TEST REPORT

DANAK
TEST Reg. No 145

LAB Scantox

Acyltransferase BL1
(*Bacillus licheniformis* BML780-KLM3' CAP50)
(GICC 3265)

***IN VITRO* MAMMALIAN**
CHROMOSOME ABERRATION TEST
PERFORMED WITH HUMAN LYMPHOCYTES

LAB Scantox Study No:	62128
Date:	16 November 2006
Author:	C. Nicholas Edwards, PhD
Number of pages:	25
Sponsors:	Genencor International Inc. (A Danisco Company) 925 Page Mill Road Palo Alto, CA 94304 USA

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study described in this report "Acyltransferase BL1 (*Bacillus licheniformis* BML780-KLM3' CAP50) (GICC 3265) - *In Vitro* Mammalian Chromosome Aberration Test performed with Human Lymphocytes" was conducted under my supervision and responsibility and in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997), which are in conformity with other international GLP regulations.

This report is a complete and accurate account of the methods employed and the data obtained.

C N Edwards — 16 November 2006

C Nicholas Edwards, PhD
Study Director
LAB Scantox

Date

QUALITY ASSURANCE STATEMENT

Study number: 62128

Study title: Acyltransferase BL1 (*Bacillus licheniformis* strain BML780-KLM3' CAP50) (GICC 3265) - *In Vitro* Mammalian Chromosome Aberration Test Performed With Human Lymphocytes

Process-based and facility inspections are carried out to cover the activities within short term studies of the type described in this study report.

A review of the study plan has been performed and reported to the Study Director:

Date of review:	**Reporting date:**
06 March 2006	06 March 2006

In accordance with LAB Scantox Quality Assurance Procedures and relevant parts of current OECD series on principles of Good Laboratory Practice and Compliance Monitoring, the study procedures applicable to this study have been inspected as follows.

Process-based inspection	**Inspection date(s)**	**Reporting date to Study Director and management**
Most recent inspection of similar study	19 December 2005 21 April 2006 28 July 2006	19 December 2005 21 April 2006 28 July 2006

The study report has been audited. As far as can be reasonably established, the methods, procedures and observations have been accurately described, and the results and data presented in the study report accurately reflect the raw data generated during the study.

The study report gives an accurate account of the methods and procedures outlined in the study plan and in the LAB Scantox Standard Operating Procedures.

Audit date of Draft Report and data:	**Reporting date:**
08, 14 and 15 November 2006	15 November 2006
Audit date of Final Report: 16 November 2006	Not applicable

Pauline Sylvest Salanti
Head of Quality Assurance
LAB Scantox

16 November 2006
Date

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MICROPTIC CYTOGENETICS GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT	**Client No./Study Ref:** 38/18 **Client Study No.:** 62128 **Study Type:** Metaphase analysis **Date:** 25 September 2006

I hereby confirm that the work conducted at Microptic Cytogenetics, in respect of this study, was in compliance with Good Laboratory Practice (GLP) as required by the United Kingdom GLP Compliance Regulations 1999 (SI 1999 No. 3106, as amended 2004, S.I. No.0994) and which are in accordance with the OECD Principles of GLP 1997 (ENV/MC/CHEM(98) 17).

NATALIE DANFORD, BSc, MPH, PhD

PRINCIPAL INVESTIGATOR

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MICROPTIC CYTOGENETICS QUALITY ASSURANCE STATEMENT	**Client No./Study Ref:** 38/18 **Client Study No.:** 62128 **Study Type:** Metaphase analysis **Date:** 25 September 2006

Quality Assurance audits were performed for Microptic Cytogenetics as a continuous process using regular scheduled inspections and audits. These were in accordance with the current Standard Operating Procedure (SOP-07) for monitoring inspections and audits.

Inspections/audits applicable to the above study type were conducted as follows:

Phases Inspected	Date of Audit/ Inspection	Date of Report to Principal Investigator/Management
Analysts	24 May 2005	01 June 2005
GLP systems	30 May 2006	31 May 2006

Any deficiencies considered to affect the quality or integrity of a study are reported to the Principal Investigator who informs the Study Director and Lead QA. No such deficiencies were found.

N. JONES
QUALITY ASSURANCE CO-ORDINATOR

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SUMMARY

This study was conducted at LAB Scantox, Hestehavevej 36A, Ejby, DK-4623 Lille Skensved, Denmark.

The test item, Acyltransferase BL1 (*Bacillus licheniformis* BML780-KLM3' CAP50) (GICC 3265), was tested in accordance with the OECD guideline "*In Vitro* Mammalian Chromosome Aberration Test" No. 473 (1997).

