FINAL REPORT

In vitro mammalian chromosome aberration test of Taglus PU Flex Thermoforming Foils in cultured human peripheral blood lymphocytes as per ISO 10993-3:2014.

STUDY CONTRACT PARTNER:
UL India Private Limited
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Bangalore - 560066, India, T : 918041384400 / F : 918028413759 / W : ul.com
UL Project Number: 4790342010

TEST FACILITY:
GLR Laboratories Private Limited
444 Gokulam Street, Mathur, Chennai - 600 068, Tamil Nadu, India.
Study No.: 073/465

STUDY SPONSOR AND APPLICANT:
Vedia solutions Div. of Laxmidental Export Pvt. Ltd
103, Akruti arcade, J P Road,
Opp A H Wadhia School,
Andheri (W), Mumbai 400053

REPORT ISSUED DATE: 31 May 2022

FINAL REPORT

PRODUCT NAME:

Taglus PU Flex Thermoforming Foils

STUDY TITLE

***In vitro* mammalian chromosome aberration test of Taglus PU Flex Thermoforming Foils in cultured human peripheral blood lymphocytes as per ISO 10993-3:2014**

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CONTENTS

STUDY DIRECTOR AUTHENTICATION STATEMENT

Study No. : 073/465

Study Title : *In vitro* mammalian chromosome aberration test of Taglus PU Flex Thermoforming Foils in cultured human peripheral blood lymphocytes as per ISO 10993-3:2014

This study was performed in accordance with the mutually agreed study plan, one study plan amendment and GLR Laboratories Private Limited's standard operating procedures, unless otherwise stated, and the study objective was achieved. I accept overall responsibility for the technical conduct of the study, as well as for the interpretation, analysis, documentation and reporting of results. This report provides a true and accurate record of the results obtained.

This study was performed in compliance with OECD Principles of Good Laboratory Practice* ENV/MC/CHEM (98)17 (Revised 1997, issued January 1998) and applicable regulatory requirements including the US Food and Drug Administration's GLP regulations, 21 CFR 58 (subparts B to G and J).

Ms. Ashwini Harke, MSc Study Director GLR Laboratories Private Limited	31 May 2022 Study Completion Date

*The identity (including the dates of manufacture and expiry, the batch/lot number) and composition of the test item are the responsibilities of the study sponsor.

QUALITY ASSURANCE STATEMENT

Study No. : 073/465

Study Title : *In vitro* mammalian chromosome aberration test of Taglus PU Flex Thermoforming Foils in cultured human peripheral blood lymphocytes as per ISO 10993-3:2014

The Quality Assurance (QA) of GLR Laboratories Private Limited verified the Study Plan, including any amendments, inspected the critical study phases, audited the raw data and report of this Study as per in-house Standard Operating Procedures (SOPs) for compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997) [ENV/MC/CHEM (98)17], and for compliance with relevant regulatory requirements.

During the Study, the following study-related inspections/audits were performed on the following dates and reported to the Study Director and Test Facility Management. Besides the below, process and facility inspections were also carried out periodically at this Test Facility by auditor(s) of the QA, as per in-house SOPs, which may have relevance to this study.

S. No.	Type of Inspection	Date(s) of Inspection	Phase(s) of Study Inspected	Date(s) of Reporting to Management, Study Director (Inspection Report No.)
1	Study Plan Verification	14 March 2022	Draft Study Plan	14 March 2022 (SBI/073/465/001)
2	Study Plan Verification	21 March 2022	Definitive Study Plan	21 March 2022 (SBI/073/465/002)
3	In-life Phase Inspection	26 March 2022	Test Item Extracts Administration	26 March 2022 (SBI/073/465/003)
4	In-life Phase Inspection	02 May 2022	Slide Scoring	02 May 2022 (SBI/073/465/004)
5	Study Plan Verification	18 May 2022	Definitive Study Plan Amendment No.01	18 May 2022 (SBI/073/465/005)
6	Report Audit	30 May 2022	Draft Report	30 May 2022 (SBI/073/465/006)
7	Report Audit	31 May 2022	Final Report	31 May 2022 (SBI/073/465/007)

The QA has determined that the methods, procedures, observations, and reported results are accurately and completely described and that the reported results are based on the Study Plan and the pertinent raw data generated during the course of the Study. The Study Director's GLP compliance statement is supported.

N. Parthiban

31 MAY 2022

Dr. Parthiban Natarajan, PhD, ERT
Head-Quality Assurance
GLR Laboratories Private Limited

Date

TEST FACILITY MANAGEMENT STATEMENT

Study No. : 073/465

Study Title : *In vitro* mammalian chromosome aberration test of Taglus PU Flex Thermoforming Foils in cultured human peripheral blood lymphocytes as per ISO 10993-3:2014

This is to certify that, the Test Facility Management appointed the Study Director and provided all necessary facilities and resources for the proper conduct of this study, in compliance with the Principles of OECD Good Laboratory Practice (GLP), as per the recommendations of the OECD (Council Act [C (97) 186 (Final)]) and as adopted in the procedures promulgated by the National GLP Compliance Monitoring Authority, Government of India.

31 May 2022

Ms. M. Yaminy, B.Com, (MBA)
Deputy Test Facility Management
GLR Laboratories Private Limited

Date

SUMMARY

The test item, Taglus PU Flex Thermoforming Foils, supplied by Vedia solutions Div. of Laxmidental Export Pvt. Ltd, was evaluated for its clastogenic potential (induction of structural chromosomal aberrations) in cultured human peripheral blood lymphocytes.

