FINAL REPORT

In vitro mammalian cell gene mutation test using the Thymidine Kinase Gene (Mouse Lymphoma Assay in L5178Y Cells) of Taglus PU Flex Thermoforming Foils as per ISO 10993-3:2014

STUDY CONTRACT PARTNER:
UL India Private Limited
Kalyani Platina, 3rd Floor, Block I, EPIP Zone, Phase II, Whitefield,
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.: 073/466

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 cell gene mutation test using the Thymidine Kinase Gene (Mouse Lymphoma Assay in L5178Y Cells) of Taglus PU Flex Thermoforming Foils as per ISO 10993-3:2014**

STUDY NUMBER:
073/466

TEST FACILITY:
GLR Laboratories Private Limited
444 Gokulam Street
Mathur, Chennai - 600 068
Tamil Nadu, India

REPORT ISSUED DATE: **31 May 2022**

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.

CONTENTS

STUDY DIRECTOR AUTHENTICATION STATEMENT 4
QUALITY ASSURANCE STATEMENT 5
TEST FACILITY MANAGEMENT STATEMENT 7
SUMMARY 8
INTRODUCTION 10
OBJECTIVE 11
STUDY DATES 11
TEST ITEM DETAILS 11
CONTROL ITEM DETAILS 12
TEST SYSTEM 12
TEST ITEM PREPARATION 14
TEST METHOD 14
ANALYSIS OF RESULTS 16
ACCEPTANCE CRITERIA 18
DATA EVALUATION 19
EVALUATION CRITERIA 19
RESULTS 19
CONCLUSION 20
REFERENCES 21
APPENDIX 1- HISTORICAL CONTROL DATA 25
PHOTOGRAPH OF THE TEST ITEM 26
RESPONSIBLE PERSONNEL 27
STATEMENT OF STUDY COMPLIANCE 27
STUDY PLAN AMENDMENT 27
STUDY PLAN DEVIATION 27
ARCHIVE STATEMENT 27
DISTRIBUTION OF REPORTS 28
ANNEXURE 1- QUALITY CONTROL AND PRODUCTION CERTIFICATE OF S9 MIX 29
ANNEXURE 2 30
ANNEXURE 3 31

STUDY DIRECTOR AUTHENTICATION STATEMENT

Study No. : **073/466**

Study Title : ***In vitro* mammalian cell gene mutation test using the Thymidine Kinase Gene (Mouse Lymphoma Assay in L5178Y Cells) of Taglus PU Flex Thermoforming Foils 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. : 073/466

Study Title : *In vitro* mammalian cell gene mutation test using the Thymidine Kinase Gene (Mouse Lymphoma Assay in L5178Y Cells) of Taglus PU Flex Thermoforming Foils 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, and 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/073/466/001)
2	Study Plan Verification	21 March 2022	Definitive Study Plan	21 March 2022 (SBI/073/466/002)
3	Inlife Phase Inspection	15 April 2022	Addition of test item extract to the cells (Main Experiment - I)	15 April 2022 (SBI/073/466/003)
4	Study Plan Verification	18 May 2022	Definitive Study Plan Amendment No.1	18 May 2022 (SBI/073/466/004)
5	Inlife Phase Inspection	21 May 2022	Scoring of TFT resistant mutant colonies (Main Experiment - II)	21 May 2022 (SBI/073/466/005)

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.)
6	Report Audit	25 May 2022	Draft Report	25 May 2022 (SBI/073/466/006)
7	Report Audit	31 May 2022	Final Report	31 May 2022 (SBI/073/466/007)

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. : 073/466

Study Title : *In vitro* mammalian cell gene mutation test using the Thymidine Kinase Gene (Mouse Lymphoma Assay in L5178Y Cells) of Taglus PU Flex Thermoforming Foils as per ISO 10993-3:2014

This is to certify that, the Test Facility Management appointed and provided the Study Director 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 ability to induce forward mutation at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells in the absence and presence of a rat liver metabolising system.

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).

Test item was extracted at a ratio of 6 cm^2/mL (as thickness of the test item was less than 0.5 mm) in RPMI medium supplemented with 10% heat inactivated horse serum (RPMI 10) at 37 ± 1 ºC for 72 h and 5 minutes, under aseptic condition. The total surface area of one test item is 441 cm^2 (as calculated in our laboratory). For main experiment 1 and 2, one test item (441 cm^2) was extracted in 73.5 mL of RPMI 10. Solvent (negative) control (RPMI 10) was also subjected to the same extraction conditions.

