

**FINAL REPORT**

Study Title

***In Vitro* Mammalian Chromosome Aberration Test**

Test Article

1-Chlorododecane

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Study Completion Date

20 May 2008

Testing Facility

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BioReliance Study Number

AC01UM.331.BTL

Sponsor

Lonza, Inc.  
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**STATEMENT OF COMPLIANCE**

Study No. AC01UM.331.BTL was conducted in compliance with the US EPA GLP Regulations (40 CFR 792) and the OECD Principles of Good Laboratory Practice [C(97)186/Final] in all material aspects with the following exception:

Analyses to determine the uniformity, concentration or stability of the test article dose formulations were not performed by the testing facility or the Sponsor.

Ramadevi Gudi  
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# Quality Assurance Statement

**Study Title:** *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

**Study Number:** AC01UM.331.BTL

**Study Director:** Ramadevi Gudi, Ph.D.

Quality Assurance performed the inspections listed below for this study. Verification of the study protocol was also performed and documented by QA. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. EPA GLP Regulations (40 CFR 792) and the OECD Principles of Good Laboratory Practice (C(97)186/Final), and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

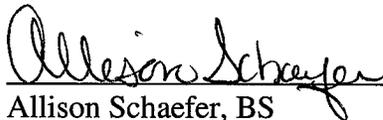
The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

**Inspect On:** 04-Sep-07 - 04-Sep-07 To Study Dir 04-Sep-07 To Mgmt 04-Sep-07  
**Phase:** Mitotic index

**Inspect On:** 27-Sep-07 - 27-Sep-07 To Study Dir 27-Sep-07 To Mgmt 01-Oct-07  
**Phase:** Draft Report and Data Audit

**Inspect On:** 16-May-08 - 16-May-08 To Study Dir 16-May-08 To Mgmt 20-May-08  
**Phase:** Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.



Allison Schaefer, BS  
QUALITY ASSURANCE

201MAY108  
DATE

***In Vitro* Mammalian Chromosome Aberration Test**

**STUDY INFORMATION**

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Study Director: **Ramadevi Gudi, Ph.D.**

BioReliance Study No.: **AC01UM.331.BTL**

Test Article I.D.: **1-Chlorododecane**

Test Article Batch Number: **N6227945 (per Certificate of Analysis)**

Test Article CAS Number: **112-52-7 (provided by Sponsor)**

Test Article Purity: **97.42% (per Certificate of Analysis)**

Test Article Molecular Weight: **204.783 (provided by Sponsor)**

Test Article Description: **Colorless liquid**

Storage Conditions: **Room temperature, protected from light**

Test Article Receipt/Login Date: **09 May 2007**

Study Initiation: **23 July 2007**

Experimental Start Date: **26 July 2007**

Experimental Completion Date: **09 September 2007**

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## SUMMARY

The test article, 1-Chlorododecane, was tested in the chromosome aberration assay using Chinese hamster ovary (CHO) cells in both the absence and presence of an Aroclor-induced S9 activation system. A preliminary toxicity test was performed to establish the dose range for the chromosome aberration assay. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article.

Ethanol was the solvent of choice based on the solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a concentration of approximately 500 mg/mL, the maximum concentration tested for solubility.

In the preliminary toxicity assay, CHO cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after treatment initiation. The maximum dose tested was 2040 µg/mL (10 mM). The test article was soluble in ethanol and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period.

Selection of dose levels for the chromosome aberration assay was based on cell growth inhibition relative to the solvent control. Substantial toxicity (i.e., at least 50% cell growth inhibition, relative to the solvent control) was observed at dose levels  $\geq 61.2$  µg/mL in all three treatment groups. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 1.56 to 50 µg/mL for the non-activated 4 and 20-hour exposure groups, and ranged from 6.25 to 100 µg/mL for the S9-activated 4-hour exposure group.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after treatment initiation. The test article was soluble in ethanol and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period.

Selection of doses for microscopic analysis (6.25, 12.5 and 35 µg/mL) was based on toxicity (the lowest dose with at least 50% reduction in cell growth and two lower doses) in the S9-activated 4-hour exposure group. Due to lack of a dose level with close to 50% reduction in cell growth, selection of doses for microscopic analysis (6.25, 12.5 and 35 µg/mL) was based on mitotic index (the lowest dose with at least 50% reduction in mitotic index and two lower doses) in the non-activated 4 and 20-hour exposure groups.

The percentage of cells with structural or numerical aberrations in the non-activated 4 and 20-hour exposure groups was not significantly increased above that of the solvent control at any dose level ( $p > 0.05$ , Fisher's Exact test).

The percentage of cells with structural aberrations in the S9-activated 4-hour exposure group (12.0%) was statistically increased above that of the solvent control at 35 µg/mL ( $p \leq 0.01$ , Fisher's Exact test). The Cochran-Armitage test was also positive for a dose response ( $p \leq 0.05$ ). The percentage of cells with numerical aberrations in the test article-treated group was not

significantly increased above that of the solvent control at any dose level ( $p>0.05$ , Fisher's Exact test).

The results of the assay are summarized in the following table:

Treatment Time	Recovery Time	Harvest Time	S9	Toxicity* at highest dose scored (35 µg/mL)	Mitotic Index Reduction **	LED <sup>1</sup> for Structural Aberrations µg/mL	LED <sup>1</sup> for Numerical Aberrations µg/mL
4 hr	16 hr	20 hr	-	30%	56%	None	None
20 hr	0 hr	20 hr	-	17%	55%	None	None
4 hr	16 hr	20 hr	+	57%	53%	35 (12.0%)	None

\* cell growth inhibition

\*\* relative to solvent control at high dose evaluated for chromosome aberrations

<sup>1</sup> LED = lowest effective dose

Based on the findings of this study, 1-Chlorododecane was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO cells in the non-activated test system. 1-Chlorododecane was concluded to be positive for the induction of structural chromosome aberrations and negative for the induction of numerical chromosome aberrations in CHO cells in the S9-activated test system.

## PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in CHO cells. A copy of the study protocol and amendment is included in Appendix I.

The study was conducted in compliance with the testing guidelines of the ICH (1996 and 1997), the OECD (1998) and the EPA (TSCA) 40 CFR 799.9537.

## CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, 1-Chlorododecane (CAS No. 112-52-7, Batch No. N6227945, Purity 97.42%), was received by BioReliance on 09 May 2007 and was assigned the code number AC01UM. The test article was characterized by Eurofins Product Safety Laboratories (Dayton, NJ) as a colorless to pale yellow liquid and the Sponsor requested that the test article should be stored at room temperature. An expiration date of 29 June 2009 was provided by the Sponsor. Upon receipt, the test article was described as a colorless liquid and was stored at room temperature, protected from light.

Eurofins Product Safety Laboratories has determined the identity, strength, purity, composition or other characteristics to define the test article. The GLP-compliant report (Characterization of Active Ingredient in a Sample of 1-Chlorododecane) is included in the study work book. A copy of the Certificate of Analysis for the test article is included in Appendix II. Based on the results of a second characterization analysis of 1-Chlorododecane (Batch No. N6227945) conducted by Eurofins Product Safety Laboratories, the test article was considered to be stable over a period of June 29, 2007 through April 24, 2008. A copy of the Stability Memo is included in Appendix II.

The solvent used to deliver 1-Chlorododecane to the test system was ethanol (CAS No. 64-17-5, Lot No. B0512128) obtained from Acros Organics. Test article dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light.