The test item, Taglus PU Flex Thermoforming Foils is a transparent sheet with a diameter, 125 mm and thickness, 0.8 mm. It is a surface device which comes in contact with mucosal membrane. The duration of contact is less than 24 hours (limited). According to ISO 10993-1:2018, this is a surface device which comes in contact with mucosal membrane and the duration of contact is up to 24 hours (limited).

The test item was extracted at a ratio of 6 cm^2/mL (as thickness of test item was less than 0.5 mm) in 10% serum supplemented RPMI-1640 (Roswell Park Memorial Institute) medium. The total surface area of one test item is approximately 441 cm^2 (as calculated in our laboratory). One test item was extracted with 73.5 mL of serum supplemented RPMI medium for 72 h and 10 minutes at 37 ± 1 °C under aseptic conditions. Solvent control was also subjected to same extraction conditions. This fulfilled the requirement of ISO 10993-12:2012 and ISO 10993-12:2021.

At the end of extraction, the extract was clear without any colour change or particulates. The pH of the extract was 7.70. No change was observed in the retrieved test item. No additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. Extract was used within 1 hour 25 minutes and was considered stable during this time.

Peripheral blood was collected from three healthy volunteers, pooled and lymphocyte cultures were prepared for testing. Neat extract of the test item (100%) was evaluated for its clastogenic potential in cultured human peripheral blood lymphocytes treated in the absence and presence of metabolic activation system (S9 mix).
The experimental design is as follows:

S9 mix	Treatment + recovery (h)	Concentration of test item extract (%)	Positive control (µg/mL culture)
-	3+17	100	Mitomycin C: 0.80
+	3+17	100	Cyclophosphamide: 12.5
-	20+0	100	Mitomycin C: 0.80

- absence, + presence

Duplicate cultures were treated with negative (solvent) control, test item extract and positive controls at appropriate concentration and for all three exposure conditions.

At the end of the treatment, cells were arrested at metaphase, harvested and slides were prepared and stained. About 1000 cells were scored for mitotic index which was used as the measurement of cytotoxicity. Since the neat extract (100%) was non-cytotoxic, it was selected for chromosomal analysis. Three-hundred well spread metaphases were analysed for chromosomal aberrations.

The chromosomal aberration frequencies in the negative (solvent) control were similar to historical data of laboratory. Positive controls induced significant increase (Fisher's exact test) in chromosomal aberrations when compared to the concurrent negative (solvent) control and were comparable to the historical data of laboratory.

Cultures treated with the test item extract in the absence and the presence of S9 mix resulted in frequencies of cells with structural aberrations which were not statistically significant when compared to the concurrent negative control. The number of aberrant cells (excluding gaps) in all treated cultures were comparable to historical data of the laboratory.

No numerical aberrations were observed in cultures treated with the test item extract both in the absence and presence of S9 mix.

Based upon the results obtained and in line with ISO 10993-3:2014, it is concluded that the given test item, Taglus PU Flex Thermoforming Foils, supplied by Vedia solutions Div. of Laxmidental Export Pvt. Ltd, does not induce chromosome aberrations in cultured human peripheral blood lymphocytes under the conditions of the study.

INTRODUCTION

Biocompatibility testing is a regulatory requirement for demonstrating the preclinical safety of medical devices. This is evaluated in line with, ISO 10993-1:2018, Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process. This standard describes the necessity to select a suitable test method for biocompatibility evaluation of medical devices.

The *in vitro* chromosomal aberration test is to identify substances that cause structural chromosomal aberrations in cultured mammalian cells. The *in vitro* chromosomal aberration test may employ cultures of established cell lines or primary cell cultures of human or rodent origin. The cells used should be selected on the basis of growth ability in culture, stability of the karyotype (including chromosome number) and spontaneous frequency of chromosomal aberrations. Cell cultures of human or other mammalian origin are exposed to the test item extracts both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used. At an appropriate predetermined interval after the start of exposure of cell cultures to the test extract, they are treated with a metaphase-arresting substance (e.g. colcemid or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromatid-type and chromosome-type aberrations.

OBJECTIVE

To evaluate the clastogenic potential (induction of structural chromosomal aberrations) of the test item in cultured human peripheral blood lymphocytes.

STUDY DATES

Study Start Date	21 March 2022
Experiment Start Date	23 March 2022
Experiment Completion Date	16 May 2022

The study completion date is the date the final report is signed by the Study Director.

TEST ITEM DETAILS

The test item, Taglus PU Flex Thermoforming Foils, was received at GLR Laboratories Private Limited on 04 March 2022 and stored at room temperature (20.1 to 24.6 °C) until use.

The following test item information provided by the Sponsor, are considered an adequate description of the characterisation, purity and stability of the test item. No additional analysis was performed in GLR Laboratories Private Limited to confirm it.

Test Item	Taglus PU Flex Thermoforming Foils
Batch / Lot No.	22022010-01
Manufacture Date	02 February 2022
Expiry Date	02 February 2025
Appearance	Transparent sheet
Ingredients	PETG (Polyethelene Tertamethylene Glycol)
Temperature Stability	37 °C
Sterility	Non-sterile
Handling procedure	The test item was handled with necessary protective clothing and all recommended safety measures were followed.

Description of the test item

The test item, Taglus PU Flex Thermoforming Foils is a transparent sheet with a diameter, 125 mm and thickness, 0.8 mm. It is a surface device which comes in contact with mucosal membrane. The duration of contact is less than 24 hours (limited). According to ISO 10993-1:2018, this is a surface device which comes in contact with mucosal membrane and the duration of contact is up to 24 hours (limited).

CONTROL ITEM DETAILS

Negative (solvent) control

RPMI-1640 (Roswell Park Memorial Institute) medium with 10% serum.