The test item was tested in primary cultures of human lymphocytes in the absence and presence of S-9 mix. The cultures were treated with formulations of the test item dissolved in cell culture medium. Two independent tests were performed. Repeat tests were also performed using lower concentrations of the test item because excessive toxicity was observed in the original tests. In the first test and repeats of this test, all cultures with and without S-9 mix were treated for three hours. In the second test and repeat of this test, the cultures were treated for 20 hours without S-9 mix and three hours with S-9 mix. All cultures were harvested 20 hours (approximately 1.5 normal cell cycles) after the start of treatment. The final concentration of S-9 homogenate used in the second test was twice as high as in the first test.

The test item caused dose-related toxicity to the test cells: reductions in mean mitotic index and reductions in the numbers of cells on the slides were observed at the higher concentrations tested. Slides from cultures treated with the test item at the following concentrations were scored for chromosomal aberrations:

First test (repeat), without S-9 mix:	64, 128 and 256 µg/ml (3 hour treatment)
(second repeat) with S-9 mix:	0.0625, 0.125 and 0.25 µg/ml (3 hour treatment)
Second test (repeat), without S-9 mix:	2, 4 and 8 µg/ml (20 hour treatment)
(repeat) with S-9 mix:	0.0156, 0.0313 and 0.0625 µg/ml (3 hour treatment).

All the concentrations in this report are expressed in terms of the weight of the lyophilized test item received. The highest concentrations scored for chromosomal aberrations in the absence and presence of S-9 mix showed the appropriate level of toxicity (greater than 50% reductions in mean mitotic index).

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with the test item in either test.

The positive control treatments produced large, statistically significant increases in the frequency of aberrant metaphases, demonstrating the sensitivity of the test and the efficacy of the S-9 mix.

Two polyploid metaphases were observed in this study, but their incidence was not dose-related and it is concluded that they were not caused by the test item. No endoreduplicated metaphases were observed.

It is concluded that Acyltransferase BL1 (*Bacillus licheniformis* BML780-KLM3' CAP50) (GICC 3265) did not cause chromosomal aberrations in this *in vitro* cytogenetic test using cultured human lymphocytes either in the absence or presence of S-9 mix.

INTRODUCTION

The mammalian cytogenetic test *in vitro* is a short term mutagenicity test for the evaluation of possible chromosome damaging effects of chemicals. This test was conducted in accordance with the OECD guideline "*In Vitro* Mammalian Chromosome Aberration Test" No. 473 (1997).

The experimental part of the study was started on 06 April 2006 and the scoring of the slides was completed on 07 September 2006.

This study was conducted at LAB Scantox, Hestehavevej 36A, Ejby, DK-4623 Lille Skensved, Denmark. The metaphase analysis was conducted by Microptic Cytogenetic Services, 2 Langland Close, Mumbles, Swansea SA3 4LY, United Kingdom.

Personnel involved in the study

Study Director: C. Nicholas Edwards, PhD
Sponsor Monitor: Dr Quang Q Bui, Genencor International Inc. (A Danisco company)
Principal Investigator for metaphase analysis: Natalie Danford, PhD, Microptic Cytogenetic Services.

General description of the test system

Cultures of growing primary human lymphocytes were exposed to the test item in the absence and presence of a metabolic activation system (S-9 mix). Two tests and repeats of these tests were performed. In the first test, all cultures with and without S-9 mix were treated for three hours and harvested 20 hours after the start of treatment. In the second test, the cultures were treated for 20 hours in the absence of S-9 mix and three hours in the presence of S-9 mix. All cultures were harvested 20 hours after the start of treatment. The sampling time of 20 hours corresponded to approximately 1.5 normal cell cycles after the start of treatment.

Some chemicals become clastogenic in this test system only when they have been changed to active metabolites by mammalian enzymes. The activation was accomplished by incubating the cells with the test item and S-9 mix for three hours. S-9 mix consists of salts, co-factors and an enzyme-rich post-mitochondrial fraction prepared from the livers of rats pre-treated with Aroclor®1254. After treatment, the cells were sedimented by centrifugation, resuspended in fresh medium, and cultured for a further 17 hours until harvest.

After incubation with demecolcine for the last two hours, the cells were harvested and metaphase preparations were made on slides. One hundred metaphases from each culture (where necessary) were analysed using a microscope, and the number and type of the observed chromosomal aberrations were recorded.

A measure of the clastogenic effect of the test item was obtained by comparing the frequency of metaphases with chromosomal aberrations in the test cultures with the negative control values.

MATERIALS AND METHODS

Test item

Acyltransferase BL1 (*Bacillus licheniformis* strain BML780-KLM3' CAP50)(GICC 3265)

Lot No:	20068010
Description:	Lyophilized powder
Intended use:	Food additive
Stability:	Lyophilized powder is stable for at least 1 year when stored frozen (see certificate of analysis in Appendix 2).