Mitomycin C (MMC; CAS No. 50-07-7, Lot No. 075K1923), was obtained from Sigma Chemical Company and was dissolved and diluted in sterile distilled water (Invitrogen Lot No. 1398445) to stock concentrations of 10 and 20 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No. 6055-19-2, Lot No. 036K1225), was obtained from Sigma Chemical Company and was dissolved and diluted in sterile distilled water to stock concentrations of 1 and 2 mg/mL for use as the positive control in the S9-activated test system. For each positive control, one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that used in the test article dilutions.

The solvent and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the solvent and positive control articles and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

## MATERIALS AND METHODS

### Test System

Chinese hamster ovary (CHO-K<sub>1</sub>) cells (repository number CCL 61) were obtained from American Type Culture Collection, Manassas, VA. In order to assure the karyotypic stability of the cell line, working cell stocks were not used beyond passage 20. The frozen lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. This cell line has an average cell cycle time of 10-14 hours with a modal chromosome number of 20. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

### Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 (Lot No. 2074) was obtained from Molecular Toxicology, Inc. (Boone, NC). Each bulk preparation of S9 was assayed for sterility and its ability to metabolize at least two pro-mutagens to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20  $\mu$ L S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin/mL, 100  $\mu$ g streptomycin/mL, 2 mM L-glutamine and 2.5  $\mu$ g/mL Amphotericin B).

### Solubility Test

A solubility test was conducted to determine the maximum soluble concentration or workable suspension using water, DMSO and ethanol. The vehicle of choice was the solvent, selected in order of preference that permitted preparation of the highest soluble stock concentration, up to 50 mg/mL in water and up to 500 mg/mL in DMSO and ethanol.

### Preliminary Toxicity Assay

The preliminary toxicity assay was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of an evaluation of test article effect on cell growth. CHO cells were seeded for each treatment condition at approximately  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask and were incubated at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO<sub>2</sub> in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 mL complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin/mL, 100  $\mu$ g streptomycin/mL, 2 mM L-glutamine and 2.5  $\mu$ g/mL Amphotericin B) for the non-activated study or 5 mL S9 reaction mixture (4 mL serum-free medium plus 1 mL of S9/cofactor pool) for the activated study, to which was added 50  $\mu$ L dosing solution of test article in solvent or solvent alone. The osmolality of the solvent and of the highest concentration of dosing solution in the treatment medium was measured. The pH of the highest concentration of dosing solution in the treatment medium was measured using test tape.

The cells were exposed to solvent alone and to nine concentrations of the test article for 4 hours in both the presence and absence of S9 activation, and for 20 hours continuously in the absence of S9 activation. The cells were incubated at  $37\pm 1^{\circ}\text{C}$  in a humidified atmosphere of  $5\pm 1\%$   $\text{CO}_2$  in air. At completion of the 4-hour exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 mL complete medium and returned to the incubator for a total of 20 hours from the initiation of treatment. At 20 hours after the initiation of treatment the cells were harvested by trypsinization and counted using a Coulter counter. The presence of test article precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

### **Chromosome Aberration Assay**

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive and solvent controls. For the chromosome aberration assay, CHO cells were seeded at approximately  $5 \times 10^5$  cells/25  $\text{cm}^2$  flask and were incubated at  $37\pm 1^{\circ}\text{C}$  in a humidified atmosphere of  $5\pm 1\%$   $\text{CO}_2$  in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 mL complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin/mL and 100  $\mu\text{g}$  streptomycin/mL, 2 mM L-glutamine, and 2.5  $\mu\text{g}/\text{mL}$  Amphotericin B) for the non-activated study or 5 mL S9 reaction mixture for the S9-activated study, to which was added 50  $\mu\text{L}$  of dosing solution of test or positive control article in solvent or solvent alone. The pH of the highest concentration of dosing solution in the treatment medium was measured using test tape.

In the non-activated study, the cells were exposed to the test article for 4 hours or continuously for 20 hours up to the cell harvest at  $37\pm 1^{\circ}\text{C}$  in a humidified atmosphere of  $5\pm 1\%$   $\text{CO}_2$  in air (Swierenga et al., 1991). In the 4-hour exposure group, after the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid<sup>®</sup> was added to duplicate flasks for each treatment condition at a final concentration of 0.1  $\mu\text{g}/\text{mL}$  and the flasks returned to the incubator until cell collection.

In the S9-activated study, the cells were exposed for 4 hours at  $37\pm 1^{\circ}\text{C}$  in a humidified atmosphere of  $5\pm 1\%$   $\text{CO}_2$  in air (Swierenga et al., 1991). After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid<sup>®</sup> was added to duplicate flasks for each treatment condition at a final concentration of 0.1  $\mu\text{g}/\text{mL}$  and the flasks were returned to the incubator until cell collection.

A concurrent toxicity test was conducted in both the non-activated and the S9-activated test systems. After cell harvest an aliquot of the cell suspension was removed from each culture, transferred to a labeled counting vial containing isotonic saline, and counted using a Coulter counter. The presence of test article precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

## **Collection of Metaphase Cells**

Two hours after the addition of Colcemid<sup>®</sup>, metaphase cells were harvested for both the non-activated and S9-activated studies by trypsinization. Cells were collected approximately 20 hours after initiation of treatment (Galloway et al., 1994). The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 mL 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 mL Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored at least overnight in fixative at approximately 2-8°C.

## **Slide Preparation**

To prepare slides, the fixed cells in 15 mL centrifuge tubes were centrifuged at approximately 800 rpm for 5 minutes. The supernatant was aspirated leaving approximately 0.2 mL above the cell pellet and 1 mL cold fresh Carnoy's fixative was added to each tube. The cells were collected by centrifugation and the supernatant was aspirated, leaving 0.1 to 0.3 mL fixative above the cell pellet (volume dependent upon size of the cell pellet). An aliquot of the cell suspension was dropped from an appropriate distance by means of a Pasteur pipet on clean microscope slides and allowed to air dry at room temperature. If necessary, the following techniques may have been used to achieve optimum spreading of metaphases: dip slide in cold water or ice, use of slide warmer or additional centrifugation steps, as needed. Each slide was identified by the study number, dose level, treatment condition, replicate tube designation and harvest date. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

## **Selection of Dose Levels for Analysis**

The selection of dose levels for analysis of chromosome aberrations in CHO cells was based upon toxicity of the test article. The highest dose level selected for evaluation was the dose which induced at least 50% toxicity, as measured by cell growth inhibition, relative to the solvent control, with a sufficient number of scorable metaphase cells. In treatment groups with lack of a dose level with close to 50% reduction in cell growth, selection of doses for microscopic analysis was based on mitotic index (the lowest dose with at least 50% reduction in mitotic index). Two additional lower dose levels were included in the evaluation.

## **Evaluation of Metaphase Cells**

To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with  $20 \pm 2$  centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that were examined and scored per duplicate flask was reduced when the percentage of aberrant cells reached a significant level (at least 10%) before 100 cells were scored. Chromatid-type

aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ( $\geq 10$  aberrations) were also recorded. Chromatid gaps (an aligned achromatic region in one chromatid, the size of which is equal to or smaller than the width of the chromatid) and isochromatid gaps (an aligned, achromatic region in both chromatids, the size of which is equal to or smaller than the width of the chromatids) were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using the microscope stage. Polyploid and endoreduplicated cells were evaluated from each treatment flask per 100 metaphase cells scored.

### **Controls**

MMC was used as the positive control in the non-activated study at final concentrations of 0.1 and 0.2  $\mu\text{g/mL}$ . CP was used as the positive control in the S9-activated study at final concentrations of 10 and 20  $\mu\text{g/mL}$ . For both positive controls, one dose level exhibiting a sufficient number of scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups.