Positive control

Chemical	Source	Lot No.	Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	Solvent	S9 mix
Mitomycin-C	Sigma-Aldrich	SLCB4710	0.08	0.80	Distilled water	-
Cyclophosphamide (CPA)	Sigma-Aldrich	LRAC0295	1.250	12.5	DMSO	+

TEST SYSTEM

Rationale for the choice of the test system

To meet the requirements of ISO 10993-3:2014 and OECD Guideline for the Testing of Chemicals No. 473 (adopted on 29 July, 2016).

Peripheral blood collection

Blood from three healthy, non-smoking female volunteers (23 - 24 years) were collected, pooled and used for this study. No volunteer suspected of any viral infection or exposed to high levels of radiation or hazardous chemicals was used. Four millilitres (4 mL) of blood were drawn from the peripheral circulation from each donor into heparinised tubes. The blood samples were pooled in equal volumes and cultures were established.

Preparation of cultures

A volume of 0.5 mL pooled blood was cultured in 9.5 mL of RPMI-1640 medium containing 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin solution and 2% phytohemagglutinin (PHA-M). Eighteen blood cultures were established and incubated at 37 ± 1 °C in 5% CO_2 and 95 % humidity for 48 hours.

Ingredients	Make and Expiry
RPMI minimal medium	HiMedia Lot No.:0000385720 Expiry Date: April 2022
Fetal bovine serum	Thermo fisher scientific Lot No.: 2384431 Expiry Date: June 2026
Penicillin / Streptomycin	HiMedia Lot no.: 0000491967 Expiry Date: September 2023
Phytohaemagglutinin (PHA-M)	HiMedia Lot no. 0000506592 Expiry Date: November 2022

Metabolic activation system (S9 mix)

Treatment was carried out both in the absence and presence of S9 mix. The S9 mix was prepared fresh and kept on ice. The mammalian liver post-mitochondrial fraction, 10% mutazyme (Make: Moltox, Lot No.: 4474, Expiry Date: 15 Jul 2023), a pre-mix which includes all the co-factors such as glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), magnesium chloride ($MgCl_2$), potassium chloride (KCl) and rat liver S9 was used at a concentration of 1% (v/v) in the final test medium. The quality control and production certificate of 10% mutazyme used, is included in the report (Appendix 1).

One millilitre of S9 mix was added to all cultures treated in the presence of S9 mix. Cultures treated in the absence of S9 mix received an equivalent volume of sterile phosphate buffer.

TEST ITEM PREPARATION

The test item was extracted at a ratio of 6 cm^2/mL (as thickness of test item was less than 0.5 mm) in 10% serum supplemented RPMI-1640 (Roswell Park Memorial Institute) medium. The total surface area of one test item is approximately 441 cm^2 (as calculated in our laboratory). One test item was extracted with 73.5 mL of serum supplemented RPMI medium for 72 h and 10 minutes at 37 ± 1 °C under aseptic conditions. Solvent control was also subjected to same extraction conditions. This fulfilled the requirement of ISO 10993-12:2012 and ISO 10993-12:2021.

At the end of extraction, the extract was clear without any colour change or particulates. The pH of the extract was 7.70. No change was observed in the retrieved test item. No additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. Extract was used within 1 hour 25 minutes and was considered stable during this time.

Neat extract of the test item (100%) was evaluated for its clastogenic potential in cultured human peripheral blood lymphocytes in the absence and presence of metabolic activation system (S9 mix).

TEST METHOD

Identification of cultures

Each experiment was performed in duplicate cultures (A and B). The cultures were suitably labelled to clearly identify the study number, cultures (A and B), treatment time, with/without S9 mix, test item concentration/positive/negative control.

Test procedure

After 48 ± 0.5 h incubation, cultures were centrifuged at 1500 rpm for 5 minutes, the supernatant was carefully removed and cells re-suspended in 10 mL of 10% RPMI-1640 media containing 1 mL of 100% test item extract/0.1 mL of positive control and 1 mL of S9 mix or sodium phosphate buffer. Cultures were incubated at 37 ± 1 °C in 5% CO_2 and 95 % humidity.

The experiment comprised of 3 h treatment (pulse treatment) with and without S9 mix followed by a 17 h recovery period (3+17) and 20 h continuous treatment (20+0). Concurrent positive and negative control cultures were maintained.

For 3 h treatment, after completion of treatment period, cells were pelleted at approximately 1500 rpm for 5 minutes, washed twice with phosphate buffered saline, resuspended in 10% RPMI-1640 media and incubated for 1.5 normal cell cycle length after the beginning of treatment (approximately 20 h). The final post-treatment volume was 10 mL per culture. Cultures receiving continuous treatment (20 h) were retained treatment medium through to harvest.

The experimental design is as follows:

S9 mix	Treatment + recovery (h)	Concentration of test item extract (%)	Positive control (µg/mL culture)
-	3+17	100	Mitomycin C: 0.80
+	3+17	100	Cyclophosphamide: 12.5
-	20+0	100	Mitomycin C: 0.80

- absence, + presence

pH and precipitation

The pH and precipitation of test item treated cultures was measured at the beginning and end of the treatment.

Harvesting

Colchicine at a final concentration of 1 µg/mL (HiMedia, Lot no. 0000468073, Expiry Date: 03 May 2026) was added to arrest dividing cells in metaphase at 2 h and 40 minutes before harvest.

At the end of 20 h, all cultures were centrifuged at 1500 rpm for 5 minutes, the supernatant was carefully removed and cells re-suspended in 5 mL of pre-warmed 0.075 M KCl (hypotonic) and incubated at 37 ± 1° C for 20 minutes to allow cell swelling.