The test item was received from Danisco A/S on 04 April 2006. The Sponsor was responsible for preparation and characterisation of the test item. A certificate of analysis for the test item was supplied by the Sponsor and is reproduced in Appendix 2. The samples of the test item were labelled with the Study No. of this study and stored in a freezer at approximately -18°C in the dark.

Preparation of test item formulations

On the day before each test, the sample of the test item was moved to a refrigerator at approximately 5°C in the dark. On the day of each test, a sub-sample of the test item was taken. The remainder of the test item was returned to storage at approximately -18°C in the dark after use. On some occasions, several sub-samples were taken at the same time and stored in a freezer at approximately -18°C in the dark until use to avoid repeated freeze-thaw cycles of the stock sample. Immediately before use, a sub-sample was dissolved in cell culture medium at a range of concentrations so that the required final test concentrations could be achieved by adding a constant volume of the formulations to the cultures. The test concentrations were expressed in terms of the weight of the lyophilized test item as received. No analyses were performed to determine the test item concentrations, homogeneity or stability achieved in the formulations or lymphocyte cultures.

Cells and culture methods

The study was performed with primary human lymphocytes obtained from two healthy, non-smoking, male volunteers from the LAB Scantox laboratory. One volunteer provided the lymphocytes for the first test and repeats of this test, and the other volunteer provided the lymphocytes for the second test and repeat of this test. Whole blood was collected in sterile glass tubes containing heparin. The lymphocytes were cultured in flasks containing whole blood (8%) and phytohaemagglutinin solution (1%, Gibco, Life Technologies A/S, Tåstrup, Denmark) in culture medium. The culture medium was RPMI 1640 medium (Gibco), supplemented with foetal calf serum (10%, Gibco) and Gentamycin (45 µg/ml, Gibco). The cells were cultured in sterile plastic culture flasks in an incubator at 37°C with an atmosphere containing 5% carbon dioxide in air.

The cell cycle times for the lymphocytes from each of the donors under the culture conditions employed in this study were determined previously. The cell cycle times for the two donors

were found to be 15.7 and 13.9 hours; thus the sampling time of 20 hours corresponded to approximately 1.5 cell cycles.

Rat liver post-mitochondrial fraction

SPF Wistar rats of the stock Mol:WIST were obtained from Taconic Europe A/S, Ejby, DK-4623 Lille Skensved, Denmark. Rats weighing approximately 200 g were used for induction of liver enzymes. A single intraperitoneal injection of Aroclor®1254 at a dose of 500 mg/kg body weight was given to each rat. The animals were killed with a high concentration of carbon dioxide 5 days after being injected and following a 16 hour period of fasting.

All steps in the preparation of the liver homogenate were performed on ice using aseptic techniques and cold, sterile solutions. The livers were removed, rinsed briefly in 0.15 M KCl, and minced in 0.15 M KCl solution (3 ml per gram wet liver). After homogenisation, the homogenate was centrifuged at 9000 g at approximately 4°C for 15 minutes. The supernatant (post-mitochondrial fraction) was collected, frozen and stored with liquid nitrogen at -196°C until use.

S-9 mix

	Test 1	Test 2
Post-mitochondrial fraction	10 %	20 %
Sodium phosphate buffer (pH 7.4)	100 mM	100 mM
$MgCl_2$/KCl	8 mM/ 33 mM	8 mM/ 33 mM
Glucose-6-phosphate	5 mM	5 mM
NADP	4 mM	4 mM

The S-9 mix was prepared shortly before use. The co-factor/buffer mix was sterilized by filtration (0.2 µm pore size) before addition of the S-9 fraction. The S-9 mix was kept on ice and warmed to approximately 37°C immediately before use. The final concentration of S-9 post-mitochondrial fraction in the treated cultures was 2% in the first test. This was increased to 4% in the second test because no large increases in the frequency of aberrant metaphases were caused by the test item in the first test.

Treatment of cultures

Human lymphocyte cultures were prepared as described above. After incubation for approximately 48 hours, the cultures were centrifuged and the supernatant was discarded. The cell pellet was resuspended in an appropriate volume of fresh culture medium and S-9 mix (1 ml) was added to appropriate cultures. Then the cultures were treated by addition of aliquots of the test item formulations (4 ml for the first test and 1 ml for the second test and all repeat tests) to give the required final concentrations of the test item. The final total volume of each culture was 5 ml. Duplicate cultures were treated at all test concentrations, with and without S-9 mix.

Two main tests and repeats of these tests were performed. In the first test and repeats of this test, all cultures were treated for three hours. In the second test and repeat of this test, cultures were treated for 20 hours without S-9 mix and three hours with S-9 mix. All cultures were harvested 20 hours (approximately 1.5 normal cell cycles) after the start of treatment.

