harlan®

HARLAN CCR STUDY 1364703

LOCAL LYMPH NODE ASSAY (LLNA)

IN MICE

WITH EXTRACTS OF

Perlazid® Vlies Duplex 60.03

REPORT

Study Completion Date:

January 16, 2012

1 COPY OF GLP-CERTIFICATE

Gute Laborpraxis/Good Laboratory Practice

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 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Harlan Cytotest Cell Research GmbH
Harlan Cytotest Cell Research GmbH
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

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

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)
6 Prüfungen zur Bestimmung von Rückständen
8 Analytische Prüfungen an biologischen Materialien

2 Toxicity studies
3 Mutagenicity studies
6 Residues
8 Analytical studies on biological materials

15.08. und 27. – 29.10.2008
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

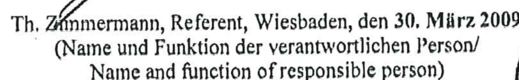
Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility 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 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 is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag



Th. Zimmermann, Referent, Wiesbaden, den 30. März 2009
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

Hess. Ministerium für Umwelt, Energie, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

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3 PREFACE

3.1 General

Title:	Local Lymph Node Assay (LLNA) in Mice with Extracts of Perlazid® Vlies Duplex 60.03
Sponsor:	Perlen Converting AG Perlenring 3 6035 Perlen Switzerland
Study Monitor:	Dr. Stefan Bokorny
Test Facility:	Harlan Cytotest Cell Research GmbH (Harlan CCR) In den Leppsteinswiesen 19 64380 Rossdorf Germany
Contracting Institute:	Harlan Laboratories Ltd. 4452 Itingen Switzerland
Reference Number:	D03695

3.2 Responsibilities

Study Director:	Dr. Julia Wieland[1]
Deputy Study Director:	Dr. Mandy Merker
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

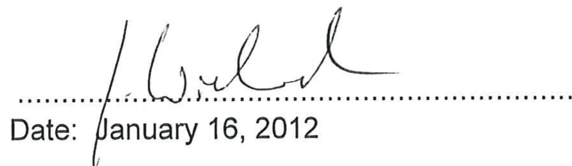
3.3 Schedule

Experimental Starting Date:	September 19, 2010
Experimental Completion Date:	September 29, 2010

[1] Due to marriage the surname of the study director changed from Vogel to Wieland, effective from May 27, 2011.

3.4 Project Staff Signatures

Study Director Dr. Julia Wieland



Date: January 16, 2012

3.5 Good Laboratory Practice

The study was performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

3.6 Guidelines

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

OECD Guidelines for Testing of Chemicals, Updated Guideline 429: Skin Sensitisation: Local Lymph Node Assay (adopted 24 April 2002).

Guideline ISO 10993, published by the International Organization for Standardization: "Biological Evaluation of Medical Devices", Part 1: "Evaluation and testing within a risk management process" (2009), Part 12: "Sample preparation and reference materials", (2007), Part 10: "Tests for irritation and delayed-type hypersensitivity" (2002 and Amd. 1, 2006) and ISO 10993 F-Dis Part 10: "Tests for irritation and skin sensitization" 2009.

3.7 Archiving

Harlan CCR will archive:

Raw data, study plan, report, and specimens (if any) for at least 3 years at the test facility's archive. Thereafter, the material will be transferred to the GLP archive of Harlan Laboratories Ltd. in Füllinsdorf, Switzerland for archiving the remaining time up to a total archiving period of 15 years. No data will be discarded without the sponsor's written consent. A sample of the test item will be archived two years after the expiration date provided by the sponsor. Thereafter the samples will be discarded without further notice.

3.8 Deviations from the Study Plan

In this study, there were no deviations to the study plan.