At the end of hypotonic treatment, cells were pelleted at 1500 rpm for 5 minutes and 5 mL cold fixative (methanol/glacial acetic acid (3:1, v/v)) was added for 10 minutes at room temperature. This procedure was repeated two times to get the clean cell pellets.

Slide preparation

Several drops of suspension were transferred on to clean microscope slides labelled with the appropriate study details (study number, cultures [A or B], treatment time, with/without S9 mix, test item concentration and positive/negative control and replicate number). Two slides were prepared from each culture. After the slides are flame-dried, cells were stained for approximately 10 minutes in Giemsa, rinsed and dried.

Selection of concentration for chromosome analysis

At least 1000 cells were scored from each culture; and mitotic index, percentage mitotic inhibition were calculated.

The Mitotic Index (MI) is a measure of the proliferative state of the culture at a particular moment in time and is calculated as follows:

$$MI = \frac{Number\ of\ cells\ in\ mitosis}{Total\ number\ of\ cells\ observed} \quad x100$$

Mitotic Inhibition (MIH %) is a measure of cytotoxicity and is calculated as follows:

Mitotic inhibition (MIH %) = [1 - (mean MI_T/mean MI_C)] x 100%

(Where T = treatment and C = negative control)

Mitotic index was used as the measurement of cytotoxicity and non-cytotoxic dose was selected for chromosomal analysis.

Slide analysis

Prior to analysis, all slides were coded using randomly generated numbers by personnel not involved in the study.

A minimum of 300 metaphase spreads, distributed equally among the replicates (150 each) were analyzed for chromosomal aberration. Only cells with 44 to 48 chromosomes were considered acceptable for analysis.

Structural aberrations were classified according to the ISCN (International System for Human Cytogenetic Nomenclature) scheme as detailed in appendix 2. Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid with no evidence of

displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

ACCEPTANCE CRITERIA

The assay is considered valid based on the following criteria:

1. The proportion of cells with structural aberrations (excluding gaps) in negative control cultures fell within the historical negative (solvent) control (normal) range.
2. At least 300 well spread metaphase were suitable for analysis at each concentration.
3. The positive control chemicals induced significant increases in the proportion of cells with structural aberrations.

DATA EVALUATION

After completion of scoring and decoding of slides, the numbers of aberrant cells in each culture was tabulated. Aberrant cells were categorised as follows:

Category 1. Cells with structural aberrations including gaps

Category 2. Cells with structural aberrations excluding gaps

Category 3. Polyploidy, endoreduplicated or hyperdiploid cells.

The total for category 2 in negative control cultures was compared with current laboratory historical negative control (normal) ranges to determine whether the assay was acceptable.

The number of cells with structural aberrations excluding gaps (category 2) in the test item treated cultures was compared with the concurrent negative control using Fisher's exact test (performed with the statistical software Graphpad Prism 9). Probability values of $p \leq 0.05$ was considered statistically significant. However statistical significance was not the only determining factor for a positive response.

There are several criteria for determining a positive result, such as a reproducible increase in the number of cells with structural chromosome aberrations. Biological relevance of the results was considered first.

An increase in the number of polyploid cells can indicate that the test item has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations. An increase

in the number of cells with endoreduplicated chromosomes can indicate that the test item has the potential to inhibit cell cycle progression.

A test item or extract for which the results do not meet the above criteria is considered non mutagenic in this system. In rare cases, results can remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* chromosome aberration test indicate that the test item induces structural chromosome aberrations in cultured mammalian somatic cells.

Negative results indicate that, under the test conditions, the test item does not induce structural chromosome aberrations in cultured mammalian somatic cells.

EVALUATION CRITERIA

The test item was considered to have the potential to induce chromosome aberrations in this assay if:

1. The assay is valid
2. A proportion of cells with structural aberrations at one or more concentrations that exceeds the historical negative control (normal) range is observed in both replicate cultures
3. There is a concentration-related trend in the proportion of cells with structural aberrations (excluding gaps).

RESULTS

pH and precipitation

Test item treatment	At the beginning of treatment		At the end of treatment	
	pH	Precipitation	pH	Precipitation
3+17 h, + S9 mix	7.70	No	7.63	No
3+17 h, -S9 mix		No	7.81	No
20+0 h, -S9 mix		No	7.90	No

Selection of doses for chromosomal aberration analysis

The mitotic indices are presented below:

Mitotic Index (MI) and MIH determination

Treatment	Mitotic index (%)											
	3+17 h, +S9				3+17 h, -S9				20+0 h, -S9			
	A	B	Mean MI	MIH*	A	B	Mean MI	MIH*	A	B	Mean MI	MIH*
Solvent	14.80	13.90	14.35	-	13.70	13.10	13.40	-	14.10	13.70	13.90	-
100	13.40	13.60	13.50	5.92	12.50	13.00	12.75	4.85	12.70	13.10	12.90	7.19

*Mitotic inhibition (%) = [1 - (mean MI_T/mean MI_C)] x 100%
(Where T = treatment and C = negative control)

(A, B refers to the duplicate cultures)

The neat extract did not cause cytotoxicity in any of the treatment regimens. Therefore, the slides were analysed for chromosomal aberration.

Analysis of chromosomal aberration data

A total of 300 cells were scored in each treatment for chromosomal aberrations. The details of chromosomal aberrations are presented in Table 1 and Table 2. The chromosomal aberration frequencies in the negative (solvent) control were similar to historical data of laboratory. Positive controls induced significant increase (fisher's exact test) in chromosomal aberrations when compared to the concurrent negative (solvent) control and were comparable to the historical data of laboratory. The assay was therefore considered valid. The historical data of laboratory is presented in Table 3.