### **Evaluation of Test Results**

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell were calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's Exact test. Fisher's Exact test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's Exact test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ( $p \leq 0.05$ ). However, values that are statistically significant but do not exceed the range of historical solvent controls may be judged as not biologically significant. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

## **Automated Data Collection Systems**

The primary computer or electronic systems used for the collection or analysis of data included, but not limited to, the following:

Oracle (Oracle Corporation), Excel 2003 (Microsoft Corporation), Kaye Lab Watch Monitoring System (Kaye GE) and LIMS Labware version 5 (configured version 1.0.3).

## **Criteria for a Valid Test**

The frequency of cells with structural chromosome aberrations in the solvent control must be within the range of the historical solvent control. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ( $p \leq 0.05$ , Fisher's Exact test) relative to the solvent control. The Historical Control Data is included in Appendix III.

## **Deviations**

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

## **Archives**

All raw data, the protocol, and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years. Raw data, the protocol and reports generated at other facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor. Following finalization of the report, residual test article will be disposed at BioReliance. Unless otherwise indicated, the slides will be discarded upon finalization of the report.

## RESULTS AND DISCUSSION

### Solubility Test

Ethanol was the solvent of choice based on the solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a concentration of approximately 500 mg/mL, the maximum concentration tested for solubility.

### Preliminary Toxicity Assay

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity test and were based upon a reduction of cell growth (cell growth inhibition) relative to the solvent control. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.204 to 2040 µg/mL in the absence (4 and 20-hour incubation) and presence (4-hour incubation) of an S9 reaction mixture. The test article was soluble in ethanol and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The osmolality in treatment medium of the highest concentration tested, 2040 µg/mL, was 275 mmol/kg. The osmolality of the solvent (ethanol) in treatment medium was 288 mmol/kg. The osmolality of the test article concentration in treatment medium is acceptable because it did not exceed the osmolality of the solvent by more than 20%. The pH of the highest concentration of test article in treatment medium was approximately 7.0.

The results of the evaluation of cell growth inhibition are presented in Tables 1-3. Substantial toxicity (i.e., at least 50% cell growth inhibition, relative to the solvent control) was observed at dose levels  $\geq 61.2$  µg/mL in all three treatment groups. Based upon the results of the toxicity study, the dose levels selected for testing in the chromosome aberration assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose levels (µg/mL)
Non-activated	4 hr	16 hr	1.56, 3.13, 6.25, 12.5, 25, 35, 50
	20 hr	0 hr	1.56, 3.13, 6.25, 12.5, 25, 35, 50
S9-activated	4 hr	16 hr	6.25, 12.5, 25, 35, 50, 75, 100

### Chromosome Aberration Assay

In the chromosome aberration assay, the test article was soluble in ethanol and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The pH of the highest concentration of test article in treatment medium was approximately 7.0.

Toxicity of 1-Chlorododecane (cell growth inhibition relative to the solvent control) in CHO cells when treated for 4 hours in the absence of S9 activation was 30% at 35 µg/mL, the highest test concentration evaluated for chromosome aberrations (Table 4). The activity of 1-Chlorododecane in the induction of chromosome aberrations is presented by treatment flask in Table 5 and summarized by group in Table 10. The mitotic index at the highest dose level

evaluated for chromosome aberrations, 35 µg/mL, was 56% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 6.25, 12.5 and 35 µg/mL. The percentage of cells with structural or numerical aberrations in the test article-treated group was not significantly increased above that of the solvent control at any dose level ( $p > 0.05$ , Fisher's Exact test). The increase in the average percentage of structurally damaged cells in the MMC (positive control) treatment group (18%) was statistically significant ( $p \leq 0.01$ , Fisher's Exact test).

Toxicity of 1-Chlorododecane (cell growth inhibition relative to the solvent control) in CHO cells when treated for 4 hours in the presence of S9 activation was 57% at 35 µg/mL, the highest test concentration evaluated for chromosome aberrations (Table 6). The activity of 1-Chlorododecane in the induction of chromosome aberrations is presented by treatment flask in Table 7 and summarized by group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 35 µg/mL, was 53% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 6.25, 12.5 and 35 µg/mL. The percentage of cells with structural aberrations in the S9-activated 4-hour exposure group was statistically increased (12.0%) above that of the solvent control at 35 µg/mL ( $p \leq 0.01$ , Fisher's Exact test). The Cochran-Armitage test was also positive for a dose response ( $p \leq 0.05$ ). The percentage of cells with numerical aberrations in the test article-treated group was not significantly increased above that of the solvent control at any dose level ( $p > 0.05$ , Fisher's Exact test). The increase in the average percentage of structurally damaged cells in the CP (positive control) treatment group (16.0%) was statistically significant ( $p \leq 0.01$ , Fisher's Exact test).

Toxicity of 1-Chlorododecane (cell growth inhibition relative to the solvent control) in CHO cells when treated for 20 hours in the absence of S9 activation was 17% at 35 µg/mL, the highest test concentration evaluated for chromosome aberrations in the non-activated 20-hour continuous exposure group (Table 8). The activity of 1-Chlorododecane in the induction of chromosome aberrations is presented by treatment flask in Table 9 and summarized by group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 35 µg/mL, was 55% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 6.25, 12.5 and 35 µg/mL. The percentage of cells with structural or numerical aberrations in the test article-treated group was not significantly increased above that of the solvent control at any dose level ( $p > 0.05$ , Fisher's Exact test). The increase in the average percentage of structurally damaged cells in the MMC (positive control) treatment group (18.0%) was statistically significant ( $p \leq 0.01$ , Fisher's Exact test).

## CONCLUSION

The positive and solvent controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, 1-Chlorododecane was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO cells in the non-activated test system. 1-Chlorododecane was concluded to be positive for the induction of structural chromosome aberrations and negative for the induction of numerical chromosome aberrations in CHO cells in the S9-activated test system.

## REFERENCES

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## DATA TABLES

TABLE 1  
PRELIMINARY TOXICITY TEST USING 1-Chlorododecane  
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4-HOUR TREATMENT, 16-HOUR RECOVERY PERIOD

Treatment µg/mL	Cell Count (x10 <sup>6</sup> )	Cell Viability (%)	Viable Cells/ Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)
Ethanol	1.87	100%	1.87	100%	NA
1-Chlorododecane 0.204	1.77	98%	1.74	93%	7%
0.612	1.50	99%	1.49	80%	20%
2.04	1.51	100%	1.51	81%	19%
6.12	1.75	99%	1.73	93%	7%
20.4	1.33	97%	1.29	69%	31%
61.2	0.06	*	0.00	0%	100%
204	0.02	*	0.00	0%	100%
612	0.04	*	0.00	0%	100%
2040	0.33	*	0.00	0%	100%

NA – Not applicable

**Treatment:** CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Viable Cells/Flask** = cell count x % viable cells

**Cell Growth Index** = (cells per flask treated group/cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

\* No live cells present.

TABLE 2  
PRELIMINARY TOXICITY TEST USING 1-Chlorododecane  
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4-HOUR TREATMENT, 16-HOUR RECOVERY PERIOD

Treatment µg/mL	Cell Count (x10 <sup>6</sup> )	Cell Viability (%)	Viable Cells/ Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)
Ethanol	1.31	100%	1.31	100%	NA
1-Chlorododecane					
0.204	1.41	99%	1.39	106%	-6%
0.612	1.12	98%	1.10	84%	16%
2.04	1.32	97%	1.28	98%	2%
6.12	1.21	97%	1.17	90%	10%
20.4	0.92	98%	0.90	69%	31%
61.2	0.54	95%	0.52	39%	61%
204	0.81	99%	0.81	61%	39%
612	0.45	*	0.00	0%	100%
2040	0.47	*	0.00	0%	100%

NA – Not applicable

**Treatment:** CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Viable Cells/Flask** = cell count x % viable cells

**Cell Growth Index** = (cells per flask treated group/cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

\* No live cells present.