4 STATEMENT OF COMPLIANCE

Harlan CCR Study:	1364703
Test Item:	Perlazid® Vlies Duplex 60.03
Study Director:	Dr. Julia Wieland
Title:	Local Lymph Node Assay (LLNA) in Mice with Extracts of Perlazid® Vlies Duplex 60.03

This study performed in the test facility of Harlan CCR GmbH was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

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

Study Director Dr. Julia Wieland

Date: January 16, 2012

5 STATEMENT OF QUALITY ASSURANCE UNIT

Harlan CCR Study: 1364703

Test Item: Perlazid® Vlies Duplex 60.03

Study Director: Dr. Julia Wieland

Title: Local Lymph Node Assay (LLNA) in Mice with Extracts of Perlazid® Vlies Duplex 60.03

The general facilities and activities of Harlan CCR GmbH are inspected periodically and the results are reported to the responsible person and the Management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Plan:	September 15/16, 2010	September 15/16, 2010
1st Amendment to Study Plan	December 20, 2010	December 20, 2010
Process Inspection Preparation:	September 20, 2010	September 20, 2010
Report:	January 12, 2011	January 12, 2011

This statement is to confirm that the present report reflects the raw data.

Head of Quality Assurance Unit for Frauke Hermann

Manuella Thomsen

Date: January 16, 2012

6 SUMMARY

In the study the test item Perlazid® Vlies Duplex 60.03 was assessed for its possible contact allergenic potential.

For this purpose the test item was extracted in a polar and a non-polar vehicle according to ISO-10993-12 for 72 ± 2 hours at 37 ± 1.5°C and a local lymph node assay was performed.

The animals did not show any clinical signs during the course of the study and no cases of mortality were observed.

In this study a Stimulation Index (S.I.) of 0.91 was determined with the undiluted non-polar extract in acetone:olive oil (4+1), and a S.I. of 0.77 was determined with the undiluted polar test item extract in ethanol:sterile water (3+7), respectively.

The test item Perlazid® Vlies Duplex 60.03 was **not a skin sensitiser** under the test conditions of this study.

7 OBJECTIVE

7.1 Aims of the Study

The purpose of this Local Lymph Node assay was to identify the contact allergenic potential of Perlazid® Vlies Duplex 60.03 after extraction in a polar and non-polar vehicle, respectively, when administered to the dorsum of both ears of mice.

This study should provide a rational basis for risk assessment to the sensitising potential of the test item in man.

7.2 Outline of the Performed Study

In order to study a possible allergenic potential of Perlazid® Vlies Duplex 60.03, two extracts were prepared according to ISO 10993-12. The test item was extracted in acetone:olive oil (4+1) as a non-polar vehicle and ethanol:sterile water (3+7) as a polar vehicle. Vehicles were chosen with regard to their applicability in the Local Lymph Node Assay. Two groups each of five female mice were treated with the undiluted test item extracts (100 %) by topical application at the dorsum of each ear (left and right) on three consecutive days. For each extract a control group of five mice was treated with the vehicle only. Five days after the first topical application, the mice were intravenously injected into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed and the draining auricular lymph nodes excised and pooled per animal. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes, which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a β-scintillation counter.

8 MATERIALS AND METHODS

8.1 Test Item

Internal Test Item Number: S 1163011

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

Identity:	Perlazid® Vlies Duplex 60.03
Batch No.:	Duplex 60.03 (Production date 06.08.10)
Purity:	> 98% according to the inorganic substances
Stability in Solvent:	Not applicable
Storage:	At room temperature, protected from light
Expiration Date:	August 06, 2012

8.2 Preparation of Test Item Extracts

A part of the test item was cut off and the weight of the cut off part was determined. The test item was extracted according to ISO 10993-12 at a weight/volume ratio of 0.2 g/mL. The extraction was performed at 37 ± 1.5 °C for 72 ± 2 hours while shaking.

The approximate amount of extraction vehicle absorbed by the test item was determined in a non-GLP pre-test. In performing the material extraction, this additional volume was determined for each piece of test item and was added to the respective amount of extraction vehicle calculated. After the extraction step, the extract was gathered using a syringe without touching the test item. For extract 1 acetone:olive oil (4+1) was used as an extraction vehicle, for extract 2 the extraction was performed in ethanol:sterile water (3+7). The vehicle controls were treated accordingly.