Structural aberrations

Cultures treated with the test item extract in the absence and the presence of S9 mix resulted in frequencies of cells with structural aberrations which were not statistically significant when compared to the concurrent negative control. The number of aberrant cells (excluding gaps) in all treated cultures were comparable to historical data.

Numerical aberrations

No numerical aberrations were observed in cultures treated with the test item extract both in the absence and presence of S9 mix.

CONCLUSION

Based upon the results obtained and in line with ISO 10993-3:2014, it is concluded that the given test item, Taglus PU Flex Thermoforming Foils, supplied by Vedia solutions Div. of Laxmidental Export Pvt. Ltd, does not induce chromosome aberrations in cultured human peripheral blood lymphocytes under the conditions of the study.

REFERENCES

1. ISO 10993-1:2018. Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process.
2. ISO 10993-3:2014. Biological evaluation of medical devices - Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity.
3. ISO 10993-12:2012. Biological evaluation of medical devices - Part 12: Sample preparation and reference materials.
4. ISO 10993-12:2021. Biological evaluation of medical devices - Part 12: Sample preparation and reference materials.
5. ISO 10993-33:2015 - Supplement to ISO 10993-3:2014: Biological evaluation of medical devices - Part 33, Guidance on tests to evaluate genotoxicity.
6. OECD Guidelines for Testing of Chemicals (TG 473, Adopted 29 July 2016).
7. OECD Principles of Good Laboratory Practice. OECD Environmental Health and Safety Publications, Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1. ENV/MC/CHEM (98)17.
8. General Requirements for the Competence of Testing and Calibration Laboratories, ISO/IEC 17025:2017.
9. Use of International Standard ISO 10993-1, “Biological Evaluation of Medical Devices - Part 1. Evaluation and Testing Within a Risk Management Process. Guidance for Industry and Food and Drug Administration Staff. September 4, 2020.

APPENDIX 1- QUALITY CONTROL AND PRODUCTION CERTIFICATE OF S9 MIX

MOLTOX 10% MUTAZYME
QUALITY CONTROL STATEMENT

LOT NO.: 4474 **SPECIES:** Rat **PREPARATION DATE:** July 15, 2021
PART NO.: 11-404L **STRAIN:** Sprague Dawley **EXPIRATION DATE:** July 15, 2023
VOLUME: 20 ml **SEX:** Male **INDUCING AGENT(s):** Phenobarbital/ β-naphthaflavone
STORAGE: At or below -20°C **TISSUE:** Liver

BIOCHEMISTRY:

- PROTEIN

3.5 mg/ml

Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard. Protein concentration of reconstituted S9 mix was mathematically derived from the concentration of S9 used in production.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	55.3
PROD	2B1, 3B2	32.1
MROD	IA2	17.9
BROD	3A, 2B	41.5

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, methyloxy- and benzyloxyresorufin-0-dealkylases (PROD, MROD, & BROD) were conducted using a modification of the methods of Burke et al., *Biochem Pharm* **34**: 3337, 1985. Fold-inductions calculated as the ratio of the sample vs. uninduced control specific activities (SA). Control SA's (pmoles/min/mg protein) were 53.0, 20.5, 24.9, & 68.7 for EROD, PROD, MROD & BROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 24 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
100.4	816

The ability of the sample to activate ethidium bromide (EtBr) EtBr/CPA/and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca et al. *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.3 - 5% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.***113**:173, 1983.).

	µl S9 per plate/number his$^+$ revertants per plate					
Promutagen	0	3.1	6.3	12.5	25	50
BP (5 µg)	98	121	176	464	634	728
2-AA (2.5 µg)	123	525	1579	2526	2461	1608

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099
www.moltox.com

Approved: Kathy Peterson 07/19/21

APPENDIX 2- ABBREVIATIONS AND CLASSIFICATION OF OBSERVATIONS

Abs	=	Aberrations
Abs +g	=	Aberrations including gaps
Abs -g	=	Aberrations excluding gaps
Gaps (G)		
Csg	=	chromosome gap
Ctg	=	chromatid gap
Chromosome deletions (chr del)		
Del	=	chromosome deletion
d min	=	double minute
F	=	isolocus fragment
Chromosome exchanges (chr exch)		
T	=	interchange between chromosomes (eg reciprocal translocation)
Inv	=	chromosome intrachange (eg pericentric inversion)
Dic	=	Dicentric
dic+f	=	dicentric with accompanying fragment
Acr	=	acentric ring
r+f	=	centric ring with accompanying fragment
R	=	centric ring
Chromatid deletions (ctd del)		
Del	=	chromatid deletion
Min	=	single minute
Chromatid exchanges (ctd exch)		
Qr	=	interchange between chromatids of different chromosomes (eg quadriradial)
Cx	=	obligate complex interchange
E	=	chromatid intrachange
tr/tr+f	=	isochromatid/chromatid interchange (triradial)/with accompanying fragment
Su	=	intra-arm intrachange with sister union of broken ends
Nud	=	intra-arm intrachange with non-union of broken ends distally
Nup	=	intra-arm intrachange with non-union of broken ends proximally
Other structural aberrations		
Pvz	=	pulverised cell
Mabs	=	multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis)
Numerical aberrations		
E	=	Endoreduplicated
H	=	Hyperdiploid (49-68 chromosomes)
P	=	Polyploid (greater than 68 chromosomes)
Hypo	=	Hypodiploidy