The concentrations of the test item that were selected for testing were:

First test, with and without S-9 mix:	156, 313, 625, 1250, 2500 and 5000 µg/ml
First test (repeat), without S-9 mix:	32, 64, 128, 256 and 512 µg/ml
with S-9 mix:	2, 4, 8, 16, 32, 64 and 128 µg/ml
First test (second repeat), with S-9 mix:	0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 µg/ml
Second test, without S-9 mix:	8, 16, 32, 64, 128, 256 and 512 µg/ml
with S-9 mix:	0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 µg/ml.
Second test (repeat), without S-9 mix:	0.25, 0.5, 1, 2, 4, 8, and 16 µg/ml
with S-9 mix:	0.00195, 0.00391, 0.00781, 0.0156, 0.0313, 0.0625 and 0.125 µg/ml.

The test concentrations were separated by factors of two and were selected with the expectation that one or more of the higher test concentrations would cause reductions in mean mitotic index of at least 50% and there would be at least two lower concentrations that showed less than 50% reduction in mean mitotic index. All the test item concentrations in this report are expressed in terms of the weight of the lyophilized test item as received.

Duplicate negative (cell culture medium) control cultures and duplicate positive control cultures were included in each test. The positive control cultures were treated with Daunomycin (0.015 µg/ml) in the absence of S-9 mix and Cyclophosphamide (6 µg/ml) in the presence of S-9 mix.

The cultures were incubated at 37°C with gentle mixing for the appropriate treatment period. After three hours of treatment the appropriate cultures were centrifuged. The supernatant treatment medium was removed and the cells were resuspended in fresh medium (5 ml). The cultures were then incubated at 37°C for a further 17 hours until harvest. In the second test, cultures without S-9 mix were treated for 20 hours until harvest. Demecolcine (final concentration 0.1 µg/ml) was added to each culture for the last two hours before harvest.

Cell harvest and slide preparation

At the 20 hour harvest time, each culture was centrifuged and the supernatant was removed. The cell pellet was resuspended in warm 0.075 M KCl (37°C, 5 ml) and incubated at 37°C for 10 minutes. The cell suspensions were then centrifuged again and the supernatants were removed.

The cells were fixed by addition of fixative (methanol/acetic acid, 3:1), washed two or three times with fresh fixative, and dropped onto clean glass slides. After drying in air, the

chromosome preparations were stained in 3% Giemsa for 10 minutes. Cover glasses were mounted with Dammarxylen®.

Determination of mitotic index and frequency of polyploidy and endoreduplication

The slides were examined using a light microscope at 100-400x magnification and the number of cells at metaphase was counted in 1000 cells from each culture. The mitotic index was calculated as the percentage of cells at metaphase. The numbers of polyploid and endoreduplicated metaphases in 200 metaphases was also counted for each culture. Polyploid metaphases were defined as metaphases with multiples of the haploid chromosome number (n), other than the diploid number (i.e. 3n, 4n, etc.). Endoreduplicated metaphases had chromosomes with 4, 8 chromatids. Marked reductions in the numbers of cells on the slides were recorded, if seen.

Metaphase analysis

Slides from cultures treated with three concentrations of the test item that showed the appropriate range of toxicity and slides from the negative and positive control cultures were given code numbers by a person who was not involved in the metaphase analysis. The code labels covered all unique identification markings on the slides to ensure that they would be scored without bias. The selected slides were sent to Microptic Cytogenetic Services, 2 Langland Close, Mumbles, Swansea SA3 4LY, United Kingdom for metaphase analysis.

One hundred metaphases with 46±2 chromosomes from most cultures were examined for the presence or absence of chromosomal aberrations, using a microscope at 1000x magnification. The scoring of the positive control slides was stopped before 100 metaphases had been scored when a large number of metaphases with aberrations had been observed (15/culture, excluding gaps). The aberrations were recorded as gaps, breaks, or exchanges, and classified as chromatid- or chromosome-type in each case. These were defined in the following way:

Gap:	small unstained lesion smaller than the width of a chromatid and with minimal misalignment of the chromatid(s)
Break:	unstained lesion larger than the width of a chromatid, or with clear misalignment
Exchange	breakage and reunion of chromatids within a chromosome, or between chromosomes
Chromatid-type:	structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids
Chromosome-type:	structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Fragments can arise from breakage and exchange events: when their origin was clear, they were recorded under that category (e.g. a dicentric chromosome with a fragment was recorded as one chromosome exchange event). When the origin of the fragment was not clear, it was recorded as a chromatid break. Metaphases with five or more aberrations (excluding gaps)

were recorded as showing multiple damage. Pulverised metaphases with gross fragmentation of the DNA were recorded separately and not included in the total of 100 scored for chromosomal aberrations because it was not possible to count the chromosomes.

The Vernier co-ordinates of at least five metaphases were recorded for each culture.

When the analysis of the selected slides was completed, the slides and original data were returned to LAB Scantox. The coding of the data was broken to allow assessment of the effect of the test item treatment.