The preparations were made freshly before each dosing occasion and applied within 24 hours after the completion of the extraction.

8.3 Dose Selection

The test item extracts were assayed undiluted, i.e. at a concentration of 100 % in order to maximise exposure.

8.4 Chemicals

^{3}H-Methyl thymidine	Hartmann Analytic (MT6032, aqueous solution) 74 GBq/mmol (2 Ci/mmol), 37 MBq/mL (1 mCi/mL)
Trichloroacetic acid	Merck 1.00807.0250 (min. 99.5 %)
Phosphate buffered saline	Fluka no. 79382 (1 tablet solved in 200 ml deionised water)

8.5 Vehicle

Extract 1: Acetone:olive oil (4+1)

Acetone

Manufacturer	Merck KGaA (64293 Darmstadt, Germany)
Supplier	VWR International GmbH (64295 Darmstadt, Germany)
Catalogue number	1.00014.1011
Batch number	K38502214-812
Purity	99.6%
Storage conditions	In the original container at room temperature (20°C ± 5°C), away from direct sunlight.

Olive oil

Supplier	Sigma/Aldrich Chemicals (82024 Taufkirchen, Germany)
Catalogue Number	O-1514
Batch number	058K0684
Storage conditions	In the original container at room temperature (20°C ± 5°C), away from direct sunlight.

Extract 2: Ethanol:sterile water (3+7)

Ethanol

Manufacturer	Merck KGaA (64293 Darmstadt, Germany)
Supplier	VWR International GmbH (64295 Darmstadt, Germany)
Catalogue number	1.009.831.1011
Batch number	K38339683-807
Purity	≥99.9%
Storage conditions	In the original container at room temperature (20°C ± 5°C).

Sterile water

Supplier	B. Braun (34212 Melsungen, Germany)
Catalogue number	6724092.00.00
Batch number	9395A164

9 TEST SYSTEM

9.1 Animal Species

Test system	Mice, CBA/CaOlaHsd
Rationale	Recognised as the recommended test system
Source	Harlan Laboratories B.V. Postbus 6174 5960 AD Horst / The Netherlands
Number of animals for the main study	20 females
Number of animals per group	5 females (nulliparous and non-pregnant)
Number of test groups	2
Number of control (vehicle) groups	2
Age	8 - 12 weeks (beginning of treatment)
Body weight	See Annex 1
Identification	The animals were distributed into the test groups at random. All animals belonging to the same experimental group were kept in one cage. The animals were identified by tail tags.
Acclimatisation	At least 5 days prior to the start of dosing under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

9.1.1 Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing:	group
Cage Type:	Makrolon Type II, with wire mesh top (EHRET GmbH, 79302 Emmendingen, Germany)
Bedding:	granulated soft wood bedding (Rettenmaier & Söhne GmbH + Co. KG, 73494 Rosenberg, Germany)
Feed:	pelleted standard diet, ad libitum (Harlan Laboratories B.V., 5960 AD Horst, Netherlands)
Water:	tap water, ad libitum, (Gemeindewerke, 64380 Rossdorf, Germany)
Environment:	temperature 22 ± 2°C relative humidity 45-65% artificial light 6.00 a.m. - 6.00 p.m.

9.2 Allocation

The animals were distributed as follows:

Group	Concentration (%)	Number of Animals per Group	Animal Numbers (Group Housing)
1 (Control Group (acetone:olive oil 4+1))	—	5	1 - 5
2 (Extract 1)	100	5	6 - 10
3 (Control Group ethanol:sterile water (3+7))	—	5	11 - 15
4 (Extract 2)	100	5	16 - 20

The sensitivity and reliability of the experimental technique employed was assessed by use of a substance, which is known to have skin sensitisation properties in CBA/CaOlaHsd mice. The validation- / positive control experiment was performed with α-hexylcinnamaldehyde in acetone:olive oil (4+1) using CBA/CaOlaHsd mice in September 2010, see Annex 2.