TABLE 1- SUMMARY OF ABERRATIONS

Experiment No.1: 3 + 17 h, +S9 mix

Treatment	Rep	Slide No.	Cells	Break		Exchanges		mabs	pvz	others	H	E	P	Gaps		Abs+g	Abs-g
				Ct	Cs	Ct	Cs							Ct	Cs		
Solvent control (RPMI medium)	A	1/2	150	0	1	1	0	0	0	0	0	0	0	0	1	3	2
	B	1/2	150	0	3	0	0	0	0	0	0	0	0	0	0	3	3
	Total		**300**	**0**	**4**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**6**	**5**
Test item extract (100%)	A	1/2	150	1	1	0	0	0	0	1	0	0	0	1	1	5	3
	B	1/2	150	0	0	0	0	0	0	1	0	0	0	1	1	3	1
	Total		**300**	**1**	**1**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**2**	**2**	**8**	**4**
Positive control CPA; 12.5 µg/mL	A	1/2	63	0	9	0	0	0	0	2	0	0	1	0	3	14	11
	B	1/2	57	1	8	2	1	0	0	2	0	0	0	0	1	15	14
	Total		**120**	**1**	**17**	**2**	**1**	**0**	**0**	**4**	**0**	**0**	**1**	**0**	**4**	**29**	**25**

CPA: Cyclophosphamide; Rep: Replicate; Ct: Chromatid; Cs: Chromosome; mabs: Multiple aberrations; pvz: Pulverization; P: Polyploidy; H: Hyper diploid; E: Endoreduplication; Abs+g: Aberrations with gap; Abs-g: Aberrations without gap

TABLE 1 (CONT.)- SUMMARY OF ABERRATIONS

Experiment No.2: 3 + 17 h, -S9 mix

Treatment	Rep	Slide No.	Cells	Break		Exchanges		mabs	pvz	others	H	E	P	Gaps		Abs+g	Abs-g
				Ct	Cs	Ct	Cs							Ct	Cs		
Solvent control (RPMI medium)	A	1/2	150	1	1	0	0	0	0	1	0	0	0	0	1	4	3
	B	1/2	150	0	2	0	0	0	0	0	0	0	0	0	1	3	2
	Total		**300**	**1**	**3**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**2**	**7**	**5**
Test item extract (100%)	A	1/2	150	0	1	0	0	0	0	0	0	0	0	1	1	3	1
	B	1/2	150	0	1	0	0	0	0	1	0	0	0	0	1	3	2
	Total		**300**	**0**	**2**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**2**	**6**	**3**
Positive control MMC; 0.80 µg/mL	A	1/2	58	2	8	1	0	0	0	2	0	0	0	0	1	14	13
	B	1/2	57	3	8	0	0	0	0	2	0	0	1	1	1	15	13
	Total		**115**	**5**	**16**	**1**	**0**	**0**	**0**	**4**	**0**	**0**	**1**	**1**	**2**	**29**	**26**

MMC: Mitomycin C; Rep: Replicate; Ct: Chromatid; Cs: Chromosome; mabs: Multiple aberrations; pvz: Pulverization; P: Polyploidy; H: Hyper diploid; E: Endoreduplication; Abs+g: Aberrations with gap; Abs-g: Aberrations without gap

TABLE 1 (CONT.)- SUMMARY OF ABERRATIONS

Experiment No.3: 20 + 0 h, -S9 mix

Treatment	Rep	Slide No.	Cells	Break		Exchanges		mabs	pvz	others	H	E	P	Gaps		Abs+g	Abs-g
				Ct	Cs	Ct	Cs							Ct	Cs		
Solvent control (RPMI medium)	A	1/2	150	1	1	0	0	0	0	0	0	0	0	0	1	3	2
	B	1/2	150	0	2	0	0	0	0	0	0	0	0	0	2	4	2
	Total		**300**	**1**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**	**7**	**4**
Test item extract (100%)	A	1/2	150	0	2	0	0	0	0	0	0	0	0	0	2	4	2
	B	1/2	150	0	1	0	0	0	0	0	0	0	0	1	2	4	1
	Total		**300**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**4**	**8**	**3**
Positive control MMC; 0.80 µg/mL	A	1/2	56	1	9	0	0	0	0	3	0	0	0	0	0	13	13
	B	1/2	53	3	4	1	1	0	0	2	0	0	0	0	0	11	11
	Total		**109**	**4**	**13**	**1**	**1**	**0**	**0**	**5**	**0**	**0**	**0**	**0**	**0**	**24**	**24**

MMC: Mitomycin C; Rep: Replicate; Ct: Chromatid; Cs: Chromosome; mabs: Multiple aberrations; pvz: Pulverization; P: Polyploidy; H: Hyper diploid; E: Endoreduplication; Abs+g: Aberrations with gap; Abs-g: Aberrations without gap

TABLE 2- ANALYSIS OF STRUCTURAL ABERRATIONS

Experiment No.1: 3 + 17 h, +S9 mix

Treatment	Replicate	Cells scored	Cells with aberrations including gaps	Cells with aberrations excluding gaps	% of cells with structural aberration (excluding gap)	p-value (Fisher's exact test)	Statistical conclusion
Solvent control (RPMI medium)	A	150	3	2	1.33	NA	NA
	B	150	3	3	2.00		
	Total	**300**	**6**	**5**	**Mean 1.67**		
Test item extract (100%)	A	150	5	3	2.00	>0.9999	NS
	B	150	3	1	0.67		
	Total	**300**	**8**	**4**	**Mean 1.33**		
Positive control CPA; 12.5 µg/mL	A	63	14	11	17.46	<0.0001	S
	B	57	15	14	24.56		
	Total	**120**	**29**	**25**	**Mean 21.01**		

