name:	C.I. Reactive Yellow 174
Product:	C.I. Reactive Yellow 174
Notification number:	88 04 0128
CAS-No.:	106359-91-5
Item No.:	1897214 from synthesis: 631178/174
Chemical name:	Trinatrium 7-(4-(6-fluor-4-(2-(2-vinylsulfonylethoxy)ethyl-amino)-1,3,5-triazin-2-yl-amino)-2-ureidophenylazo)-naphthalin-1,3,6-trisulfonat
Active components:	75.4%
Colour:	yellow
Physical state:	powder
Purity:	75.4%
Stability:	19.01.2010
Storage:	at room temperature
Safety Precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety

Preparation of the Vehicle

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil).

(Acetone; CAS 67-64-1, Merck, Lot K32732014; Olive Oil Highly refined, Sigma GmbH, Lot 115K6046)

Preparation of the Test Item

The test item was assayed at four concentrations.

A solubility test was performed first to detect the highest possible application solution.

Due to results of the solubility test and in consultation with the sponsor, the applicated concentrations of the homogenous suspension were:

15%, 9%, 3% and 1% (w/v).

The preparations were made immediately prior to each dosing. The two components of the vehicle were confected first. Afterwards the test item was weighed out and the appropriate amount of vehicle was added.

Stability of the Test Item in the Vehicle

Analysis of Stability was performed at the contract laboratory BioProof AG, Weihenstephanerstr. 28, 81673 München, Germany.

Controls

The vehicle served as negative control.

P-Phenylenediamine (CAS 106-50-3, Sigma GmbH, purity > 98%) at a concentration of 1% (w/v) in AOO (3+1 (v/v) Acetone/Olive Oil) served as positive control.

Other Materials

^{3}H-methyl thymidine (TRK 300, 25 Ci/mmol; Lot B264; Amersham Pharmacia Biotech), diluted to a working concentration of 80µCi/mL

NaCl 0.9%, B. Braun Melsungen AG, Lot 5361A191

Trichloroacetic acid (TCA), BSL BIOSERVICE Lot 240106

Phosphate buffered saline (PBS), BSL BIOSERVICE Lot 080206

Test Animals

Mice, CBA/Ca01aHsd, female, age 6 – 12 weeks, 5 mice per test group.

The animals were derived from a controlled full barrier maintained breeding system (SPF).

Source: Harlan Winkelmann GmbH, D-33178 Borchen.

According to Art. 9.2, No.7 of the German Act on Animal Welfare the animals are bred for experimental purposes.

Animal Husbandry

The animals were barrier maintained (semi-barrier) in an air conditioned room

- Temperature: 22 ± 3 °C
- Rel. humidity: 55 ± 10%
- Artificial light, sequence being 12 hours light, 12 hours dark
- Air change: at least 10 x / hour
- Feeding ad libitum, ssniff R/M-H, 10 mm V1534-000 complete diet for rats/mice, totally-pathogen-free (TPF)
- Free access to tap water (drinking water, municipal residue control, microbiol. controlled periodically)
- The animals were kept in groups in Macrolon-cages on Lignocel bedding
- Certificates of food, water and bedding are filed at BSL Bioservice
- Adequate acclimatisation period

Preparation of the Animals

The animals were randomly selected.

Identification was ensured by cage number and individual marking (tail).

Clinical Observation

Prior to the application and once a day thereafter all animals were observed in order to detect special clinical signs or reactions to treatment.

Weight Assessment

The animals were weighed prior to the application and at the end of the test period.

Dose Groups

4 Test Groups (4 different concentrations), 1 Positive Control Group and 1 Negative Control Group (vehicle) were tested.

Test Regime

Topical Application

Before the first application the thickness of the ears of all animals were determined by using a digital caliper gage. Then each mouse was treated by topical application of 25µL of the selected solution to the entire dorsal surface of each ear.

Topical applications were performed once daily over three consecutive days.

Administration of ^{3}H-methyl thymidine

Five days after the first topical application treatment all mice were dosed with 20µCi ^{3}H-methyl thymidine by intravenous injection (tail vein) of 250µL of ^{3}H-methyl thymidine, diluted to a working concentration of 80µCi/mL.

Preparation of cell suspension

Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed. Before excising the lymph nodes the thickness of the ears of all animals was determined. The draining "auricular lymph nodes" were excised, pooled for each animal (2 lymph nodes per animal, if technically possible), weighed and collected in PBS. A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were resuspended with PBS. This washing procedure was repeated.