At the end of extraction, the extracts were clear without any colour change or particulates. No additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. No changes were observed in retrieved test items. Pre and post treatment pH of the extract were 7.62 and 7.73, respectively in main experiment 1 and 7.66 and 7.79, respectively in main experiment 2. The extract was used within 10 minutes of preparation and was considered stable during this time.

<u>Main experiment 1:</u>
Mouse lymphoma cell cultures were treated with 100 % test item extract for 3 h in the absence and presence of S9, to determine cytotoxicity (plating for viability) and mutant frequency. Duplicate cultures were set for test item, positive controls (Methyl Methane Sulphonate, MMS - 20 µg/mL, without S9 and Benzo[a]pyrene, B(a)P -3 µg/mL, with S9) and solvent (RPMI 10) control.

No cytotoxicity was observed in cultures treated with 100% test item extract. Mutant frequencies (MF) in solvent control cultures fell within acceptable ranges (118.05 and 110.65 mutants per 10^6 viable cells observed for 3 h treatment in the absence and presence of S9 respectively). Mutant frequencies observed in 100% test item extract in the absence and presence of S9 following 3 h treatment was within the normal range (95.30 x 10^6 and

72.82 x 10^6, respectively). Clear increases in mutation were induced by the positive control chemicals, MMS- 352.70 x 10^6 and B(a)P- 468.44 x 10^6.

As 100% test item extract did not induce any increase in mutant frequencies when treated for 3 h in the absence and presence of S9, the test item was further evaluated in the absence of S9 for 24 h (main experiment 2).

Main experiment 2:
Mouse lymphoma cell cultures were treated with 100 % test item extract for 24 h in the absence of S9, to determine cytotoxicity (plating for viability) and mutant frequency. Duplicate cultures were set for test item, positive (Methyl Methane Sulphonate, MMS - 20 µg/mL, without S9)] and solvent (RPMI 10) control.

No cytotoxicity was observed in cultures treated with 100% test item extract and mutant frequency was 66.95 x 10^6. Mutant frequencies (MF) in solvent control culture fell within acceptable ranges (95.12 mutants per 10^6 viable cells). Clear increase in mutation was induced by the positive control chemical, MMS– 547.27 x 10^6.

In both experiments 1 and 2 (3 h +/- S9; and 24 h without S9), the relative total growth (RTG) for the positive control was greater than 10% when compared to solvent control. The mean cloning efficiency (CE) of the negative control from the mutation experiment observed between 65% to 120%. Therefore, the study was considered valid. No increases in mutant frequencies compared to the concurrent solvent controls were observed at 100% test item extract. For the solvent controls, the proportion of small colony mutants in the absence and presence of S9 in main experiment 1 were 17.96% and 16.66% respectively, and 26.93% in main experiment 2. Marked increase in both small and large colony mutants were observed following treatment with the positive control chemicals MMS and B[a]P indicating a clear positive result.

Based upon the results obtained in this study and in line with ISO 10993-3:2014, it is concluded that under the test conditions, the given test item Taglus PU Flex Thermoforming Foils, supplied by Vedia solutions Div. of Laxmidental Export Pvt. Ltd., does not induce forward mutation in mouse lymphoma L5178Y cells both in the presence and absence of an exogenous metabolic activation system.

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 test selection necessary to evaluate the biocompatibility of medical devices.

The Mouse Lymphoma Tk Assay (MLA) is part of an *in vitro* test and one of the most commonly used mammalian cell mutagenesis system; the L5178Y $TK^{+/-}$ mouse lymphoma -TK assay detects the mutations at the thymidine kinase locus caused by base pair changes, frameshift and small deletions. Mutant cells, deficient in TK due to the forward mutation in the TK locus (from TK^{+} to TK^{-}), are resistant to the cytotoxic effect of pyrimidine analogues such as trifluorothymidine (TFT). The mutagenicity of the test agents is indicated by the increase in the number of mutants after treatment.

The mutation system works by placing treated cells under selective pressure so that only mutant cells are able to survive. The TK locus is autosomal and the L5178Y cell line is heterozygous $(TK^{+/-})$, producing the enzyme thymidine kinase. This enzyme is a salvage enzyme for nucleic acid breakdown products but if a toxic base analogue (5-trifluorothymidine) is present in the medium, the enzyme will incorporate the analogue into the cells. Thus, the cells die unless the enzyme is rendered inactive, by mutation. Resistance to 5-trifluorothymidine (TFT) results from a lack of thymidine kinase (TK) activity. Thus, the mutants $(TK^{+/-})$ are unable to use the toxic analogue and survive in its presence.