TABLE 3  
PRELIMINARY TOXICITY TEST USING 1-Chlorododecane  
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20-HOUR CONTINUOUS TREATMENT

Treatment μg/mL	Cell Count (x10 <sup>6</sup> )	Cell Viability (%)	Viable Cells/ Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)
Ethanol	1.31	100%	1.31	100%	NA
1-Chlorododecane 0.204	1.51	98%	1.48	113%	-13%
0.612	0.90	98%	0.88	68%	32%
2.04	1.23	97%	1.20	91%	9%
6.12	1.24	97%	1.21	92%	8%
20.4	1.56	100%	1.56	119%	-19%
61.2	0.80	*	0.00	0%	100%
204	0.57	*	0.00	0%	100%
612	0.73	*	0.00	0%	100%
2040	0.55	*	0.00	0%	100%

NA – Not applicable

**Treatment:** CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Viable Cells/Flask** = cell count x % viable cells

**Cell Growth Index** = (cells per flask treated group/cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

\* No live cells present.

TABLE 4  
 CHROMOSOME ABERRATION ASSAY: CONCURRENT TOXICITY TEST USING 1-Chlorododecane  
 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4-HOUR TREATMENT, 16-HOUR RECOVERY PERIOD

Treatment µg/mL	Flask	Cell Count Averages (x10 <sup>6</sup> )	Cell Viability (%)	Mean Cells per Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)	Mitotic Index (%)	Mitotic Inhibition (%)																																																																																																						
Ethanol	A	1.01	98%	1.34	100%	NA	7.9%	NA																																																																																																						
	B	1.73	98%						1-Chlorododecane 1.56	A	1.19	98%	1.33	99%	1%	8.0%	-1%	B	1.54	97%	3.13	A	1.21	98%	1.44	107%	-7%	6.9%	13%	B	1.72	99%	6.25	A	1.27	99%	1.42	105%	-5%	7.2%	9%	B	1.59	99%	12.5	A	1.39	98%	1.31	97%	3%	6.4%	19%	B	1.32	95%	25	A	1.54	98%	1.36	101%	-1%	6.0%	24%	B	1.24	97%	35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%
1-Chlorododecane 1.56	A	1.19	98%	1.33	99%	1%	8.0%	-1%																																																																																																						
	B	1.54	97%						3.13	A	1.21	98%	1.44	107%	-7%	6.9%	13%	B	1.72	99%	6.25	A	1.27	99%	1.42	105%	-5%	7.2%	9%	B	1.59	99%	12.5	A	1.39	98%	1.31	97%	3%	6.4%	19%	B	1.32	95%	25	A	1.54	98%	1.36	101%	-1%	6.0%	24%	B	1.24	97%	35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%						
3.13	A	1.21	98%	1.44	107%	-7%	6.9%	13%																																																																																																						
	B	1.72	99%						6.25	A	1.27	99%	1.42	105%	-5%	7.2%	9%	B	1.59	99%	12.5	A	1.39	98%	1.31	97%	3%	6.4%	19%	B	1.32	95%	25	A	1.54	98%	1.36	101%	-1%	6.0%	24%	B	1.24	97%	35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																		
6.25	A	1.27	99%	1.42	105%	-5%	7.2%	9%																																																																																																						
	B	1.59	99%						12.5	A	1.39	98%	1.31	97%	3%	6.4%	19%	B	1.32	95%	25	A	1.54	98%	1.36	101%	-1%	6.0%	24%	B	1.24	97%	35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																														
12.5	A	1.39	98%	1.31	97%	3%	6.4%	19%																																																																																																						
	B	1.32	95%						25	A	1.54	98%	1.36	101%	-1%	6.0%	24%	B	1.24	97%	35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																																										
25	A	1.54	98%	1.36	101%	-1%	6.0%	24%																																																																																																						
	B	1.24	97%						35	A	0.68	97%	0.95	70%	30%	3.5%	56%	B	1.27	97%	50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																																																						
35	A	0.68	97%	0.95	70%	30%	3.5%	56%																																																																																																						
	B	1.27	97%						50	A	0.06	*	0.00	0%	100%	0.0%	100%	B	0.04	*	MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																																																																		
50	A	0.06	*	0.00	0%	100%	0.0%	100%																																																																																																						
	B	0.04	*						MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A	B	0.93	98%	MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																																																																														
MMC 0.1	A	1.01	97%	0.95	70%	30%	N/A	N/A																																																																																																						
	B	0.93	98%						MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%	B	0.70	98%																																																																																										
MMC 0.2	A	0.72	99%	0.70	52%	48%	6.6%	16%																																																																																																						
	B	0.70	98%																																																																																																											

NA – Not applicable

**Treatment:** CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Mean Viable Cells/Flask** = cell count x % viable cells, reported as mean of Flasks A and B.

**Cell Growth Index** = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

\* No live cells present.

**Mitotic Index** = (Cells in mitosis/500 cells scored) x 100, reported as mean of Flasks A and B.

**Mitotic Inhibition** = (Mean of treatment mitotic index – mean of control mitotic index)/mean of control mitotic index, expressed as a percentage.

N/A: Mitotic index not measured at this dose level.

TABLE 5  
**CHROMOSOME ABERRATION ASSAY: CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH 1-Chlorododecane  
 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION**

Treatment ( $\mu\text{g/mL}$ )	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells		Total Number of Structural Aberrations				Severely Damaged Cells	Average Aberrations Per Cell	
			Numerical	Structural	Numerical	Structural	Chromatid		Chromosome				
							Br	Ex	Br	Dic Ring			
Ethanol	A	8.2	100	100	1	0	0	0	0	0	0	0	0.000
	B	7.6	100	100	1	0	0	0	0	0	0	0	0.000
1-Chlorododecane 6.25	A	7.0	100	100	0	0	0	0	0	0	0	0	0.000
	B	7.4	100	100	2	0	0	0	0	0	0	0	0.000
12.5	A	6.8	100	100	1	0	0	0	0	0	0	0	0.000
	B	6.0	100	100	2	0	0	0	0	0	0	0	0.000
35	A	3.8	100	100	2	1	0	2	0	0	0	0	0.020
	B	3.2	100	100	2	0	0	0	0	0	0	0	0.000
MMC, 0.2	A	6.0	100	50	1	16	0	4	5	0	0	1	0.200
	B	7.2	100	50	3	20	0	3	7	0	0	0	0.200

**Treatment:** CHO cells were treated for 4 hours at  $37\pm 1^\circ\text{C}$  in the absence of an exogenous source of metabolic activation. Additional dose levels of 1.56, 3.13 and 25  $\mu\text{g/mL}$  were included in the assay as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose level 50  $\mu\text{g/mL}$  was not analyzed due to excessive toxicity.