Evaluation of results

The test data is considered to be valid as the negative control cultures showed a low frequency of metaphases with aberrant chromosomes and the positive controls showed clear increases in the frequency of metaphases with aberrant chromosomes. The data obtained for the control cultures was consistent with the historical control data for this laboratory (see Appendix 1).

The number of metaphases with aberrant chromosomes at each test concentration was compared to the concurrent negative control value. When appropriate, statistical analysis was performed using Fischer's Exact Test. The statistical analysis was performed with SAS® procedures (version 8.2) described in SAS/STAT® User's Guide, SAS OnlineDoc®, 1999, SAS Institute Inc., Cary, North Carolina 27513, USA. Gap-type aberrations were recorded during metaphase analysis and reported in the tables, but they were omitted from the statistical analysis because gaps are of questionable biological significance.

It would have been concluded that the test item had shown clastogenic activity in this study if all of the following criteria had been met:
- increases in the frequency of metaphases with aberrant chromosomes were observed at one or more test concentrations
- the increases were reproducible between replicate cultures and between tests (when treatment conditions were the same)
- the increases were statistically significant
- the increases were not associated with large changes in pH or osmolarity of the treated cultures

The historical negative control data for this laboratory (see Appendix 1) was also considered in the evaluation. Evidence of a dose-response relationship would have been considered to support the conclusion.

The test item would have been considered to have given a negative response if no reproducible, statistically significant increases were observed.

Results which failed to meet the stated criteria for a negative or positive response would have been considered to be equivocal.

Archives

For a period of 10 years LAB Scantox will be responsible for the archiving of the following material relating to the study:

Study plan, study plan amendment, correspondence, test item receipts, all original data including the metaphase analysis data recorded by Microptic Cytogenetic Services, microscope slides and the final report.

At the end of the storage period LAB Scantox will contact the Sponsor for instructions whether the material should be transferred, retained or destroyed.

RESULTS

The results of the tests are presented in Tables 1 to 7.

The test item caused dose-related toxicity in the absence and presence of S-9 mix in all tests: dose-related reductions in mean mitotic index and reductions in the numbers of cells observed on the slides at the higher dose levels were observed. A dose-related amount of haemolysis was observed in most cultures treated with the test item at 128 µg/ml and above without S-9 mix and 0.5 µg/ml and above with S-9 mix in each test. Slides from cultures treated with the test item at the following concentrations were selected for the scoring of chromosomal aberrations:

First test (repeat), without S-9 mix:	64, 128 and 256 µg/ml (3 hour treatment)
(second repeat) with S-9 mix:	0.0625, 0.125 and 0.25 µg/ml (3 hour treatment)
Second test (repeat), without S-9 mix:	2, 4 and 8 µg/ml (20 hour treatment)
(repeat) with S-9 mix:	0.0156, 0.0313 and 0.0625 µg/ml (3 hour treatment).

The test item caused reductions in mean mitotic index of 52 to 54% at the highest concentrations scored for chromosomal aberrations, compared to the solvent control values. This level of toxicity meets the requirements of the OECD 473 guideline for the highest concentration to be scored for aberrations (greater than 50% reduction in mean mitotic index).

In general, one hundred metaphases from each selected culture were scored for the presence or absence of aberrant chromosomes. The scoring of most of the positive control cultures was halted before 100 metaphases had been examined when large numbers of metaphases with chromosomal aberrations (15/culture, excluding gaps) had been observed for each, clearly demonstrating the effectiveness of the positive control treatments.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Acyltransferase BL1 (*Bacillus licheniformis* BML780-KLM3' CAP50)(GICC 3265) in either test (see Tables 1 to 4).

The frequencies of metaphases with chromosome aberrations in the negative and positive control cultures were within the normal ranges and compatible with the historical control data for this laboratory (see Appendix 1).

The positive control treatments produced large, statistically significant increases in the frequency of aberrant metaphases in both tests, demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

Two polyploid metaphases were observed in this study, but their incidence was not dose-related and it is concluded that they were not caused by the test item. No endoreduplicated metaphases were observed.

CONCLUSION

It is concluded that Acyltransferase BL1 (*Bacillus licheniformis* BML780-KLM3' CAP50) (GICC 3265) did not cause chromosomal aberrations in this *in vitro* cytogenetic test using cultured human lymphocytes either in the absence or presence of S-9 mix.

REFERENCE

1. OECD Guideline for the Testing of Chemicals No. 473: *In Vitro* Mammalian Chromosome Aberration Test. Adopted 21 July 1997.