After the final wash each pellet was resuspended in approx. 1 mL 5% TCA at approx. 4 °C overnight for precipitation of macromolecules. Each precipitate was once washed again, resuspended in 10 mL scintillation fluid, transferred into scintillation vials and stored at room temperature overnight.

Determination of incorporated ^{3}H-methyl thymidine

The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ^{3}H-methyl thymidine levels were also measured (5% TCA). Determination of radioactivity was performed individually for each animal.

Evaluation of Results

The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals (STIMULATION INDEX). Before DPM/NODE values were determined, background values were subtracted.

EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation {EC3 = c + [(3-d) / (b-d)] x (a-c)}, between two points of the stimulation indices axis, one above (a,b) and one below (c,d) the stimulation index of three. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

A substance is regarded as a 'sensitizer' in the LLNA if at least one concentration of the test item results in a 3 fold or greater increase in ^{3}H-methyl thymidine - incorporation into lymph node cells of the lymph nodes of the test group animals, relative to that recorded for the lymph nodes of control group animals **(Stimulation Index equal to or greater than 3.0).**

Deviation to the Project Protocol

There was no deviation to the project protocol.

Results

Four concentrations were chosen to gain a wide spectrum for the test design:

Due to results of a solubility test and in consultation with the sponsor, the test item was assayed at concentrations of 15%, 9%, 3% and 1% (w/v). Additionally a positive control for verifying the functionality of the current test run was carried along.

The vehicle was AOO (3+1 (v/v) Acetone/Olive Oil). Stability of the test item in the vehicle was proven (details see Annex to this report).

Each mouse was treated by topical application of the prepared test item to the entire dorsal surface of each ear once daily over three consecutive days.

Five days after the first topical application all mice were injected intravenously with ^{3}H-methyl thymidine.

Directly prior to the first application and shortly before excising the lymph nodes the thickness of both ears from all animals was measured. This is to exclude irritating properties of the test item, which may lead to false positiv results.

Mean Ear thickness at:	day 1	day 6
of the 15% group was	**0.21 mm**	**0.22 mm**
of the 9% group was	**0.21 mm**	**0.21 mm**
of the 3% group was	**0.20 mm**	**0.21 mm**
of the 1% group was	**0.22 mm**	**0.22 mm**
of the negative control group was	**0.20 mm**	**0.20 mm**
of the positive control group was	**0.19 mm**	**0.20 mm**

Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed and the draining "auricular lymph nodes" were excised and weighed individually.

The mean weights of the lymph nodes	
for the 15% group was	**4.7 mg**
for the 9% group was	**4.5 mg**
for the 3% group was	**3.7 mg**
for the 1% group was	**3.9 mg**
for the negative control-group was	**2.6 mg**
for the positive control-group was	**5.5 mg**

A single cell suspension of the lymph node cells for each animal was prepared. The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations per minute (DPM). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was calculated as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test item / negative control, was calculated for each concentration.

The stimulation index at a concentration	of	15%	was	**7.8**
The stimulation index at a concentration	of	9%	was	**5.5**
The stimulation index at a concentration	of	3%	was	**3.3**
The stimulation index at a concentration	of	1%	was	**4.2**
The stimulation of the positive control (Phenylenediamine) at a concentration	of	1%	was	**9.9**

All animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the study.

At the daily clinical observation the animals did not show any visible clinical symptoms.

Conclusions

The EC3 value could not be calculated as the stimulation indices of all concentrations were above 3. This finding was confirmed by the second endpoint, the weight of the lymph nodes, as all of the test groups showed increased lymph node weights compared to the control group.

Consequently, according OECD 429 the test item C.I. Reactive Yellow 174 has shown skin sensitizing properties under the given experimental conditions.