Two types of TFT-resistant mutant colonies are selected and these are designated as large and small (slow-growing) colonies. Molecular analysis has indicated that the large colonies tend to represent events within the gene (base-pair substitutions and deletions) whereas small colony mutants often involve large genetic changes frequently visible as chromosome aberrations. Thus, in this system, gene mutations within the *tk* gene (11 to 13 kilobases) and chromosomal events involving the gene may be detected. The TK system has a high spontaneous mutant frequency and because of the high numbers of cells that can be treated and sampled it is the most satisfactory mammalian cell mutation assay from the statistical point of view.

OBJECTIVE

To evaluate the potential of the test item to induce forward mutation at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells in the absence and presence of a rat liver metabolising system.

STUDY DATES

Study Start Date	21 March 2022
Experiment Start Date (Cell line retrieval from liquid nitrogen)	11 April 2022
Experiment Completion Date	22 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 used.

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 at 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 (Roswell Park Memorial Institute) medium with 10% horse serum (RPMI 10).

Justification for solvent used	Use of cell culture medium supplemented with 10% heat inactivated horse serum is recommended in ISO 10993-3:2014. This contains both polar and non-polar components.

Positive control

The positive control chemicals were used as shown in the following table:

Chemical	Source	Lot/batch no.	Expiry date	Stock concentration* (mg/mL)	Final concentration (µg/mL)	S9
Methyl Methane sulphonate (MMS)	Sigma- Aldrich	MKCD8572	24 December 2022	2.0	20	-
Benzo(a)pyrene (B(a)P)	Sigma Aldrich	BCBX0204	July 2022	0.3	3	+

*Solvent: dimethyl sulfoxide (DMSO).

The control items were handled with necessary protective clothing and all recommended safety measures were followed.

TEST SYSTEM

Cell Cultures

L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma cell line (L5178Y) derived from a methylcholanthrene-induced thymic lymphoma from a DBA-2 mouse was used for this study. L5178Y $TK^{+/-}$ mouse lymphoma cells were obtained from ATCC

(Lot no.70029119). The mycoplasma free cells were cryopreserved in liquid nitrogen (-196 ^{o}C) until the commencement of the experiment. Vial no. P-3-5 was used for main experiment 1 and Vial no. P-3-9 was used for main experiment 2. The vial was thawed rapidly, the cells diluted in RPMI 10 and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air. When cells were growing well, subcultures were established in an appropriate number of flasks.

The test system was suitably labelled to clearly identify the study number, test item, positive and negative control groups.

Metabolic activation system

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.: 4490, Expiry Date: August 20, 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 (Annexure 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 KCl.

Growth media

Three types of RPMI 1640 medium were prepared as follows:

Growth media Composition	**Make, Lot /Batch No. and Expiry**	**Final concentration in:**		
RPMI medium	Himedia, Lot No.:0000491273 Expiry Date: July 2024	**RPMI A**	**RPMI 10**	**RPMI 20**
Horse serum (heat inactivated)	Thermo Fisher Scientific Lot No.:2382770 Expiry Date: May 2023	0%	10%	20%
Penicillin / Streptomycin/ Amphotericin B	Lonza Lot No.:20F305302 Expiry Date: January 2023	100 Units/mL / 100 µg/mL/ 2.5 µg/mL	100 Units/mL / 100 µg/mL/ 2.5 µg/mL	100 Units/mL / 100 µg/mL/ 2.5 µg/mL
Pluronic (0.5 mg/mL)	ThermoFisher scientific Lot No.:2337221 Expiry Date: November 2022	0.1%	0.1%	-

Heat inactivated horse-serum was used in order to eliminate a factor which degrades TFT.

TEST ITEM PREPARATION

Test item was extracted at a ratio of 6 cm^2/mL (as thickness of the test item was less than 0.5 mm) in RPMI medium supplemented with 10% heat inactivated horse serum (RPMI 10) at 37 ± 1 ºC for 72 h and 5 minutes, under aseptic condition. The total surface area of one test item is 441 cm^2 (as calculated in our laboratory). Solvent (negative) control (RPMI 10) was also subjected to the same extraction conditions. This fulfilled the requirement of ISO 10993-12:2012 and ISO 10993-12:2021.

The details of extracts preparation are as follows:

Experiment	Extraction vehicle	Surface area (cm^2)	Volume of vehicle (mL)	Extract preparation start time	Extract preparation end time	Condition of extracts	pH
Main Experiment 1	RPMI 10	441[#]	73.5	09:35 a.m. on 12 April 2022	09:40 a.m. on 15 April 2022	Clear solution; no particulates and colour change	7.62 (before extraction) and 7.73 (after extraction)
Main Experiment 2	RPMI 10	441[#]	73.5	12:05 a.m. on 03 May 2022	12:10 a.m. on 06 May 2022	Clear solution; no particulates and colour change	7.66 (before extraction) and 7.79 (after extraction)

[#]One (1) test item was used for each extraction.

No additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. No change was observed in retrieved test item. The extract was used within 10 minutes of preparation and was considered stable during this time.

TEST METHOD

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), experiment number, with/without S9 mix, test item/positive/negative control.

Main experiment 1

Main experiment was performed both in the absence and presence of S9 mix (3 h treatment) with neat extract (100%). As per ISO 10993-33:2015 - Supplement to ISO 10993-3:2014 the recommended maximum test concentration is 100%.

Concentrations selected for main experiment 1 and 2

3 h, -S9	3 h, +S9	24 h, -S9
Solvent	Solvent	Solvent
100% test item extract	100% test item extract	100% test item extract
MMS, 20 µg/mL	B(a)P, 3 µg/mL	MMS, 20 µg/mL

For main experiment 1 in the absence and presence of S9 (3-h treatment), approximately 10^6 cells were resuspended in 20 mL of medium containing 2 mL of 100% test item extract/0.2 mL of positive control and 1 mL of S9 mix or KCl. The cultures were incubated at 37 ± 1 °C for 3 h.

The test item extract (100 %) was administered to the test system within 10 minutes of preparation and were considered stable during this time. Following 3 h incubation at 37 ± 1 °C, cultures were centrifuged at 1000 rpm for 5 minutes, supernatant removed and resuspended further in 20 mL RPMI 10/tube until next day (24 h).

At the end of the 24 h, all cultures (3 h) were mixed gently and D_0 cell counts were taken; the cell density was adjusted to 2 x 10^5 cells/mL. All cultures were incubated for a further 21 h.

At the end of the Day 2, all cultures (3 h) were mixed gently and D_2 cell counts were taken; the cell density was adjusted to 1 x 10^4 cells/mL and plated for (a) viability (to determine cytotoxicity from relative total growth) and (b) TFT resistance (to determine mutant frequency).

a. Plating for viability

Samples from the above were diluted to 8 cells/mL as follows:

	Initial concentration (cells/mL)	Dilution (mL)		Intermediate concentration (cells/mL)	Dilution (mL)		Final concentration (cells/mL)
	(A)	(A)	Medium	(B)	(B)	RPMI 20	(C)
Viability	1 x l0^4	0.5	9.5	5 x 10^2	0.8	49.2	8

Using a multichannel pipette, 0.2 mL of culture (From C - 8 cells/mL) was placed into each well of a 96-well microtiter plate (at an average of 1.6 cells per well). The plates were incubated at 37 ± 1 ºC with 5% CO_2 until scoreable (8 days for main experiment 1 and 7 days for main experiment 2). Wells containing viable clones were identified and counted macroscopically.

b. Plating for TFT resistance

The cell count of the cultures was adjusted to 1 x 10^4 cells/mL as per the calculations showed in plating for viability section. TFT (300 µg/mL) was diluted into these suspensions to give a final concentration of 3 µg/mL. TFT acts as the selective agent(s) for determination of numbers of mutants.

Using a multichannel pipette, 0.2 mL of culture was placed into each well of four 96-well microtiter plates (384 wells at 2 x 10^3 cells/well/culture). Plates were incubated at 37 ± 1 °C with 5% CO_2 until scoreable (12 days) and wells containing clones were identified and counted macroscopically.

In addition, the number of wells containing small and large colonies were scored for the negative and positive controls. The colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies (the small colony mutant detection (by Mouse Lymphoma Assay in L5178Y Cells) was validated in our laboratory separately. Small colonies are defined as less than a quarter of the diameter of the well, while large colonies are more than a quarter of the diameter of the well.

Main experiment 2

As the test item extract did not induce any mutation when treated for 3 h in the absence and presence of S9, the test item extract (100%) was evaluated without using metabolic activation for 24 h treatment period in main experiment 2. The test item extract was administered to the test system within 10 minutes of preparation and were considered stable during this time. The methodology was similar to that described above. No cytotoxicity was observed in 100% test item extract.

ANALYSIS OF RESULTS

Treatment of data

All calculations were performed manually.

Suspension Growth (SG) was a measure of the growth in suspension during treatment and the expression period.

Suspension Growth (SG) was calculated as follows:

Suspension growth = a x b x c

$$\text{Where a} = \left(\frac{D_0 \text{ post - treatment cell count}}{\text{Pre - treatment cell density}} \right)$$

$$\text{Where b} = \left(\frac{D_1 \text{ cell count}}{\text{Cell count set up on } D_0 \text{ post - treatment}} \right)$$

$$\text{Where c} = \left(\frac{D_2 \text{ cell count}}{\text{Cell count set up on } D_1} \right)$$

Note: for three-hours treatments a is assumed to equal 1

Usually the denominators for b and c are 2 x 10^5 cells/mL. However, if cytotoxicity causes the cell count to be lower than 2 x 10^5 cells/mL following treatment and/or if the cells do not grow during part of the expression period, it can be lower. In these cases, the respective cell count values were entered into the calculation above.