Test 1 (repeat)
Results for treatments without S-9 mix

Treatment (µg/ml)	Mitotic index	Redn. in mean MI (%)	No. aberrant metaphases	Number and types of aberrations observed								Pol	E
				Gaps		Breaks		Exchanges		M	Pul		
				Ct	Cs	Ct	Cs	Ct	Cs				
Vehicle	5.7 6.9	0	2 - 2	2		2 1	1						
TA (512)	1.8f 1.6f	73	These slides were not selected for metaphase analysis										
TA (256)	2.9f 3.1f	52	3 NS 0	6 1	1	3							
TA (128)	4.1 5.3	25	6 NS 3	6 3	1	7 2			1				
TA (64)	6.0 6.6	0	4 NS 5	2 3	2	6 7		2				1	
TA (32)	6.3 5.8	4	These slides were not selected for metaphase analysis										
PC (0.015)	6.3 5.6	6	15a ** 15b	3 4		13 16	3 1	3 2	1				

Key:

Mitotic index	Percentage of cells at metaphase
Redn. in mean MI (%)	Percentage reduction in mean mitotic index for duplicate cultures compared to the negative control
No. aberrant metaphases	Number of metaphases with aberrant chromosomes (excluding gaps) (Statistical analysis was performed on the total from both cultures) NS Not statistically significant, $p>0.05$, compared to negative control ** Statistically significant, $p<0.01$, compared to negative control
Ct	Chromatid-type aberration
Cs	Chromosome-type aberration
M	Number of metaphases with multiple aberrations (>5, excluding gaps)
Pul	Number of pulverised metaphases
Pol	Number of polyploid metaphases, 200 metaphases scored where possible
E	No. endoreduplicated metaphases, 200 metaphases scored where possible
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control – Daunomycin
a	Only 71 metaphases were scored for this culture (15 aberrant = 21%)
b	Only 65 metaphases were scored for this culture (15 aberrant = 23%)
f	Fewer cells on slide

Test 1 (second repeat)
Results for treatments with S-9 mix

Treatment (µg/ml)	Mitotic index	Redn. in mean MI (%)	No. aberrant metaphases	Number and types of aberrations observed								Pol	E
				Gaps		Breaks		Exchanges		M	Pul		
				Ct	Cs	Ct	Cs	Ct	Cs				
Vehicle	5.6 5.3	0	1 1 -		1	1 1							
TA (4)	0.3f 0.2f	95	These slides were not selected for metaphase analysis										
TA (2)	0.2f 0.0f	98	These slides were not selected for metaphase analysis										
TA (1)	0.8f 0.9f	84	These slides were not selected for metaphase analysis										
TA (0.5)	1.2f 0.9f	81	These slides were not selected for metaphase analysis										
TA (0.25)	2.5 2.5	54	1 0 NS	1		1							
TA (0.125)	3.0 3.7	39	1 2 NS	2 2	1	1 2							
TA (0.0625)	5.5 5.7	3 Inc	0 1 NS			1							
PC (6)	2.9 3.4	42	15a 15b **	5 1		20 14	3 4	2 4					

Key:

Mitotic index	Percentage of cells at metaphase
Redn. in mean MI (%)	Percentage reduction in mean mitotic index for duplicate cultures compared to the negative control (Inc = Increase)
No. aberrant metaphases	Number of metaphases with aberrant chromosomes (excluding gaps) (Statistical analysis was performed on the total from both cultures) NS Not statistically significant, $p>0.05$, compared to negative control ** Statistically significant, $p<0.01$, compared to negative control
Ct	Chromatid-type aberration
Cs	Chromosome-type aberration
M	Number of metaphases with multiple aberrations (>5, excluding gaps)
Pul	Number of pulverised metaphases
Pol	Number of polyploid metaphases, 200 metaphases scored where possible
E	No. endoreduplicated metaphases, 200 metaphases scored where possible
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control - Cyclophosphamide
a	Only 49 metaphases were scored for this culture (15 aberrant = 31%)
b	Only 27 metaphases were scored for this culture (15 aberrant = 56%)
f	Fewer cells on slide

Test 2 (repeat)
Results for treatments without S-9 mix

Treatment (µg/ml)	Mitotic index	Redn. in mean MI (%)	No. aberrant metaphases		Number and types of aberrations observed								Pol	E
					Gaps		Breaks		Exchanges		M	Pul		
					Ct	Cs	Ct	Cs	Ct	Cs				
Vehicle	6.5 6.5	0	0 1	-	5			1						
TA (16)	1.6f 1.9f	73	These slides were not selected for metaphase analysis											
TA (8)	3.4f 2.9f	52	0 0	NS	1 1									
TA (4)	5.4f 3.5f	32	0 1	NS	5 1			1						
TA (2)	4.5 4.8	28	0 0	NS	3 1									
TA (1)	5.7 6.3	8	These slides were not selected for metaphase analysis											
TA (0.5)	6.1 6.6	2	These slides were not selected for metaphase analysis											
TA (0.25)	6.8 6.4	2 Inc	These slides were not selected for metaphase analysis											
PC (0.015)	4.9 6.1	15	15a 15b	**	10 10		4 6	6 2	11 9	1	1			