**Mitotic index** = number mitotic figures x 100/500 cells counted.

**%Aberrant Cells:** numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

**Chromatid breaks (Br)** include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

**Chromosome breaks (Br)** include breaks and acentric fragments; Dic = dicentric chromosome.

**Severely damaged cells** includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

**Average aberrations per cell:** severely damaged cells and pulverizations were counted as 10 aberrations.

**TABLE 6**  
**CHROMOSOME ABERRATION ASSAY: CONCURRENT TOXICITY TEST USING 1-Chlorododecane**  
**IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION**

**4-HOUR TREATMENT, 16-HOUR RECOVERY PERIOD**

Treatment µg/mL	Flask	Cell Count Averages (x10 <sup>6</sup> )	Cell Viability (%)	Mean Cells per Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Ethanol	A	1.10	99%	1.02	100%	NA	8.5%	NA
	B	0.97	98%					
1-Chlorododecane 6.25	A	0.67	99%	0.79	78%	22%	7.2%	15%
	B	0.92	100%					
12.5	A	0.81	98%	0.60	59%	41%	6.1%	28%
	B	0.43	98%					
25	A	0.43	97%	0.52	51%	49%	5.9%	31%
	B	0.63	99%					
35	A	0.52	98%	0.44	43%	57%	4.0%	53%
	B	0.37	99%					
50	A	0.48	99%	0.40	40%	60%	2.2%	74%
	B	0.35	96%					
75	A	0.32	94%	0.35	35%	65%	1.7%	80%
	B	0.45	92%					
100	A	0.46	86%	0.43	42%	58%	1.9%	78%
	B	0.58	80%					
CP 10	A	0.81	99%	0.59	58%	42%	4.5%	47%
	B	0.37	98%					
CP 20	A	0.96	96%	0.82	80%	20%	N/A	N/A
	B	0.74	97%					

NA – Not applicable

**Treatment:** CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Mean Viable Cells/Flask** = cell count x % viable cells, reported as mean of Flasks A and B.

**Cell Growth Index** = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

**Mitotic Index** = (Cells in mitosis/500 cells scored) x 100, reported as mean of Flasks A and B.

**Mitotic Inhibition** = (Mean of treatment mitotic index – mean of control mitotic index)/mean of control mitotic index, expressed as a percentage.

N/A: Mitotic index not measured at this dose level.

TABLE 7  
**CHROMOSOME ABERRATION ASSAY: CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH 1-Chlorododecane  
 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION**

Treatment ( $\mu\text{g/mL}$ )	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell	
			Numerical	Structural	Numerical	Structural	Caps	Chromatid		Chromosome				
								Br	Ex	Br	Dic			Ring
Ethanol	A	9.0	100	100	1	0	0	0	0	0	0	0	0	0.000
	B	8.0	100	100	2	1	0	0	1	0	0	0	0	0.010
1-Chlorododecane 6.25	A	7.0	100	100	1	0	0	0	0	0	0	0	0	0.000
	B	7.4	100	100	1	0	0	0	0	0	0	0	0	0.000
12.5	A	6.0	100	100	6	1	0	0	0	0	1	0	0	0.010
	B	6.2	100	100	3	2	0	1	1	0	0	0	0	0.020
35	A	4.2	100	100	1	11	0	8	1	0	2	0	0	0.110
	B	3.8	100	100	2	13	0	11	3	0	0	1	0	0.150
CP, 10	A	4.0	100	50	1	18	0	4	9	0	0	0	0	0.260
	B	5.0	100	50	1	14	0	4	5	0	0	0	0	0.180

**Treatment:** CHO cells were treated for 4 hours at  $37\pm 1^\circ\text{C}$  in the presence of an exogenous source of metabolic activation. An additional dose level of  $25 \mu\text{g/mL}$  was included in the assay as a safeguard against excessive toxicity at higher dose levels but was not required for microscopic examination. Dose levels 50, 75 and  $100 \mu\text{g/mL}$  were not analyzed due to excessive toxicity.

**Mitotic index** = number mitotic figures x 100/500 cells counted.

**%Aberrant Cells:** numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

**Chromatid breaks (Br)** include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

**Chromosome breaks (Br)** include breaks and acentric fragments; Dic = dicentric chromosome.

**Severely damaged cells** includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

**Average aberrations per cell:** severely damaged cells and pulverizations were counted as 10 aberrations.

**TABLE 8**  
**CHROMOSOME ABERRATION ASSAY: CONCURRENT TOXICITY TEST USING 1-Chlorododecane**  
**IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION**

**20-HOUR CONTINUOUS TREATMENT**

Treatment µg/mL	Flask	Cell Count Averages (x10 <sup>6</sup> )	Cell Viability (%)	Mean Cells per Flask (x10 <sup>6</sup> ) §	Cell Growth Index (%)	Cell Growth Inhibition (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Ethanol	A	1.01	98%					
	B	1.09	99%	1.04	100%	NA	9.1%	NA
<b>1-Chlorododecane</b>								
1.56	A	1.15	99%					
	B	1.08	98%	1.10	106%	-6%	8.4%	8%
3.13	A	1.12	97%					
	B	0.98	98%	1.02	99%	1%	8.6%	5%
6.25	A	1.46	97%					
	B	1.10	98%	1.25	120%	-20%	6.7%	26%
12.5	A	1.22	98%					
	B	1.16	98%	1.17	113%	-13%	6.6%	27%
25	A	1.41	99%					
	B	0.96	97%	1.16	112%	-12%	5.1%	44%
35	A	0.87	98%					
	B	0.86	99%	0.85	83%	17%	4.1%	55%
50	A	0.68	*					
	B	0.69	*	0.00	0%	100%	0.5%	95%
MMC 0.1	A	1.09	95%					
	B	1.01	96%	1.00	97%	3%	5.5%	40%
MMC 0.2	A	0.98	97%					
	B	1.04	98%	0.99	95%	5%	N/A	N/A

NA – Not applicable

**Treatment:** CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.

**Cell Viability:** determined by trypan blue dye exclusion.

**Mean Viable Cells/Flask** = cell count x % viable cells, reported as mean of Flasks A and B.

**Cell Growth Index** = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.

**Cell Growth Inhibition** = 100% - % cell growth index; not calculated for negative controls.

§ The reported values were rounded off to two significant numbers.

\* No live cells present.

**Mitotic Index** = (Cells in mitosis/500 cells scored) x 100, reported as mean of Flasks A and B.

**Mitotic Inhibition** = (Mean of treatment mitotic index – mean of control mitotic index)/mean of control mitotic index, expressed as a percentage.

N/A: Mitotic index not measured at this dose level.

TABLE 9  
**CHROMOSOME ABERRATION ASSAY: CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH 1-Chlorododecane  
 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION**

Treatment (µg/mL)	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells	Total Number of Structural Aberrations						Severely Damaged Cells	Average Aberrations Per Cell	
			Numerical	Structural		Gaps	Chromatid		Chromosome		Ring			
							Br	Ex	Br	Dic				
Ethanol	A	9.4	100	100	2	0	0	0	0	0	0	0	0	0.000
	B	8.8	100	100	0	0	0	0	0	0	0	0	0	0.000
1-Chlorododecane 6.25	A	7.0	100	100	0	0	0	0	0	0	0	0	0	0.000
	B	6.4	100	100	1	1	0	0	0	0	1	0	0	0.010
12.5	A	7.2	100	100	2	0	0	0	0	0	0	0	0	0.000
	B	6.0	100	100	0	0	0	0	0	0	0	0	0	0.000
35	A	4.0	100	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.2	100	100	1	1	0	0	0	0	1	0	0	0.010
MMC, 0.1	A	5.0	100	50	0	20	0	4	7	1	0	0	0	0.240
	B	6.0	100	50	1	16	0	4	3	1	0	0	0	0.160

**Treatment:** CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation. Additional dose levels of 1.56, 3.13 and 25 µg/mL were included in the assay as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose level 50 µg/mL was not analyzed due to excessive toxicity.