Relative suspension growth (RSG) was a measure of the growth in suspension during treatment and the expression period relative to the mean control.

Relative suspension growth (RSG) was calculated as follows:

$$\text{RSG (\%)} = \left(\frac{\text{Individual SG value}}{\text{Mean control SG value}} \right) \text{x } 100$$

Viability was the measure of the cells ability to clone i.e. Cloning efficiency (CE).

Cloning Efficiency (CE) is calculated as follows:
For microtitre plate tests, calculations are based on P(0), the proportion of wells in which a colony has not grown:

$$\text{P (0)} = \left(\frac{\text{Number of wells with no colony}}{\text{Total number of wells}} \right)$$

The Cloning Efficiency (CE) for each culture was calculated according to the following calculation:

$$\text{CE} = \left(\frac{\text{-In P (0)}}{\text{Number of cells per well *}} \right) \text{x } 100$$

* Number of cells per well was 1.6 cells per well on average on all viability plates

Relative Total Growth (RTG) is the measure of cytotoxicity relative to the control, that takes into account all cell growth and cell loss during the treatment period and the 2-day expression period (RSG), and the cells' ability to clone 2 days after treatment (viability).

Relative Total Growth was calculated as follows:

$$RTG = RSG \times \left(\frac{\text{Individual Viability Value}}{\text{Mean Control Viability Value}} \right)$$

Mutant frequency was calculated as follows:

$$MF = \left(\frac{- \text{ In P (0) for mutant plates}}{\text{Number of cells per well* x (viability/100)}} \right)$$

* Number of cells per well was 2000 cells per well on average on all mutant plates.

Small and large colony mutant frequencies was calculated in an identical manner, using the relevant number of empty wells for small and large colonies, as appropriate.

The increases in mutant frequencies (total wells with clones), by comparison with concurrent controls, was carried out. The control mutant frequency was compared with each test item extract treatment.

ACCEPTANCE CRITERIA

The assay is considered valid as all the following criteria are met:

1. The mean mutant frequencies in the negative (solvent) control cultures fell within the normal range (50 to 170 mutants per 10^6 viable cells).
2. At least one positive control showed an absolute increase in mean total MF of at least 300 x 10^{-6} (at least 40% of this should be in the small colony MF).
3. The RTG for the positive controls was greater than 10%.
4. The mean CE of the negative controls from the Mutation Experiments was between the range 65% to 120% on Day 2.

5. The mean suspension growth of the negative controls from the mutation experiments was between the range 8 to 32 following 3-hour treatments or between 32 and 180 following 24-hour treatments.

DATA EVALUATION

Individual plate counts from all experiments was recorded separately (SG, RSG, CE, RTG and MF). Control counts was compared with the accepted normal ranges from our laboratory for numbers of spontaneous revertant on solvent control plates. As the data from our laboratory was consistent with ranges of spontaneous revertant per plate, it was considered acceptable elsewhere.

EVALUATION CRITERIA

The test article was considered to be mutagenic in this assay if:

1. The assay is valid
2. A significant increase in MF in one or more doses is considered as a positive response.
3. Any observed response was reproducible under the same treatment conditions.

RESULTS

The individual plate counts, cytotoxicity and mutant detection observed for main experiment 1 and 2 are shown in Table 1 – Table 3. Historical data is given in Appendix 1.

Main experiment 1 (3 h, +/- S9)

No cytotoxicity was observed in cultures treated with 100% test item extract. Mutant frequencies (MF) in solvent control cultures fell within acceptable ranges (118.05 and 110.65 mutants per 10^6 viable cells observed for 3 h treatment in the absence and presence of S9 respectively). Mutant frequencies observed in 100% test item extract in the absence and presence of S9 following 3 h treatment was within the normal range (95.30 x 10^6 and 72.82 x 10^6, respectively). Clear increases in mutation were induced by the positive control chemicals, MMS- 352.70 x 10^6 and B(a)P- 468.44 x 10^6.

As 100% test item extract did not induce any increase in mutant frequencies when treated for 3 h in the absence and presence of S9, the test item was further evaluated in the absence of S9 for 24 h (main experiment 2).