Key:

Mitotic index	Percentage of cells at metaphase
Redn. in mean MI (%)	Percentage reduction in mean mitotic index for duplicate cultures compared to the negative control (Inc = Increase)
No. aberrant metaphases	Number of metaphases with aberrant chromosomes (excluding gaps) (Statistical analysis was performed on the total from both cultures) NS Not statistically significant, $p>0.05$, compared to negative control ** Statistically significant, $p<0.01$, compared to negative control
Ct	Chromatid-type aberration
Cs	Chromosome-type aberration
M	Number of metaphases with multiple aberrations (>5, excluding gaps)
Pul	Number of pulverised metaphases
Pol	Number of polyploid metaphases, 200 metaphases scored where possible
E	No. endoreduplicated metaphases, 200 metaphases scored where possible
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control - Daunomycin
a	Only 70 metaphases were scored for this culture (15 aberrant = 21%)
b	Only 45 metaphases were scored for this culture (15 aberrant = 33%)
f	Fewer cells on slide

Test 2 (repeat)
Results for treatments with S-9 mix

Treatment (µg/ml)	Mitotic index	Redn. in mean MI (%)	No. aberrant metaphases	Number and types of aberrations observed								Pol	E
				Gaps		Breaks		Exchanges		M	Pul		
				Ct	Cs	Ct	Cs	Ct	Cs				
Vehicle	6.6 5.6	0	0 1 -	2					1				
TA (0.125)	1.9f 2.2f	66	These slides were not selected for metaphase analysis										
TA (0.0625)	3.0f 2.8f	52	0 1 NS	4 3		1							
TA (0.0313)	4.6f 3.2f	36	0 0 NS	1 3									
TA (0.0156)	6.5 6.2	4 Inc	3 0 NS	2 2		2		1					
TA (0.00781)	5.5 5.9	7	These slides were not selected for metaphase analysis										
TA (0.00391)	6.0 6.0	2	These slides were not selected for metaphase analysis									1	
TA (0.00195)	6.2 5.6	3	These slides were not selected for metaphase analysis										
PC (6)	4.0 3.5	39	13 ** 15a	9 3		14 18	1 1	1					

Key:

Mitotic index	Percentage of cells at metaphase
Redn. in mean MI (%)	Percentage reduction in mean mitotic index for duplicate cultures compared to the negative control (Inc = Increase)
No. aberrant metaphases	Number of metaphases with aberrant chromosomes (excluding gaps) (Statistical analysis was performed on the total from both cultures) NS Not statistically significant, $p>0.05$, compared to negative control ** Statistically significant, $p<0.01$, compared to negative control
Ct	Chromatid-type aberration
Cs	Chromosome-type aberration
M	Number of metaphases with multiple aberrations (>5, excluding gaps)
Pul	Number of pulverised metaphases
Pol	Number of polyploid metaphases, 200 metaphases scored where possible
E	No. endoreduplicated metaphases, 200 metaphases scored where possible
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control - Cyclophosphamide
a	Only 56 metaphases were scored for this culture (15 aberrant = 27%)
f	Fewer cells on slide

Main test 1 - Mitotic indices

Without S-9 mix			With S-9 mix		
Treatment (µg/ml)	Mitotic index		Treatment (µg/ml)	Mitotic index	
	Individual values	Redn. in mean MI (%)		Individual values	Redn. in mean MI (%)
Vehicle	7.2 7.5	0	Vehicle	5.6 7.5	0
TA (5000)	n n	100	TA (5000)	n n	100
TA (2500)	n n	100	TA (2500)	n n	100
TA (1250)	0.0f 0.0f	100	TA (1250)	n n	100
TA (625)	0.1f 0.1f	99	TA (625)	n n	100
TA (313)	0.7f 0.5f	92	TA (313)	n n	100
TA (156)	5.1f 3.9f	39	TA (156)	0.0f 0.0f	100
PC (0.015)	5.9 5.1	25	PC (6)	5.3 4.7	24

Key:

Mitotic index	Percentage of cells at metaphase
Individual values	Values for each of the duplicate cultures
Redn. in mean MI (%)	Reduction in mean mitotic index for duplicate cultures compared to the negative control (Negative control = 0%; Inc = Increase)
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control: Daunomycin -S-9 mix, Cyclophosphamide +S-9 mix
n	No cells on slide
f	Fewer cells on slide than on the vehicle control slides

No polyploidy or endoreduplicated metaphases were observed on these slides.