**Mitotic index** = number mitotic figures x 100/500 cells counted.

**%Aberrant Cells:** numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

**Chromatid breaks (Br)** include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

**Chromosome breaks (Br)** include breaks and acentric fragments; Dic = dicentric chromosome.

**Severely damaged cells** includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

**Average aberrations per cell:** severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 10  
SUMMARY

Treatment µg/mL	S9 Activation	Treatment Time	Mean Mitotic Index	Cells Scored		Aberrations Per Cell (Mean +/- SD)		Cells With Aberrations	
				Numerical	Structural			Numerical (%)	Structural (%)
Ethanol	-S9	4	7.9	200	200	0.000	±0.000	1.0	0.0
1-Chlorododecane									
6.25	-S9	4	7.2	200	200	0.000	±0.000	1.0	0.0
12.5	-S9	4	6.4	200	200	0.000	±0.000	1.5	0.0
35	-S9	4	3.5	200	200	0.010	±0.141	2.0	0.5
MMC, 0.2	-S9	4	6.6	200	100	0.200	±0.449	2.0	18.0**
Ethanol	+S9	4	8.5	200	200	0.005	±0.071	1.5	0.5
1-Chlorododecane									
6.25	+S9	4	7.2	200	200	0.000	±0.000	1.0	0.0
12.5	+S9	4	6.1	200	200	0.015	±0.122	4.5	1.5
35	+S9	4	4.0	200	200	0.130	±0.379	1.5	12.0**
CP, 10	+S9	4	4.5	200	100	0.220	±0.561	1.0	16.0**
Ethanol	-S9	20	9.1	200	200	0.000	±0.000	1.0	0.0
1-Chlorododecane									
6.25	-S9	20	6.7	200	200	0.005	±0.071	0.5	0.5
12.5	-S9	20	6.6	200	200	0.000	±0.000	1.0	0.0
35	-S9	20	4.1	200	200	0.005	±0.071	0.5	0.5
MMC, 0.1	-S9	20	5.5	200	100	0.200	±0.449	0.5	18.0**

**Treatment:** Cells from all treatment conditions were harvested 20 hours after the initiation of the treatments.

**Aberrations per Cell:** Severely damaged cells were counted as 10 aberrations.

**Percent Aberrant Cells:** \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; using Fisher's Exact test.

**APPENDIX I**

**Study Protocol and Amendments**

QA Reviewed

Act 30 July 2007  
Init. Date

**PROTOCOL AMENDMENT 1**

**SPONSOR:** Lonza, Inc.

**TEST ARTICLE I.D.:** 1-Chlorododecane

**BIORELIANCE STUDY NO:** AC01UM.331.BTL

**PROTOCOL TITLE:** *In Vitro* Mammalian Chromosome Aberration Test

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1. **LOCATION:** Page 10, Section 12.0 Regulatory Requirements\Good Laboratory Practice, second paragraph

**AMENDMENT:** Delete reference to "United States Food and Drug Administration (FDA)" and add "United States Environmental Protection Agency (US EPA) GLP Standards 40CFR 792".

**REASON FOR THE AMENDMENT:** To specify appropriate GLP guidelines to be followed.

**APPROVALS:**

Ramadevi Gudi  
BIORELIANCE STUDY DIRECTOR

30 Jul 2007  
DATE

Valentine D. Wagner, III  
BIORELIANCE STUDY MANAGEMENT

30 Jul 2007  
DATE

J.P.V.M.  
SPONSOR REPRESENTATIVE

2 Aug 2007  
DATE

PROTOCOL AMENDMENT 2

QA Reviewed  
CRH 07MAY08  
LAD 07MAY08  
Init. Date

SPONSOR: Lonza, Inc.

TEST ARTICLE I.D.: 1-Chlorododecane

BIORELIANCE STUDY NO: AC01UM.331.BTL

PROTOCOL TITLE: *In Vitro* Mammalian Chromosome Aberration Test

- 
1. LOCATION: Page 10, Section 12.0 Regulatory Requirements\Good Laboratory Practice

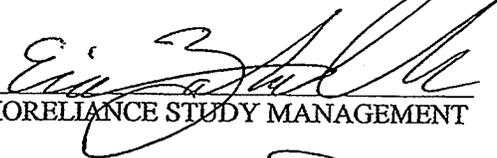
AMENDMENT: Add "EPA (TSCA) testing guideline 40 CFR 799.9537".

REASON FOR THE AMENDMENT: To specify appropriate testing guidelines.

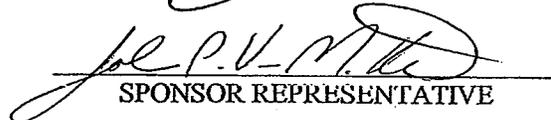
APPROVALS:

  
\_\_\_\_\_  
BIORELIANCE STUDY DIRECTOR

06 May 2008  
DATE

  
\_\_\_\_\_  
BIORELIANCE STUDY MANAGEMENT

5/6/08  
DATE

  
\_\_\_\_\_  
SPONSOR REPRESENTATIVE

5/15/08  
DATE

QA Reviewed

DB 25 July 2007

LCB 25 July 2007

Init. Date

Received by RA/OA 23-JUL-2007

BioReliance Study Number: AC01UM.331.BTL

***In Vitro* Mammalian Chromosome Aberration Test**

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Sponsor Name: Lonza, Inc  
2.2 Address: 90 Boroline Road  
Allendale, NJ 07401  
2.3 Representative: John P. Van Miller, Ph.D., DABT  
Toxicology Regulatory Services, Inc.  
Phone: 434-977-5957  
Fax: 434-977-0899  
Email: jvanmiller@toxregserv.com

3.0 TEST AND CONTROL ARTICLES

3.1 Test Article Name: 1-Chlorododecane  
CAS No.: 112-52-7  
Storage Temperature: Ambient  
Storage Parameters: Unless otherwise indicated, all test articles will be stored in the dark and solids will be stored with desiccant.  
Purity: An adjustment for purity or active ingredient will not be made unless indicated by the Sponsor.  
Molecular Weight: 204.783  
Batch No.: N6227945  
Appearance: To be documented in the study records  
Expiration Date: To be provided by the Sponsor

Protocol No. SPGT331 23-July-2007 1 of 12

- 3.2 Controls: Solvent: Test Article Solvent (or Vehicle)  
Positive: Mitomycin C (MMC)  
Cyclophosphamide (CP)

3.3 Characterization and Stability of the Test Article and Test Article Mixtures

BioReliance will not perform analysis of the test article or dosing solutions unless details of the analysis are provided below. The Sponsor will be directly responsible for determination and documentation of the analytical purity, composition and stability of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

Since the in-life portion of this study is less than four weeks in duration, BioReliance will not retain a reserve sample of the test article.

3.5 Residual Test Article and Dosing Preparations

Dosing preparations, excluding those saved for concentration or homogeneity analysis, will be disposed of following administration to the test system. Following finalization of the report, residual test article will be discarded unless otherwise indicated by the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Toxicology Testing Facility  
BioReliance
- 4.2 Address: 9630 Medical Center Drive  
Rockville, MD 20850
- 4.3 Study Director: Ramadevi Gudi, Ph.D.  
Phone: 301-610-2169  
Fax: 301-738-2362  
E-mail: rama.gudi@invitrogen.com

5.0 TEST SCHEDULE

- 5.1 Proposed Experimental Initiation Date: 24 July 2007
- 5.2 Proposed Experimental Completion Date: 18 Sept 2007
- 5.3 Proposed Report Date: 02 Oct 2007

## 6.0 TEST SYSTEM

The CHO-K<sub>1</sub> cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K<sub>1</sub> cells were obtained from the American Type Culture Collection (repository number CCL 61), Manassas, VA. The stability of the modal chromosome number of the cell line is routinely checked and the cell line is routinely tested and determined to be free from mycoplasma contamination. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

## 7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The chromosome aberration assay will be conducted using standard procedures (Evans, 1976), by exposing cultures of CHO cells to a minimum of four concentrations of the test article as well as to positive and solvent controls. In the non-activated test system, treatment will be for 4 hours and for 20 hours; in the S9 activated test system, exposure will be for 4 hours (Swierenga et al., 1991). To ensure evaluation of first division metaphase cells the dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 20 hours (1.5 normal cell cycles) after the initiation of treatment (Galloway et al., 1994). The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group. In the event of a positive response in the 4 hour non-activated study, the prolonged exposure non-activated study may not be scored. The test article will also be assessed for its ability to induce numerical chromosome aberrations.