Main experiment 2 (24 h, -S9)

No cytotoxicity was observed in cultures treated with 100% test item extract and mutant frequency was 66.95 x 10^6. Mutant frequencies (MF) in solvent control culture fell within acceptable ranges (95.12 mutants per 10^6 viable cells). Clear increase in mutation was induced by the positive control chemical, MMS– 547.27 x 10^6.

In both experiments 1 and 2 (3 h +/- S9; and 24 h without S9), the relative total growth (RTG) for the positive control was greater than 10% when compared to solvent control. The mean cloning efficiency (CE) of the negative control from the mutation experiment observed between 65% to 120%. Therefore, the study was considered valid. No increases in mutant frequencies compared to the concurrent solvent controls were observed at 100% test item extract. For the solvent controls, the proportion of small colony mutants in the absence and presence of S9 in main experiment 1 were 17.96% and 16.66% respectively, and 26.93% in main experiment 2. Marked increase in both small and large colony mutants were observed following treatment with the positive control chemicals MMS and B[a]P indicating a clear positive result.

CONCLUSION

Based upon the results obtained in this study and in line with ISO 10993-3:2014, it is concluded that under the test conditions, the given test item Taglus PU Flex Thermoforming Foils, supplied by Vedia solutions Div. of Laxmidental Export Pvt. Ltd., does not induce forward mutation in mouse lymphoma L5178Y cells both in the presence and absence of an exogenous metabolic activation system.

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 Guideline for Testing of Chemicals (No.490, *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, 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. ISO/IEC 17025:2017: General Requirements for the Competence of Testing and Calibration Laboratories.

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, June 16, 2016.

Table 1

Individual plate counts, cytotoxicity and mutation detection in main experiment 1 (3 h treatment, -S9)

Plating for viability

Dose (%)	Cell counts (x 10^5 cells/per mL)			SG	RSG	P(0)	CE /viability	RTG
	Day 0	Day 1	Day 2					
Solvent (A)	2.00	9.03	9.35	21.10	99.51	0.18	106.38	101.17
Solvent (B)	2.00	9.05	9.42	21.31	100.48	0.19	102.91	98.82
100% (A)	2.00	9.11	9.39	21.38	100.82	0.30	75.90	73.13
100% (B)	2.00	9.07	9.24	20.95	98.78	0.30	74.82	70.62
MMS (A)	2.00	8.97	9.19	20.60	97.16	0.26	85.35	79.25
MMS (B)	2.00	9.01	9.15	20.61	97.17	0.24	87.96	81.68

SG - Suspension growth, RSG - Relative suspension growth, CE- Cloning Efficiency, RTG -Relative total growth

Dose (%)	Number of wells with colonies	Number of wells scored
Solvent (A)	157	192
Solvent (B)	155	192
100% (A)	135	192
100% (B)	134	192
MMS (A)	143	192
MMS (B)	145	192

Plating for TFT resistance

Dose (%)	Number of wells scored	Number of wells with colonies				Total number of wells with colonies	Small colonies	Large colonies	P(0)	Mutant Frequency (x 10^6)
		A	B	C	D					
Solvent (A)	382	20	22	19	23	84	16	68	0.78	116.71
Solvent (B)	381	19	23	21	20	83	14	69	0.78	119.38
Mean										**118.05**
100% (A)	381	14	12	13	11	50	12	38	0.87	94.02
100% (B)	379	12	13	11	15	51	15	36	0.86	96.59
Mean										**95.30**
MMS (A)	380	43	43	44	43	173	123	50	0.54	355.84
MMS (B)	381	44	42	44	45	175	127	48	0.54	349.55
Mean										**352.70**

Table 2

Individual plate counts, cytotoxicity and mutation detection in main experiment 1 (3 h treatment, +S9)

Plating for viability

Dose (%)	Cell counts (x 10^5 cells/per mL)			SG	RSG	P(0)	CE /Viability	RTG
	Day 0	Day 1	Day 2					
Solvent (A)	2.00	9.15	9.45	21.60	99.73	0.18	106.38	98.04
Solvent (B)	2.00	9.17	9.48	21.70	100.27	0.17	110.06	101.97
100% (A)	2.00	8.92	9.21	20.50	94.76	0.26	85.35	74.73
100% (B)	2.00	9.02	9.23	20.80	96.03	0.24	87.96	78.05
B(a)P (A)	2.00	9.34	9.63	22.50	103.74	0.30	75.90	72.76
B(a)P (B)	2.00	9.27	9.58	22.20	102.43	0.29	78.14	73.95

SG - Suspension growth, RSG - Relative suspension growth, CE- Cloning Efficiency, RTG -Relative total growth