9.3 Experimental Design and Procedures

9.3.1 Topical Application

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear (left and right) with 100% extract 1 or 2. The application volume, 25 µL, was spread over the entire dorsal surface (∅ ~ 8 mm) of each ear once daily for three consecutive days. Two further groups of mice were treated with an equivalent volume of the relevant vehicle alone (control animals).

9.3.2 Administration of ^{3}H-Methyl Thymidine

^{3}H-methyl thymidine (^{3}HTdR) was purchased from Hartmann Analytic, 38124 Braunschweig, Germany (specific activity, 2 Ci/mmol; concentration, 1 mCi/mL).

Five days after the first topical application, all mice were administered with 250 µl of 79.0 µCi/mL ^{3}HTdR (corresponds to 19.8 µCi ^{3}HTdR per mouse) by intravenous injection via a tail vein.

9.3.3 Determination of Incorporated ^{3}HTdR

Approximately five hours after treatment with ^{3}HTdR all mice were euthanised by intraperitoneal injection of Pentobarbital-Natrium (Release®, WDT, 30827 Garbsen, Germany).

The draining lymph nodes were rapidly excised and pooled per animal (2 nodes per animal). Single cell suspensions (in phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200 µm mesh size). After washing two times with phosphate buffered saline (approx. 10 mL) the lymph node cells were resuspended in 5 % trichloroacetic acid (approx. 3 mL) and incubated at approximately +4 °C for at least 18 hours for precipitation of macromolecules. The precipitates were then resuspended in 5 % trichloroacetic acid (1 mL) and transferred to plastic scintillation vials with 10 mL of 'Ultima Gold' scintillation liquid (Perkin Elmer (LAS) GmbH, 63110 Rodgau, Germany) and thoroughly mixed.

The level of ^{3}HTdR incorporation was then measured on a β-scintillation counter (Tricarb 2900 TR, Perkin Elmer (LAS) GmbH, 63110 Rodgau, Germany). Similarly, background ^{3}HTdR levels were also measured in two 1ml-aliquots of 5 % trichloroacetic acid. The β-scintillation counter expresses ^{3}HTdR incorporation as the number of radioactive disintegrations per minute (DPM).

9.3.4 Interpretation of Raw Data

The proliferative response of lymph node cells is expressed as the number of radioactive disintegrations per minute per lymph node s of each animal (DPM/animal) and as the ratio of ^{3}HTdR incorporated into lymph node cells of lymph nodes of test animals relative to that recorded for lymph nodes of control animals (Stimulation Index; S.I.). Before DPM/animal values were determined, mean scintillation-background DPM was subtracted from test and control raw data.

A test item is regarded as a sensitiser in the LLNA if exposure to at least one test item extract resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the stimulation index.

9.4 Observations

In addition to the sensitising reactions the following observations and data were recorded during the test and observation period:

Mortality / Viability	Once daily (week day) from experimental start to necropsy.
Body weights	Prior to the first application and prior to treatment with ^{3}HTdR.
Clinical signs (local / systemic)	Clinical signs were recorded at least once daily from experimental start to necropsy. Especially the treatment sites were observed carefully.

9.5 Statistical Analysis

The mean values and standard deviations were calculated in the body weight tables and for the DPM values (group mean DPM ± standard deviation).

10 RESULTS

Table 1: Calculation and Results of Individual Data Extract 1 (acetone:olive oil (4+1))

Test item extract concentration		DPM values measured	DPM–BG per animal (2 lymph nodes) a)	S.I. b)
% (v/v)	Animal No.			
---	BG I	15	---	---
---	BG II	22	---	---
0	1	648	630	---
0	2	857	839	---
0	3	1323	1305	---
0	4	870	852	---
0	5	827	809	---
100	6	886	868	1.0
100	7	988	970	1.1
100	8	647	629	0.7
100	9	1154	1136	1.3
100	10	441	423	0.5

BG = Background (1 ml 5% trichloroacetic acid) in duplicate
S.I. = Stimulation Index
a) = values corrected for mean background value (BGI and BGII).
b) = Stimulation Indices relative to the mean value (DPM/animal) of the control group

Table 2: Calculation and Results of Individual Data Extract 2 (ethanol:sterile water (3+7))