### 7.1 Solubility Determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test article, the solvents to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

### 7.2 Preliminary Toxicity Test for Selection of Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon post-treatment toxicity (cell growth inhibition relative to the solvent control) and solubility of the test article. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article. The highest concentration tested will be

5 mg/ml or 10 mM whichever is lower for freely soluble test articles, or the maximum concentration resulting in a workable suspension for poorly soluble test articles not to exceed 5 mg/ml. If the molecular weight of the test article is not provided, the highest concentration will be 5000 µg/mL, unless limited by workability/solubility of the test article. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest dose level, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 4 hours in the absence and presence of S9 and for 20 hours in the absence of S9. Just prior to trypsinization the cell cultures will be visually inspected for the extent of monolayer confluency relative to the solvent control. Twenty hours after treatment initiation the cells will be harvested by trypsinization and counted using an automatic cell counter and the cell viability will be assessed using trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control.

Whenever possible, the high dose to evaluate chromosome aberrations will be selected to give at least 50% toxicity (cell growth inhibition relative to the solvent control) irrespective of solubility but not to exceed 5 mg/ml or 10 mM. At least two additional dose levels, demonstrating minimal or no toxicity, will be included. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested in the chromosome aberration assay will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined by direct visual inspection. In the event the test article demonstrates a dose-responsive increase in toxicity at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration that results in at least 50% toxicity. In the event that neither cytotoxicity nor insolubility is observed in the preliminary test, the highest dose in the chromosome aberration assay will be 5 mg/ml or 10 mM whichever is lower. If the osmolality of the treatment medium is considered excessive, the Sponsor will be consulted. The doses levels once determined for the definitive chromosome aberration assay will be documented in the raw data and report

### 7.3 Frequency and Route of Administration

Target cells will be treated for 4 hours in the absence and presence of S9, and for 20 hours in the absence of S9, by incorporation of the test article-solvent mixture into the treatment medium. This technique has been demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1976).

If the Sponsor is aware of specific metabolic requirements, then this information will be utilized in the preparation of the study design. Verification of a clear positive response is not required. Negative results will not be confirmed when justification can be provided. Equivocal results may be confirmed, upon consultation with the Sponsor, and may employ a modification of the study design. This guidance is based

on the OECD Guideline 473 (1997) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1996).

#### 7.4 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 homogenate was purchased (Molecular Toxicology, Inc. Boone, NC) and stored frozen at -60°C or colder until used. Each batch of S9 was assayed for sterility and its ability to metabolize at least two pro-mutagens to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride (MgCl<sub>2</sub>), 6 mM potassium chloride (KCl), 1mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum free medium.

#### 7.5 Controls

##### 7.5.1 Solvent (or Vehicle) Control

The solvent for the test article will be used as the solvent control. For solvents other than water, physiological buffer, or medium, the final concentration in treatment medium will not exceed 1%.

##### 7.5.2 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 µg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S9-activated study.

#### 7.6 Preparation of Target Cells

Exponentially growing CHO-K<sub>1</sub> cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml, 100 µg streptomycin/ml and 2.5 µg/mL Amphotericin B) for each treatment condition at approximately 5 x 10<sup>5</sup> cells/25 cm<sup>2</sup> flask. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO<sub>2</sub> in air for 16-24 hours.

#### 7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the BioReliance study number and a code system to designate the treatment condition

and test phase.

#### 7.8 Treatment of Target Cells

Unless otherwise instructed by the Sponsor, test article dosing solutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light. Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S9 reaction mixture (4 mL culture medium + 1 mL of S9 cofactor pool) for the S9-activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, or medium is used as the solvent.

In the non-activated study, the cells will be treated for 4 hours and for 20 hours; in the S9-activated study the cells will be treated for 4 hours. Treatment will be carried out at  $37 \pm 1^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 1\%$   $\text{CO}_2$  in air. After the 4 hour treatment period in the non-activated and the S9-activated studies, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

A concurrent toxicity test to determine cell growth inhibition relative to the solvent control will be conducted in both the non-activated and the S9-activated studies.

#### 7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after initiation of treatment. This post-treatment harvest time represents approximately 1.5 normal cell cycles and was selected to ensure that the cells are analyzed in the first division metaphase after initiation of treatment. Two hours prior to cell harvest, Colcemid<sup>®</sup> will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and may be stored overnight or longer at approximately 2-8°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control (% toxicity). To prepare slides, the cells will be collected by centrifugation and if necessary, the cells will be resuspended in fresh fixative. The suspension of fixed cells will be applied to glass microscope slides and air-dried. The slides will be identified by the experiment number, treatment condition and harvest date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

#### 7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) be determined and recorded for each coded treatment group selected for scoring chromosome aberrations. Slides will be coded using random numbers by an individual not involved with the scoring process. In the event of a positive response in the 4 hour non-activated study, the prolonged exposure non-activated study may not be scored. Metaphase cells with  $20 \pm 2$  centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that will be examined and scored per duplicate flask may be reduced if the percentage of aberrant cells reaches a significant level before 100 cells are scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ( $\geq 10$  aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The percent polyploid and endoreduplicated cells will be evaluated per 100 cells for each dose level analyzed for structural aberrations.

Unless otherwise indicated, the slides will be discarded after the finalization of the report.

#### 7.11 Automated Data Collection Systems

The primary computer or electronic systems used for the collection or analysis of data will include but not limited to the following:

Oracle (Oracle Corporation), Excel 2003 (Microsoft Corporation), LIMS System (BioReliance) and Kaye Lab Watch Monitoring System (Kaye GE).

### 8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

#### 8.1 Solvent Control

The frequency of cells with structural chromosome aberrations in the solvent control must be within the range of the historical solvent control.

## 8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ( $p \leq 0.05$ , Fisher's exact test) relative to the solvent control.

## 9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent control and will be presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ( $p \leq 0.05$ ). However, values that are statistically significant but do not exceed the range of historic negative or solvent controls may be judged as not biologically significant. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

## 10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. Unless alternate arrangements are made, the report will be initially issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. The report will include:

Results presented will include, but not be limited to:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Source of cells, karyotype features (modal chromosome number) and suitability of the cell type used, absence of mycoplasma, cell cycle length, passage number.

- Test conditions: composition of medium; CO<sub>2</sub> concentration; incubation time; cell seeding density; solvent and solvent selection rationale; concentration of test article and concentration selection rationale; composition and acceptability criteria for the metabolic activation (S9) system; duration of treatment; duration of treatment with and concentration of Colcemid®; type of metabolic activation system used; positive and solvent controls; methods of slide preparation; number of cell cultures; criteria for scoring aberrations and criteria for considering studies positive, negative.
- Results: description of precipitation; pH and osmolality of the treatment medium; cell growth inhibition relative to the solvent control; mitotic index and number of metaphases analyzed; type and number of aberration (structural and numerical) given separately for each treated and control culture; concentration-response relationship; statistical analysis; historical control data.
- Discussion
- Appendices: Historical Control Data (negative and positive controls with ranges, means and standard deviations), copy of protocol and any amendment, and, if provided by the Sponsor, copies of the analyses that characterized the test article, its stability and the stability and strength of the dosing preparations.
- Statement of Compliance
- Quality Assurance Statement

If an electronic copy of the protocol, the report or another study document is provided by BioReliance, the executed paper document is considered the official master document. If there is a discrepancy between an electronic copy and the corresponding master document, the master document will be considered the official document.