Dose (%)	Number of wells with colonies	Number of wells scored
Solvent (A)	157	192
Solvent (B)	159	192
100% (A)	143	192
100% (B)	145	192
B(a)P (A)	135	192
B(a)P (B)	137	192

Plating for TFT resistance

Dose (%)	Number of wells scored	Number of wells with colonies				Total number of wells with colonies	Small colonies	Large colonies	P(0)	Mutant Frequency (x 10^6)
		A	B	C	D					
Solvent (A)	381	20	22	21	19	82	14	68	0.78	113.90
Solvent (B)	380	21	20	19	20	80	13	67	0.79	107.38
Mean										**110.65**
100% (A)	379	10	11	11	12	44	11	33	0.88	72.28
100% (B)	380	11	10	13	12	46	12	34	0.88	73.34
Mean										**72.82**
B(a)P (A)	378	51	46	48	50	195	114	81	0.48	477.85
B(a)P (B)	377	50	46	46	51	193	116	77	0.49	459.01
Mean										**468.44**

Table 3

Individual plate counts, cytotoxicity and mutation detection in main experiment 2 (24 h treatment, -S9)

Plating for viability

Dose (%)	Cell counts (x 10^5 cells/per mL)			SG	RSG	P(0)	CE /viability	RTG
	Day 0	Day 1	Day 2					
Solvent (A)	8.23	8.45	8.77	76.24	98.47	0.18	108.20	99.30
Solvent (B)	8.31	8.57	8.83	78.61	101.53	0.18	106.38	100.67
100% (A)	8.19	8.29	8.49	72.05	93.07	0.22	93.54	81.12
100% (B)	8.21	8.35	8.53	73.10	94.41	0.23	90.68	79.79
MMS (A)	8.48	8.69	8.98	82.72	106.84	0.30	74.82	74.50
MMS (B)	8.55	8.75	9.13	85.38	110.28	0.29	78.14	80.31

SG - Suspension growth, RSG - Relative suspension growth, CE- Cloning Efficiency, RTG -Relative total growth

Dose (%)	Number of wells with colonies	Number of wells scored
Solvent	158	192
Solvent	157	192
100%	149	192
100%	147	192
MMS	134	192
MMS	137	192

Plating for TFT resistance

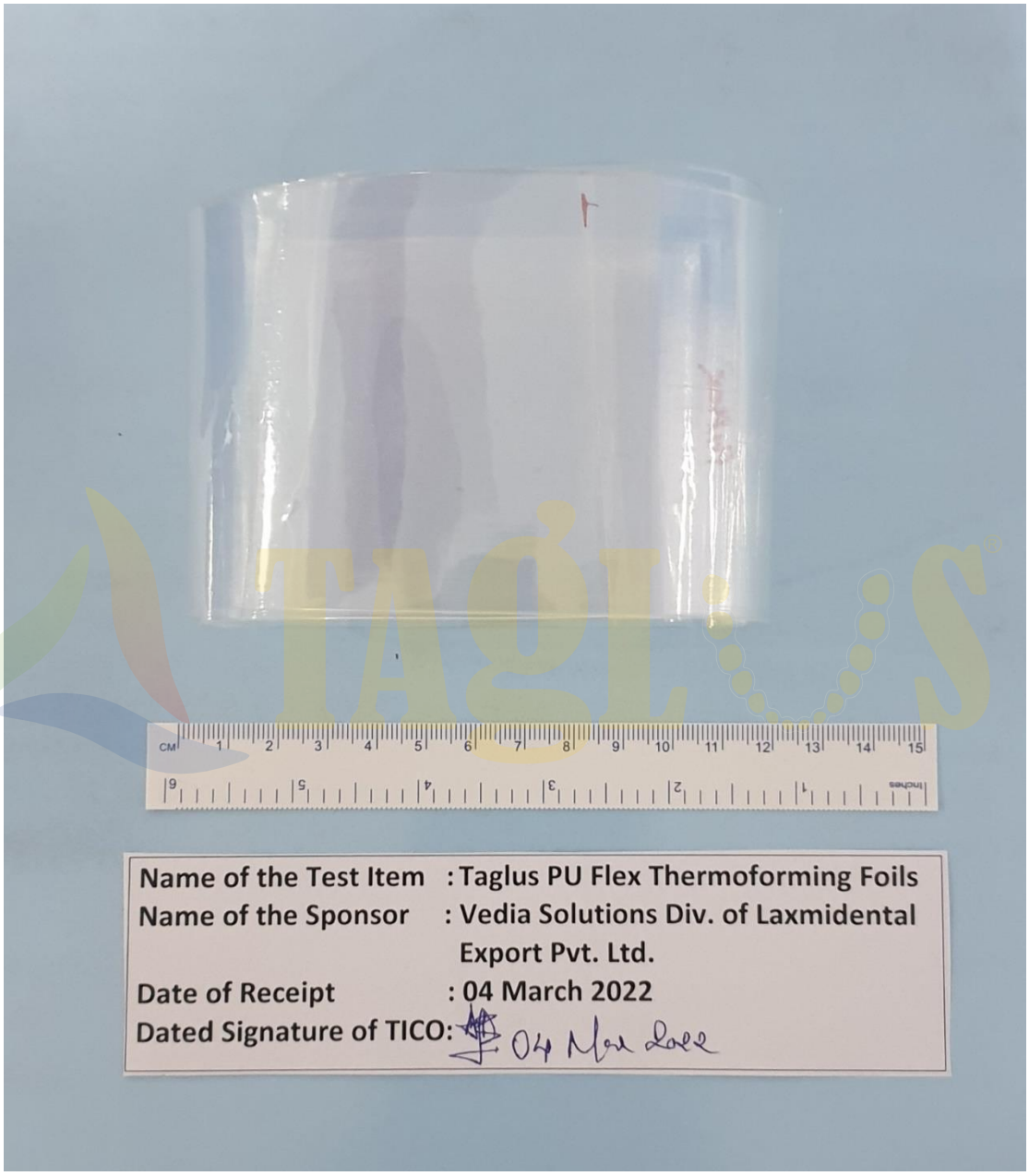
Dose (%)	Number of wells scored	Number of wells with colonies				Total number of wells with colonies	Small colonies	Large Colonies	P(0)	Mutant Frequency (x 10^6)
		A	B	C	D					
Solvent (A)	383	15	18	17	19	69	18	51	0.82	91.80
Solvent (B)	381	19	17	19	17	72	20	44	0.81	98.44
Mean										**95.12**
100% (A)	380	13	09	10	11	43	13	30	0.89	64.21
100% (B)	379	10	13	10	12	45	12	33	0.88	69.70
Mean										**66.95**
MMS (A)	378	51	53	55	57	216	121	95	0.43	566.25
MMS (B)	379	53	51	53	56	213	122	91	0.44	528.28
Mean										**547.27**