Test item extract concentration		DPM values measured	DPM–BG per animal (2 lymph nodes) a)	S.I. b)
% (v/v)	Animal No			
---	BG I	15	---	---
---	BG II	22	---	---
0	11	250	232	---
0	12	479	461	---
0	13	752	734	---
0	14	767	749	---
0	15	717	699	---
100	16	397	379	0.7
100	17	586	568	1.0
100	18	530	512	0.9
100	19	549	531	0.9
100	20	230	212	0.4

BG = Background (1 ml 5% trichloroacetic acid) in duplicate
S.I. = Stimulation Index
a) = values corrected for mean background value (BGI and BGII).
b) = Stimulation Indices relative to the mean value (DPM/animal) of the control group

Table 3: Calculation of Stimulation Indices per Dose Group

Test item concentration	Group calculation Mean DPM per animal (2 lymph nodes) [a]	SD	**S.I.**
Vehicle (Acetone:Olive Oil (4+1))	886.5	250.2	**1.00**
100% Extract 1	804.7	281.8	**0.91**
Vehicle (Ethanol:Sterile Water (3+7))	574.5	224.5	**1.00**
100% Extract 2	439.9	146.2	**0.77**

[a] Mean DPM/animal was determined by dividing the sum of the measured values from lymph nodes of all animals within a group by the number of animals in that group (5 animals)

10.1 Viability / Mortality

No deaths occurred during the study period.

10.2 Clinical Signs

No symptoms of local toxicity at the ears of the animals and no systemic findings were observed during the study period.

10.3 Body Weights

The body weight of the animals, recorded prior to the first application and prior to treatment with ^{3}HTdR, was within the range commonly recorded for animals of this strain and age.

The individual body weight values are included in Annex 1.

11 DISCUSSION

In order to study a possible allergenic potential of Perlazid® Vlies Duplex 60.03, two extracts were prepared according to ISO 10993-12. The test item was extracted in acetone:olive oil (4+1) as a non-polar vehicle and ethanol:sterile water (3+7) as a polar vehicle. Vehicles were chosen with regard to their applicability in the Local Lymph Node Assay. Two groups each of five female mice were treated with the undiluted test item extracts (100 %) by topical application at the dorsum of each ear on three consecutive days. For each extract a control group of five mice was treated with the respective vehicle only. Five days after the first topical application, the mice were intravenously injected into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed and the draining auricular lymph nodes excised and pooled per animal. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes, which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a β-scintillation counter.

All treated animals survived the scheduled study period and no signs of toxicity were observed.

A test item is regarded as a sensitiser in the LLNA if the exposure to one or both test item extracts results in a 3-fold or greater increase in incorporation of ^{3}HTdR compared with concurrent controls, as indicated by the Stimulation Index (S.I.).

In this study a Stimulation Index (S.I.) of 0.91 was determined with the undiluted non-polar extract in acetone:olive oil (4+1), and a S.I. of 0.77 was determined with the undiluted polar test item extract in ethanol:sterile water (3+7), respectively.

11.1 Conclusion

The test item Perlazid® Vlies Duplex 60.03 was **not a skin sensitiser** under the test conditions of this study.

12 REFERENCES

1) Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. Contact Dermatitis, 21, 215-220.

2) Kimber I. and Basketter D.A. (1992). The murine local lymph node assay. A commentary on collaborative studies and new directions. Food and Chemical Toxicology, 30, 165-169.

3) Basketter D.A., Gerberick G.F., Kimber I. and Loveless S.E. (1996). The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. Food and Chemical Toxicology, 34, 985-997.

4) Chamberlain M. and Basketter D.A. (1996). The local lymph node assay: status of validation. Food and Chemical Toxicology, 34, 999-1002.

5) Basketter D.A., Lea L.J., Cooper K., Stocks J., Dickens A., Pate I., Dearman R.J. and Kimber I. (1999). Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: a Statistical Evaluation. Food and Chemical Toxicology, 37, 1-8.