#### 11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years. Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

## 12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

The study will be conducted in compliance with the most recent version of the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58 and the Organisation for Economic Co-operation and Development Principles of Good Laboratory Practice, C(97)186/Final and the study protocol.

At BioReliance, an in-process phase, the raw data, and report(s) will be inspected per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA statement will be included in the final report. This statement will list the study-specific phases inspected, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test article or its mixtures.

Raw data, the protocol and reports generated at facilities other than BioReliance will be QA audited per the contractual arrangements between that facility and the Sponsor.

Alterations of this protocol may be made as the study progresses. All protocol modifications and rationale for the change(s) will be documented, signed, dated and approved by the Study Director, BioReliance QA and the Sponsor/Sponsor Monitor. All protocol amendments will be delivered to the Sponsor and all Principal Investigators (if any) via mail, electronic file transfer or fax transmission, as well as internally at the Test Facility, on or as close as possible to the effective date of the amendment.

Deviations from the protocol (i.e., unplanned changes) will be documented in a deviation report or a note to file will be generated. A deviation report will be signed by the Study Director and BioReliance QA. All deviations will be identified in the study report.

## 13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), *Chemical Mutagens, Principles and Methods for their Detection*, vol. 4. Plenum Press, New York, NY.

Galloway, S.M., M.J. Aardema, M. Ishidate Jr., J.L. Ivett, D.J. Kirkland, T. Morita, P. Mosesso and T. Sofuni (1994) Report from working group on in vitro tests for chromosomal aberrations, *Mutation Research* 312(3):241-261.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

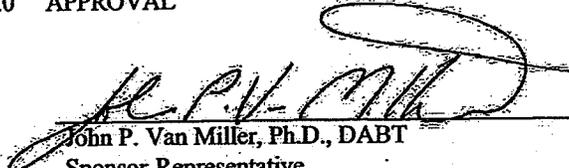
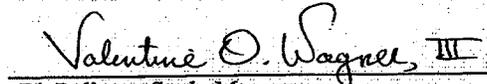
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Swierenga S.H.H., J.A. Heddle, E.A. Sigal, J.P.W. Gilman, R.L. Brillinger, G.R. Douglas and E.R. Nestmann (1991) Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese lung and human lymphocyte cultures, *Mutation Research* 246:301-322.

14.0 APPROVAL

 _____ John P. Van Miller, Ph.D., DABT Sponsor Representative	<u>7/25/07</u> Date
 _____ Ramadevi Gudi, Ph.D. BioReliance Study Director	<u>23 Jul 2007</u> Date
 _____ Valentine O. Wagner, III BioReliance Study Management	<u>23 Jul 2007</u> Date

**APPENDIX II**

**Certificate of Analysis and Stability Memo**

# CERTIFICATE OF ANALYSIS

**Product:** 1-Chlorododecane

**Batch #:** N6227945

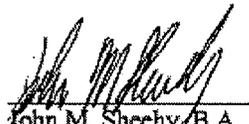
**PSL Reference No.:** 070416-3R

**Date of Analysis:** June 29, 2007

**Result:**

1-Chlorododecane    97.42%

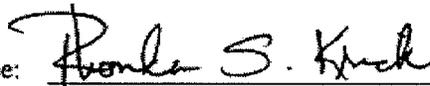
Approval:

  
John M. Sheehy, B.A.,  
Analytical Services  
Product Safety Labs

Date

8/16/07

QA Release:

  
Rhonda Krick, B.S.  
Quality Assurance  
Product Safety Labs

Date

08/17/07

PSL GLP Study # 22018



# Toxicology Regulatory Services

May 8, 2008

Valentine (Skip) O. Wagner III, M.S.  
Study Director  
BioReliance  
9630 Medical Center Drive  
Rockville, MD 20850

Ramadevi Gudi, Ph.D.  
Study Director  
BioReliance  
9630 Medical Center Drive  
Rockville, MD 20850

Re: 1-Chlorododecane: Confirmation of Stability

Dear Skip and Rama:

Following is a summary of the analyses that have been performed on behalf of Lonza Inc. in order to confirm stability of the referenced test article throughout the duration of testing conducted at BioReliance, e.g. Bacterial Reverse Mutation Assay and In Vitro Chromosomal Aberration Assay (Study Nos. AC01UM.503.BTL and AC01UM.331.BTL, respectively).

Characterization of the active ingredient in a test sample of 1-Chlorododecane was conducted at Eurofins|Product Safety Laboratories (E|PSL) as described in the table below.

E PSL Study No.	Test Article Identification	Batch No.	Date of Analysis	% Active Ingredient (mean)
22018	1-Chlorododecane (CAS No. 112-52-7)	#N6227945	June 29, 2007	97.42
24870	1-Chlorododecane (CAS No. 112-52-7)	#N6227945	April 24, 2008	98.8

The purity of the test sample remained within specification and no degradation of the material occurred between analyses. Based on these results, stability of 1-Chlorododecane (Batch No. N6227945) is confirmed for the period of June 29, 2007 through April 24, 2008.

Please contact me if you have any questions or require additional information.

Regards,

John P. Van Miller, Ph.D., DABT  
Toxicology Consultant to Lonza Inc.

2365 HUNTERS WAY, CHARLOTTESVILLE, VIRGINIA 22911 ■ TELEPHONE: 434-977-5957 ■ FACSIMILE: 434-977-0899

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**APPENDIX III**  
**Historical Control Data**

IN VITRO MAMMALIAN CYTOGENETIC TEST USING  
CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES  
STRUCTURAL ABERRATIONS  
2004-2006

NON-ACTIVATED TEST SYSTEM

<b>Historical Values</b>	<b>Solvent (%)</b>	<b>Positive Control<sup>2</sup> (%)</b>
Mean	0.6	20.5
±SD <sup>1</sup>	0.8	7.3
Range	0.0-5.5	8.0-56.0

S9-ACTIVATED TEST SYSTEM

<b>Historical Values</b>	<b>Solvent (%)</b>	<b>Positive Control<sup>3</sup> (%)</b>
Mean	0.7	22.8
±SD <sup>1</sup>	0.9	10.9
Range	0.0-5.0	8.0-84.3

<sup>1</sup> SD = standard deviation.

<sup>2</sup> Positive control for non-activated studies, Mitomycin C (MMC, 0.1-0.2 µg/mL).

<sup>3</sup> Positive control for S9-activated studies, cyclophosphamide (CP, 10-20 µg/mL).

IN VITRO MAMMALIAN CYTOGENETIC TEST USING  
CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES  
COMBINED NUMERICAL ABERRATIONS  
(POLYPLOID AND ENDOREDUCATED CELLS)  
2004-2006

NON-ACTIVATED TEST SYSTEM

<b>Historical Values</b>	<b>Solvent (%)</b>	<b>Positive Control<sup>2</sup> (%)</b>
Mean	2.1	2.0
±SD <sup>1</sup>	1.2	1.2
Range	0.0-6.5	0.0-6.5

S9-ACTIVATED TEST SYSTEM

<b>Historical Values</b>	<b>Solvent (%)</b>	<b>Positive Control<sup>3</sup> (%)</b>
Mean	2.8	2.4
±SD <sup>1</sup>	1.7	1.5
Range	0.0-10.0	0.0-6.0

<sup>1</sup> SD = standard deviation.

<sup>2</sup> Positive control for non-activated studies, Mitomycin C (MMC, 0.1-0.2 µg/mL).

<sup>3</sup> Positive control for S9-activated studies, cyclophosphamide (CP, 10-20 µg/mL).