CPA= Cyclophosphamide, NA = not applicable, NS = Not Significant, S = Significant

Experiment No.2: 3 + 17 h, -S9 mix

Treatment	Replicate	Cells scored	Cells with aberrations including gaps	Cells with aberrations excluding gaps	% of cells with structural aberration (excluding gap)	p-value (Fisher's exact test)	Statistical conclusion
Solvent control (RPMI medium)	A	150	4	3	2.00	NA	NA
	B	150	3	2	1.33		
	Total	**300**	**7**	**5**	**Mean 1.67**		
Test item extract (100%)	A	150	3	1	0.67	0.7247	NS
	B	150	3	2	1.33		
	Total	**300**	**6**	**3**	**Mean 1.00**		
Positive control MMC; 0.80 µg/mL	A	58	14	13	22.41	< 0.0001	S
	B	57	15	13	22.81		
	Total	**115**	**29**	**26**	**Mean 22.61**		

MMC= Mitomycin C, NA = not applicable, NS = Not Significant, S = Significant

TABLE 2 (CONT.)- ANALYSIS OF STRUCTURAL ABERRATIONS

Experiment No.3: 20 + 0 h, -S9 mix

Treatment	Replicate	Cells scored	Cells with aberrations including gaps	Cells with aberrations excluding gaps	% of cells with structural aberration (excluding gap)		p-value (Fisher's exact test)	Statistical conclusion
Solvent control (RPMI medium)	A	150	3	2		1.33	NA	NA
	B	150	4	2		1.33		
	Total	**300**	**7**	**4**	**Mean**	**1.33**		
Test item extract (100%)	A	150	4	2		1.33	>0.9999	NS
	B	150	4	1		0.67		
	Total	**300**	**8**	**3**	**Mean**	**1.00**		
Positive control MMC; 0.80 µg/mL	A	56	13	13		23.21	< 0.0001	S
	B	53	11	11		20.75		
	Total	**109**	**24**	**24**	**Mean**	**21.98**		