6) Steiling W., Basketter D.A., Berthold K., Butler M., Garrigue J-L., Kimber I., Lea L.J., Newsome C., Roggeband R., Stropp G., Waterman S. and Wiemann C. (2001): Skin Sensitisation Testing - New Perspectives and Recommendations. Food and Chemical Toxicology, 39, 293-301.

13 DISTRIBUTION OF THE REPORT

Sponsor	1x copy, 1x PDF
Study Director	1x original

14 ANNEX 1

14.1 Table of Body Weights

Individual animal weights at the start of the experiment

Dose Group	Animal No.	Initial Weight (g)	Mean		SD	Range
Negativ Control	1	19.8				
A:OO (4+1)	2	18.9				
	3	19.3				
	4	19.4				
	5	21.2	19.7	±	0.9	21.2 - 18.9
Test Item	6	21.3				
Dose: 100.000 [%]	7	19.2				
Extrakt 1	8	20.7				
	9	20.5				
	10	20.0	20.3	±	0.8	21.3 - 19.2
Negativ Control	11	19.7				
30% ETOH	12	19.2				
	13	19.9				
	14	17.5				
	15	19.0	19.1	±	0.9	19.9 - 17.5
Test Item	16	17.7				
Dose: 100.000 [%]	17	19.5				
Extrakt 2	18	19.3				
	19	20.4				
	20	19.4	19.3	±	1.0	20.4 - 17.7
Summary			19.6	±	1.0	17.5 - 21.3

Individual animal weights prior administration of ^{3}H-methyl thymidine

Dose Group	Animal No.	Initial Weight (g)	Mean		SD	Range
Negativ Control	1	22.0				
A:OO (4+1)	2	20.8				
	3	19.0				
	4	20.5				
	5	22.2	20.9	±	1.3	22.2 - 19.0
Test Item	6	21.1				
Dose: 100.000 [%]	7	20.1				
Extrakt 1	8	21.0				
	9	20.5				
	10	20.4	20.6	±	0.4	21.1 - 20.1
Negativ Control	11	20.2				
30% ETOH	12	20.0				
	13	18.9				
	14	20.2				
	15	21.3	20.1	±	0.9	21.3 - 18.9
Test Item	16	19.0				
Dose: 100.000 [%]	17	20.6				
Extrakt 2	18	18.7				
	19	20.0				
	20	20.9	19.8	±	1.0	20.9 - 18.7
Summary			20.4	±	1.0	18.7 - 22.2

15 ANNEX 2

15.1 Results of the GLP Positive Control

Experiment performed in September 2010.

Positive control substance: α-Hexylcinnamaldehyde

Vehicle: acetone:olive oil (4+1 v/v)

Test item concentration % (w/v)	Group	Measurement DPM	Calculation			Result
			DPM-BG [a)]	number of lymph nodes	DPM per lymph node [b)]	**S.I.**
---	BG I	15	---	---	---	---
---	BG II	11	---	---	---	---
0	1	2291	2278	8	284.8	**1.00**
5	2	2421	2408	8	301.0	**1.06**
10	3	6596	6583	8	822.9	**2.89**
25	4	14711	14698	8	1837.3	**6.45**

BG = Background (1 ml 5% trichloroacetic acid) in duplicate

1 = Control Group

2-4 = Test Group

S.I. = Stimulation Index

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, DPM/node was determined by dividing the measured value by the number of lymph nodes pooled

	Test item concentration %	S.I.
Test Group 3	10 (a)	2.89 (b)
Test Group 4	25 (c)	6.45 (d)
EC3 = (a-c) [(3-d)/(b-d)] + c = **10.5 %** (w/v)		

EC3 = Estimated concentration for a S.I. of 3.

a,b,c,d = Co-ordinates of the two pairs of data lying immediately above and below the S.I. value of 3 on the LLNA dose response plot.

15.2 Historical positive control data

These values represent the data of the last 10 positive controls (November 2009 – September 2010):

	Stimulation Index at 25% (w/v) (α-HCA in acetone:olive oil (4:1 v/v))
Mean ± SD	6.31 ± 1.34
No. of experiments	10