Table 1: Weight Gain (g)

Group	*Animal No.*	*Start of study*	*End of study*	*Weight gain*
	1	16	16	0
C.I. Reactive	2	16	17	1
Yellow 174	3	17	17	0
15%	4	16	16	0
in AOO	5	18	18	0
	6	17	17	0
C.I. Reactive	7	17	18	1
Yellow 174	8	18	19	1
9%	9	16	17	1
in AOO	10	16	16	0
	11	16	17	1
C.I. Reactive	12	18	18	0
Yellow 174	13	16	17	1
3%	14	17	17	0
in AOO	15	16	17	1
	16	18	18	0
C.I. Reactive	17	17	17	0
Yellow 174	18	19	19	0
1%	19	16	16	0
in AOO	20	16	17	1
	26	17	18	1
Negative	27	17	17	0
control	28	16	16	0
AOO	29	16	16	0
	30	17	18	1
Positive	21	17	17	0
control	22	17	18	1
1%	23	16	17	1
in AOO	24	17	18	1
	25	18	19	1

Table 2a: Radioactive determination of the test substance groups.
If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
30	383.0	4	367.7	**Negative**		26	99.8	97.8	48.9	
31	282.0	6	265.1	**Control**		27	72.0	69.9	35.0	
32	322.0	6	302.7			28	82.2	80.2	40.1	
33	230.0	9	209.3			29	56.8	54.8	27.4	
34	193.0	11	171.8			30	46.6	44.6	22.3	
MV	**282.0**		**263.3**			**MV**	**71.5**	**69.5**	**34.7**	**1.0**
SD	**67.0**		**68.9**			**SD**	**18.7**	**18.7**	**9.4**	
85	2008.0	2	1967.8	**C.I.**	15	1	534.2	532.2	266.1	7.7
86	2632.0	1	2605.7	**Reactive**		2	707.3	705.3	352.6	10.2
87	2207.0	2	2162.9	**Yellow 174**		3	587.1	585.1	292.5	8.4
88	1570.0	3	1522.9			4	413.4	411.4	205.7	5.9
89	1747.0	2	1712.1			5	464.7	462.7	231.4	6.7
MV	**2032.8**		**1994.3**			**MV**	**541.3**	**539.3**	**269.7**	**7.8**
SD	**370.4**		**375.4**			**SD**	**101.9**	**101.9**	**51.0**	**1.5**
90	737.0	2	722.3	**C.I.**	9	6	196.1	194.0	97.0	2.8
91	270.0	5	256.5	**Reactive**		7	69.6	67.6	33.8	1.0
92	1770.0	1	1752.3	**Yellow 174**		8	475.7	473.6	236.8	6.8
93	2089.0	1	2068.1			9	561.4	559.4	279.7	8.1
94	2351.0	1	2327.5			10	631.8	629.8	314.9	9.1
MV	**1443.4**		**1425.3**			**MV**	**386.9**	**384.9**	**192.4**	**5.5**
SD	**802.9**		**799.3**			**SD**	**217.0**	**217.0**	**108.5**	**3.1**
97	478.0	2	468.4	**C.I.**	3	11	127.2	125.1	62.6	1.8
98	1022.0	1	1011.8	**Reactive**		12	274.6	272.6	136.3	3.9
99	1058.0	1	1047.4	**Yellow 174**		13	284.3	282.3	141.2	4.1
100	733.0	2	718.3			14	195.0	193.0	96.5	2.8
101	1061.0	1	1050.4			15	285.1	283.1	141.6	4.1
MV	**870.4**		**859.3**			**MV**	**233.2**	**231.2**	**115.6**	**3.3**
SD	**231.2**		**231.4**			**SD**	**62.8**	**62.8**	**31.4**	**0.9**
102	1080.0	1	1069.2	**C.I.**	1	16	290.2	288.2	144.1	4.1
103	1040.0	1	1029.6	**Reactive**		17	279.5	277.5	138.7	4.0
104	757.0	1	749.4	**Yellow 174**		18	203.4	201.4	100.7	2.9
105	1398.0	1	1384.0			19	375.7	373.7	186.8	5.4
106	1113.0	1	1101.9			20	299.1	297.1	148.5	4.3
MV	**1077.6**		**1066.8**			**MV**	**289.6**	**289.6**	**144.8**	**4.2**
SD	**204.0**		**202.0**			**SD**	**54.8**	**54.8**	**27.4**	**0.8**
150	22.0	50	11.0	**Background**			3.0			
151	14.0	100	0.0	**Szinti and**			0.0			
152	28.0	39	17.1	**TCA**			4.6			
153	20.0	65	7.0				1.9			
154	15.0	87	2.0				0.5			
MV			**7.4**			**MV**	**2.0**	**0.0**	**0.0**	**0.0**
SD			**6.2**			**SD**	**1.7**			