APPENDIX 1- HISTORICAL CONTROL DATA

3 h - without S9		
	Solvent (RPMI medium)	Positive Control (MMS, 20 µg/mL)
Average MF value	106.72	388.87
Standard deviation	15.16	18.63
Minimum value	92.43	364.39
Maximum value	132.15	416.82
3 h - with S9		
	Solvent (RPMI medium)	Positive Control (B(a)P, 3 µg/mL)
Average MF value	113.23	531.03
Standard deviation	19.98	100.7
Minimum value	98.31	447.41
Maximum value	147.46	702.24
24 h - without S9		
	Solvent (RPMI medium)	Positive Control (MMS, 20 µg/mL)
Average MF value	99.27	488.09
Standard deviation	16.69	72.49
Minimum value	81.34	376.50
Maximum value	117.90	560.35

Data obtained from the studies performed in the year 2021.

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

STATEMENT OF STUDY COMPLIANCE

The study will be 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 will be performed in accordance with GLR Laboratories Private Limited standard operating procedures (SOPs). The study will be 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 study plan deviation occurred during the conduct of the study.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, the study plan with its amendments (if any) 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- QUALITY CONTROL AND PRODUCTION CERTIFICATE OF S9 MIX

MOLTOX®
Molecular Toxicology, Inc.

10% Mutazyme
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
AGE: 5 – 6 weeks
WEIGHT: 175 – 199 g
TISSUE: Liver

Part Number Information
LOT NO.: 4490
PART NO.: 11-402L
VOLUME: 20 mL
STORAGE: At or below -20°C

PREP DATE: August 20, 2021
EXPIRY: August 20, 2023
INDUCING AGENT: Aroclor 1254, (Monsanto KL615), 500 mg/kg i.p

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983. **For Research Purposes Only**

BIOCHEMISTRY:
- PROTEIN: 3.6 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
BROD	2B1, 2B2	58.4
EROD	1A1, 1A2	88.7
MROD	1A1, 1A2	57.2
PROD	2B1, 2B2	30.4

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/ mg protein) were 50.8, 46.2, 22.0 & 17.6 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:
- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microorganisms 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 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
210.8	1198

The ability of the sample to activate ethidium bromide (EtBr) 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 0.6 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted 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))	110	307	527	805	1009	1092
2-AA (2.5 µg)	84	1694	2022	2145	1705	1220

Approved: 08/27/21

MOLECULAR TOXICOLOGY, INC. www.moltox.com (828) 264-9099

ANNEXURE 2

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 3

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