MMC= Mitomycin C, NA = not applicable, NS = Not Significant, S = Significant

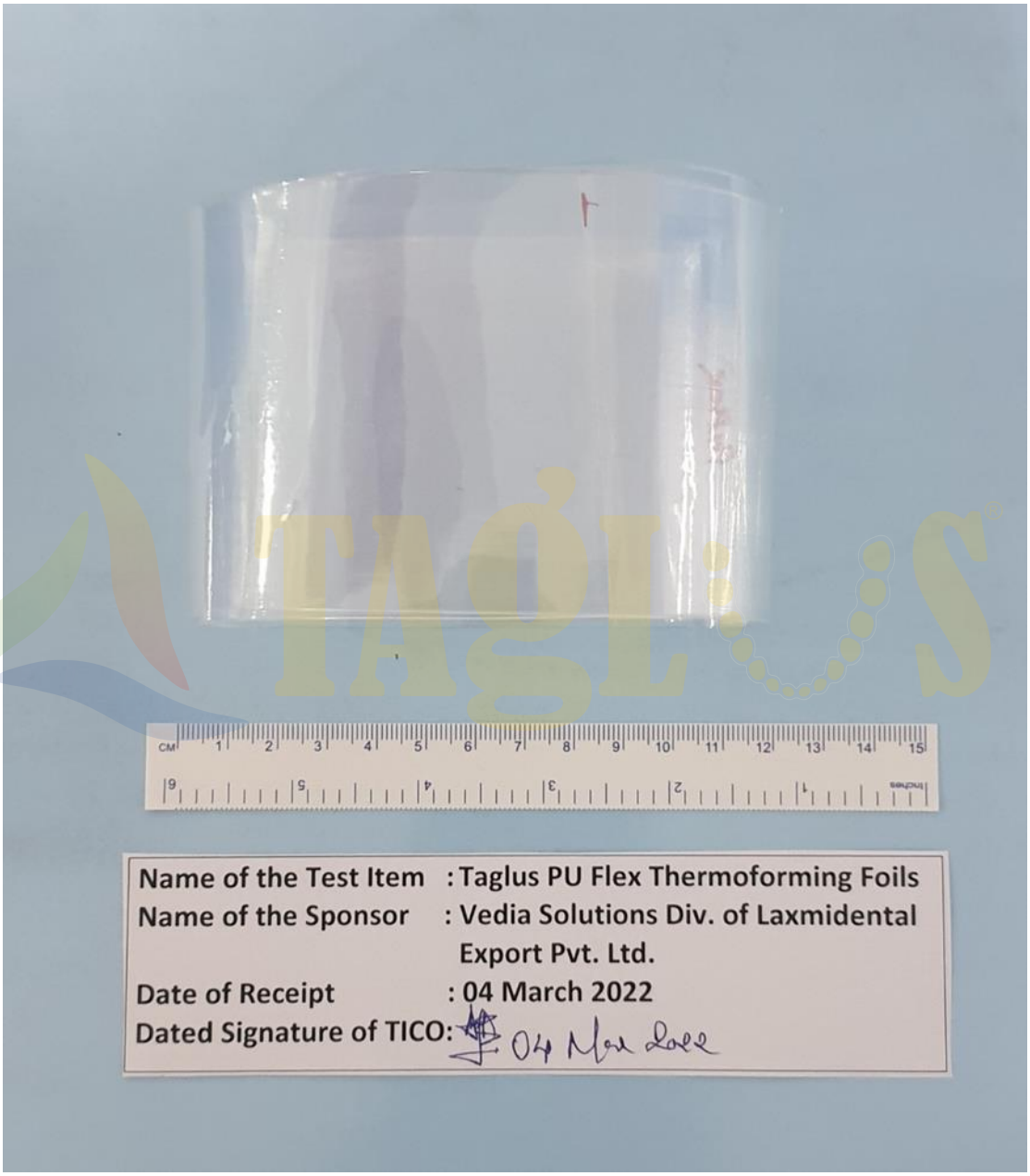
TABLE 3- HISTORICAL CONTROL DATA

Solvent control (RPMI medium) and positive controls

Controls	Treatment period	Percentage of cells with chromosome aberrations (excluding gaps)
		Mean ± 2SD
Solvent control (RPMI Medium)	3-h treatment, +S9 mix	0.3 ± 0.5
	3-h treatment, -S9 mix	0.5 ± 0.7
	20-h treatment, -S9 mix	0.5 ± 0.4
Positive controls	3-h treatment, +S9 mix (Cyclophosphamide,12.5 µg/mL)	15 ± 1.5
	3-h treatment, -S9 mix (Mitomycin C, 0.80 µg/mL)	20 ± 4.4
	20-h treatment, -S9 mix (Mitomycin C, 0.80 µg/mL)	18 ± 5.4

SD: Standard deviation
Data obtained from the studies performed in the year 2021.

REPRESENTATIVE PHOTOGRAPH OF THE TEST ITEM



RESPONSIBLE PERSONNEL

Ms. Ashwini Harke, MSc	Study Director
Dr. M. Fouziya Fathima, Pharm. D	Study Scientist
Ms. S. Koezhily, MSc	Study Scientist
Mr. K. Sakthivel, MSc	Phlebotomist

STATEMENT OF STUDY COMPLIANCE

This study was performed in compliance with:

- OECD Principles of Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17.
- US Food and Drug Administration's GLP regulations, 21 CFR Part 58 (subparts B to G and J).
- ISO/IEC 17025: 2017 (general requirements for the competence of testing and calibration laboratories).

All procedures were performed in accordance with GLR Laboratories Private Limited Standard Operating Procedures (SOPs). The study was subjected to Quality Assurance evaluation by the GLR Laboratories Private Limited Quality Assurance Unit (QAU) in accordance with SOPs.

STUDY PLAN AMENDMENT

One study plan amendment was made to change the representation of the metric units of the test item dimensions.

STUDY PLAN DEVIATION

No deviations occurred during the conduct of the study.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, slides, the study plan with its amendments and the final report will be retained for a period of 9 years after issue of the final report in the archives of GLR Laboratories Private Limited. The archived sample of test item will be retained for 2 years beyond its date of expiry. At the end of the archival

period the study sponsor will be contacted to determine whether the archived contents should be either retained for a further period, returned to the sponsor, or destroyed by GLR Laboratories as per in-house standard operating procedure in compliance with the principles of GLP. Sponsors will be notified of the financial implications, if any, of each of these options at that time.

DISTRIBUTION OF REPORTS

Two originals of the study report are prepared and distributed as mentioned below:

1. Sponsor.
2. Archive (GLR Laboratories Private Limited).

ANNEXURE 1

सत्यमेव जयते

GOVERNMENT OF INDIA
Department of Science and Technology
National Good Laboratory Practice (GLP) Compliance Monitoring Authority (NGCMA)

Certificate of GLP Compliance

This is to certify that

GLR Laboratories Private Limited
444, Gokulam Street, Mathur
Madhavaram, Chennai-600068 (Tamil Nadu), India

is a GLP certified test facility in compliance with the NGCMA's Document No. GLP-101 "Terms & Conditions of NGCMA for obtaining and maintaining GLP certification by a test facility" and OECD Principles of GLP.

The test facility conducts the below-mentioned tests/ studies:

- **Toxicity Studies**
- **Mutagenicity Studies**

The specific areas of expertise, test items and test systems are listed in the annexure overleaf.

Validity: March 13, 2020 – April 3, 2022

Certificate No. : GLP/C-132A/2020
Issue Date : 13-03-2020

(Dr. Neeraj Sharma)
Head, NGCMA

ANNEXURE 2

GLR Laboratories Pvt Ltd
committed to scientific & service excellence

Declaration of

Compliance to Principles of Good Laboratory Practice and GLP Certification status of GLR Laboratories

This is to declare that there is no change in the status of GLP certification of GLR Laboratories Private Limited.

The present 'Certification of GLP Compliance' of GLR Laboratories (Certificate Number: GLP/C-132A/2020) is valid up to 03 April 2022. As stated in the "Terms and Conditions of NGCMA for Obtaining and Maintaining GLP Certification by a Test Facility" (Document No.: GLP-101; Issue No.: 08; Issue Date: October 25, 2019) of the National GLP Compliance Monitoring Authority (NGCMA) of India (Department of Science and Technology, Government of India), the tenure of this certification is extendable up to three months, i.e., up to 03 July 2022, as GLR Laboratories has successfully completed the recertification inspection by the NGCMA during the dates 26 to 28 Mar 2022, well within the tenure of present certification. The renewed GLP compliance certificate of GLR Laboratories, based on the inspection and action taken report, will be issued by the NGCMA from the present validity period of 03 April 2022 extending up to the next three-year period, i.e., 02 April 2025, without any break or change in the tenure of GLP certification.

16 MAY 2022

(Dr. Parthiban Natarajan)
Head Quality Assurance & Assistant Director
GLR Laboratories Pvt Ltd.

16 May 2022

(Dr. S. S. Murugan)
Test Facility Management
GLR Laboratories Pvt Ltd.

Date: 16 May 2022

OECD-GLP | ISO/IEC 17025 | Drug Controller Approved Laboratory

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