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value,
SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA= counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 2b: Radioactive determination of the positive control group of the resent study. If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
30	383.0	4	367.7	**Negative Control**		26	99.8	97.8	48.9	
31	282.0	6	265.1			27	72.0	69.9	35.0	
32	322.0	6	302.7			28	82.2	80.2	40.1	
33	230.0	9	209.3			29	56.8	54.8	27.4	
34	193.0	11	171.8			30	46.6	44.6	22.3	
MV	**282.0**		**263.3**			**MV**	**71.5**	**69.5**	**34.7**	**1.0**
SD	**67.0**		**68.9**			**SD**	**18.7**	**18.7**	**9.4**	
25	2179.0	1	2157.2	**P-Phenylene-**	1	1	585.6	583.6	291.8	8.4
26	3639.0	1	3602.6	**diamine**		2	977.9	975.9	488.0	14.0
27	2534.0	1	2508.7			3	681.0	679.0	339.5	9.8
28	2467.0	1	2442.3			4	663.0	660.9	330.5	9.5
29	2074.0	1	2053.3			5	557.3	555.3	277.7	8.0
MV	**2578.6**		**2552.8**			**MV**	**692.9**	**690.9**	**345.5**	**9.9**
SD	**557.3**		**551.8**			**SD**	**149.8**	**149.8**	**74.9**	**2.2**
150	22.0	50	11.0	**Background**			3.0			
151	14.0	100	0.0	**Szinti and**			0.0			
152	28.0	39	17.1	**TCA**			4.6			
153	20.0	65	7.0				1.9			
154	15.0	87	2.0				0.5			
MV			**7.4**			**MV**	**2.0**	**0.0**	**0.0**	**0.0**
SD			**6.2**			**SD**	**1.7**			

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value,
SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA= counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 2c: Radioactive determination of the latest BSL BIOSERVICE positive control

Study number 060114-3
P-Phenylenediamine (CAS 106-50-3, Sigma GmbH, purity > 98%); Lot 69H3638)
1% (w/v) in AOO (3+1 (v/v) Acetone/Olive Oil)
Date of Certificate: April 27, 2006

If not noted individually, results include both lymph nodes of an animal.

POS	CPMA	LUM	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
18	366.0	4	351.4	**Negative**		16	95.4	94.3	47.2	
19	323.0	4	310.1	**Control**		17	84.2	83.1	41.6	
20	666.0	2	652.7			18	177.2	176.1	88.1	
21	180.0	7	167.4			19	45.4	44.4	22.2	
22	320.0	4	307.2			20	83.4	82.3	41.2	
MV	**371.0**		**357.7**			**MV**	**97.1**	**96.1**	**48.0**	**1.0**
SD	**160.3**		**160.1**			**SD**	**43.4**	**43.4**	**21.7**	
78	3210.0	0	3210.0	**P-Phenylen**	1	1	871.3	870.3	435.1	9.1
79	2417.0	1	2392.8	**diamine**		2	649.5	648.5	324.2	6.8
80	2043.0	1	2022.6			3	549.0	548.0	274.0	5.7
81	1633.0	1	1616.7			4	438.8	437.8	218.9	4.6
82	3544.0	0	3544.0			5	962.0	961.0	480.5	10.0
MV	**2569.4**		**2557.2**			**MV**	**694.1**	**693.1**	**346.6**	**7.2**
SD	**712.4**		**720.7**			**SD**	**195.6**	**195.6**	**97.8**	**2.0**
85	29.0	48	15.1	**Background**			4.1			
86	15.0	80	3.0	**Szinti and**			0.8			
87	11.0	100	0.0	**TCA**			0.0			
88	15.0	100	0.0				0.0			
89	18.0	94	1.1				0.3			
MV			**3.8**			**MV**	**1.0**	**0.0**	**0.0**	**0.0**
SD			**5.7**			**SD**	**1.6**			

Szinti = scintillation fluid; TCA = trichloroacetic acid; MV = Mean Value,
SD = Standard Deviation; DPM = disintegrations per minute, CPM = counts per minute chemiluminescence adjusted; CPMA= counts per minute including chemiluminescence; LUM = % chemiluminescence