Note: No slides from this test were selected for metaphase analysis: there was too much toxicity

Main test 1 (repeat) - Mitotic indices

With S-9 mix		
Treatment (µg/ml)	Mitotic index	
	Individual values	Redn. in mean MI (%)
Vehicle	4.9 4.7	0
TA (128)	0.2f 0.3f	95
TA (64)	1.0f 0.3f	86
TA (32)	0.4f 1.2f	83
TA (16)	0.8f 0.9f	82
TA (8)	1.7f 2.3f	58
TA (4)	1.2f 2.0f	67
TA (2)	2.2f 2.1f	55
PC (6)	1.9 1.4	66

Key:

Mitotic index	Percentage of cells at metaphase
Individual values	Values for each of the duplicate cultures
Redn. in mean MI (%)	Reduction in mean mitotic index for duplicate cultures compared to the negative control (Negative control = 0%; Inc = Increase)
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control: Cyclophosphamide
f	Fewer cells on slide than on the vehicle control slides

No polyploidy or endoreduplicated metaphases were observed on these slides.

Note: No slides from this test were selected for metaphase analysis : there was too much toxicity

Main test 2 - Mitotic indices

Without S-9 mix			With S-9 mix		
Treatment (µg/ml)	Mitotic index		Treatment (µg/ml)	Mitotic index	
	Individual values	Redn. in mean MI (%)		Individual values	Redn. in mean MI (%)
Vehicle	5.2 4.7	0	Vehicle	7.2 6.2	0
TA (512)	0.0f 0.0f	100	TA (4)	0.0f 0.0f	100
TA (256)	0.1f 0.3f	96	TA (2)	0.0f 0.1f	99
TA (128)	0.2f 0.2f	96	TA (1)	0.2f 0.0f	99
TA (64)	0.1f 0.2f	97	TA (0.5)	0.1f 0.1f	99
TA (32)	0.4f 2.9f	67	TA (0.25)	1.0f 1.4f	82
TA (16)	1.8f 2.0f	62	TA (0.125)	0.8f 0.5f	90
TA (8)	1.3f 1.7f	70	TA (0.0625)	1.2f 1.0f	84
PC (0.015)	4.3 4.9	7	PC (6)	0.4f 2.9	75

Key:

Mitotic index	Percentage of cells at metaphase
Individual values	Values for each of the duplicate cultures
Redn. in mean MI (%)	Reduction in mean mitotic index for duplicate cultures compared to the negative control (Negative control = 0%; Inc = Increase)
Vehicle	Vehicle control – Cell culture medium
TA	Acyltransferase BL1 (*B. licheniformis* BML780-KLM3' CAP50) (GICC 3265)
PC	Positive control: Daunomycin -S-9 mix, Cyclophosphamide +S-9 mix
f	Fewer cells on slide than on the vehicle control slides

No polyploidy or endoreduplicated metaphases were observed on these slides.

Note: No slides from this test were selected for metaphase analysis : there was too much toxicity

Historical control data
(Previous ten studies)

Treatment		Frequency of metaphases with aberrant chromosomes excluding gaps (%)				Number of cultures
		Mean	SD	Minimum	Maximum	
Negative control	-S9	0.9	1.0	0	3	42
Daunomycin 0.015 µg/ml	-S9	30.3	14.4	4	65	40
Negative control	+S9	1.6	1.4	0	5	42
Cyclophosphamide 6 µg/ml	+S9	38.4	14.7	11	75	40

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CERTIFICATE OF ANALYSIS

Name of Test Article: **ACYLTRANSFERASE BL1**

Production/Strain Name: *Bacillus licheniformis* **BML780-KLM3' CAP50.**

Production Site: Rochester, USA

Genencor International Culture Collection Number: GICC 3265

Designation of Lot Tested: 20068010

Description: Lyophilized Powder

Analytical studies 3-5 listed below were conducted in accordance with GLP regulations and ISO 9002 standards.

RESULTS:

1. Activity: 21512 LATU/g

2. Dry Matter: 89%

3. Microbial analysis: Microbial analysis conducted on the liquid test material prior to lyophilization by GCOR, Rochester, NY

Analysis	Results
Total viable count	< 1CFU/ml
Coliform	< 1CFU/ml
E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Production strain	negative
Anaerobic sulfite reducers	negative
Antibiotic activity assay	negative

4. Mycotoxin analysis: Not applicable

5. Heavy metals analysis (conducted on the liquid test material prior to lyophilization at Silliker Laboratories)

Analysis	Results
Heavy metals as Pb	< 30 ppm
Arsenic	< 3 ppm
Lead	< 0.5 ppm
Mercury	< 0.5 ppm
Cadmium	< 5 ppm

6. Stability Data : Lyophilized powder is stable for at least 1 year when stored frozen

Bio-Analytical Representative:

Jorn Borch Soe Date: 25 August 2006

Jorn Borch Soe

Study Sponsor's Representative

Hanne Valsted Thygesen Date: 25 Aug 2006

Hanne Valsted Thygesen

Study Monitor's Representative:

Quang Q. Bui Date: Aug 28, 2006

Quang Q. Bui