Table 3: Individual weight of each lymph node and means of animals and groups

Group	*Animal No.*	*right lymph node(mg)*	*left lymph node (mg)*	*Mean of individual animal (mg)*	*Mean of test group (mg)*
	1	4.3	4.4	4.4	
C.I. Reactive	2	5.3	4.2	4.8	
Yellow 174	3	4.0	3.9	4.0	**4.7**
15%	4	6.5	5.2	5.9	
in AOO	5	5.6	3.5	4.6	
Mean value		**5.1**	**4.2**	**4.7**	
Standard Deviation		**0.9**	**0.6**	**0.6**	
	6	2.6	3.5	3.1	
C.I. Reactive	7	4.3	2.9	3.6	
Yellow 174	8	4.2	6.0	5.1	**4.5**
9%	9	4.6	4.9	4.8	
in AOO	10	6.0	5.5	5.8	
Mean value		**4.3**	**4.6**	**4.5**	
Standard Deviation		**1.1**	**1.2**	**1.0**	
	11	3.0	3.7	3.4	
C.I. Reactive	12	4.6	3.6	4.1	
Yellow 174	13	3.4	3.9	3.7	**3.7**
3%	14	4.3	3.3	3.8	
in AOO	15	3.3	4.1	3.7	
Mean value		**3.7**	**3.7**	**3.7**	
Standard Deviation		**0.6**	**0.3**	**0.2**	
	16	4.5	3.6	4.1	
C.I. Reactive	17	6.0	3.8	4.9	
Yellow 174	18	4.6	3.0	3.8	**3.9**
1%	19	2.7	3.4	3.1	
in AOO	20	3.3	4.3	3.8	
Mean value		**4.2**	**3.6**	**3.9**	
Standard Deviation		**1.1**	**0.4**	**0.6**	
	26	2.8	3.5	3.2	
Negative	27	2.7	2.6	2.7	
control	28	2.7	2.6	2.7	**2.6**
AOO	29	2.2	2.2	2.2	
	30	2.1	2.5	2.3	
Mean value		**2.5**	**2.7**	**2.6**	
Standard Deviation		**0.3**	**0.4**	**0.3**	
Positive	21	4.2	5.3	4.8	
control	22	5.1	7.7	6.4	
1%	23	4.6	6.2	5.4	**5.5**
in AOO	24	6.1	5.7	5.9	
	25	4.6	5.2	4.9	
Mean value		**4.9**	**6.0**	**5.5**	
Standard Deviation		**0.7**	**0.9**	**0.6**	

Table 4:
Individual measurement of ear thickness and means of animals and groups

Group	*Animal No.*	*Measurement of ear thickness right first*	*right second*	*left first*	*left second*	*Mean of first test group*	*Mean of second test group*
	1	0.21	0.21	0.22	0.22		
C.I. Reactive	2	0.18	0.19	0.19	0.20		
Yellow 174	3	0.21	0.22	0.21	0.22	**0.21**	**0.22**
15%	4	0.23	0.23	0.22	0.23		
in AOO	5	0.22	0.22	0.21	0.21		
	6	0.22	0.22	0.21	0.21		
C.I. Reactive	7	0.22	0.23	0.21	0.22		
Yellow 174	8	0.20	0.20	0.18	0.19	**0.21**	**0.21**
9%	9	0.22	0.22	0.21	0.22		
in AOO	10	0.22	0.22	0.20	0.20		
	11	0.18	0.19	0.19	0.20		
C.I. Reactive	12	0.18	0.19	0.19	0.20		
Yellow 174	13	0.22	0.22	0.21	0.21	**0.20**	**0.21**
3%	14	0.21	0.22	0.22	0.23		
in AOO	15	0.21	0.21	0.21	0.21		
	16	0.22	0.22	0.21	0.21		
C.I. Reactive	17	0.21	0.21	0.22	0.22		
Yellow 174	18	0.23	0.23	0.21	0.22	**0.22**	**0.22**
1%	19	0.22	0.23	0.23	0.23		
in AOO	20	0.22	0.23	0.20	0.21		
	26	0.20	0.21	0.19	0.20		
Negative	27	0.23	0.23	0.21	0.21		
control	28	0.18	0.19	0.19	0.20	**0.20**	**0.20**
AOO	29	0.19	0.19	0.20	0.20		
	30	0.17	0.18	0.19	0.19		
Positive	21	0.22	0.22	0.20	0.21		
control	22	0.18	0.19	0.18	0.18		
1%	23	0.17	0.18	0.17	0.18	**0.19**	**0.20**
in AOO	24	0.22	0.22	0.20	0.20		
	25	0.19	0.20	0.20	0.21		

Distribution of the Report

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Annex

Analytical Report: 19 pages