FINAL REPORT

Bacterial Reverse Mutation (AMES) test of Taglus Premium Thermoforming Foils as per ISO 10993-3:2014

STUDY CONTRACT PARTNER:
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UL Project Number: 4790342013

TEST FACILITY:
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444 Gokulam Street, Mathur, Chennai - 600 068, Tamil Nadu, India.
Study No: 073/459

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: 04 May 2022

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PRODUCT NAME:

Taglus Premium Thermoforming Foils

STUDY TITLE

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CONTENTS

STUDY DIRECTOR AUTHENTICATION STATEMENT

Study No. : **073/459**

Study Title : **Bacterial Reverse Mutation (AMES) test of Taglus Premium Thermoforming Foils as per ISO 10993-3:2014**

This study was performed in accordance with the mutually agreed study plan 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	04 May 2022 Study Completion Date

*The identity and composition of the test item were the responsibilities of the sponsor.

QUALITY ASSURANCE STATEMENT

Study No. : **073/459**

Study Title : **Bacterial Reverse Mutation (AMES) test of Taglus Premium 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, 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 No.)
1	Study Plan Verification	14 March 2022	Draft Study Plan	14 March 2022 (SBI/073/459/001)
2	Study Plan Verification	21 March 2022	Definitive Study Plan	21 March 2022 (SBI/073/459/002)
3	In-life Phase Inspection	19 April 2022	Addition of Test Item Extracts to Cells	19 April 2022 (SBI/073/459/003)
4	In-life Phase Inspection	22 April 2022	Scoring of Revertant Colonies	22 April 2022 (SBI/073/459/004)
5	Report Audit	28 April 2022	Draft Report	28 April 2022 (SBI/073/459/005)
6	Report Audit	04 May 2022	Final Report	04 May 2022 (SBI/073/459/006)

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 — 04 MAY 2022

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

Date

TEST FACILITY MANAGEMENT STATEMENT

Study No. : 073/459

Study Title : Bacterial Reverse Mutation (AMES) test of Taglus Premium Thermoforming Foils 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.

Dr. S. S. Murugan, PhD
Test Facility Management
Managing Director
GLR Laboratories Private Limited

Date

SUMMARY

The mutagenic potential of the test item, Taglus Premium Thermoforming Foils, supplied by Vedia Solutions Div. of Laxmidental Export Pvt. Ltd. was evaluated by examining its ability to revert histidine - requiring five strains of *Salmonella typhimurium* in the absence and presence of an exogenous metabolic activation system.

The test item, Taglus Premium Thermoforming Foils is a transparent circular disk with a diameter, 125 mm and thickness, 0.8 mm (as stated by sponsor). 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 3 cm^2/ mL (as the thickness of the test item was greater than 0.5 mm) in the polar (physiological saline) and non-polar (DMSO - dimethyl sulfoxide) solvents, respectively at 37 ± 1 °C for 71 h and 15 minutes. The total surface area of the test item is 245 cm^2 (as calculated in our laboratory). Polar extract was prepared by extracting one test item (245 cm^2) in 81.67 mL of physiological saline. Similarly, non-polar extract was prepared by extracting one test item (245 cm^2) in 81.67 mL of DMSO. Solvent controls were also subjected to similar extraction conditions. This fulfilled the requirement of ISO 10993-12:2012 and ISO 10993-12:2021.

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. After extraction, all extracts were transferred to sterile containers and added to the test system within 3 h.

Suspensions of bacterial cells were exposed to the neat extracts of the test item and controls both in the presence and absence of S9 mix, using the direct plate incorporation method. Triplicates were maintained for all treatments and controls. Frequencies of revertant colonies were evaluated in all treatments after an incubation period of 70 h and 45 minutes.

The mean number of revertant colonies in negative (solvent) control were similar to historical data of the laboratory. The positive control induced an increase in the number of revertant colonies when compared to negative (solvent) control; the average revertant colonies were similar to historical data of the laboratory.

No cytotoxicity (clearing or diminution of the background lawn or reduction in revertant numbers) was observed in plates treated with the neat extract of the test item in polar and non-polar solvents, with and without S9 mix, in any of the tester strains. The mean number of revertant colonies observed in these plates were comparable to the negative (solvent) control and historical data of the laboratory.

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 Premium Thermoforming Foils supplied by Vedia Solutions Div. of Laxmidental Export Pvt. Ltd. is non-mutagenic in the bacterial reverse mutation (AMES) test.

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 bacterial reverse mutation test (Ames test) is a rapid, reliable and economical method of evaluating the mutagenic potential of a test article by measuring genetic activity in one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of an exogenous metabolic activation system. A large database has been accumulated with this assay, confirming its ability to detect genetically active compounds of most chemical classes with around 80-90% sensitivity and specificity.

The following bacterial strains were used in this study:

Organism	Strain	Type of mutation in the histidine gene
S. typhimurium	TA98	frame-shift
S. typhimurium	TA100	base-pair substitution
S. typhimurium	TA1535	base-pair substitution
S. typhimurium	TA1537	frame-shift
S. typhimurium	TA102	base-pair substitution

With the exception of strain TA102, these strains require biotin as well as histidine for growth. In strain TA102, the critical mutation in the histidine gene is located on a multicopy plasmid pAQ1. This strain is particularly sensitive to the activities of oxidative and cross-linking mutagens. The pKM101 plasmid derivatives (TA98, TA100 and TA102) have increased sensitivity to certain mutagens as the pKM101 plasmid codes for an error-prone DNA repair system.

When exposed to a mutagen, some of the bacteria in the treated population undergo genetic changes which revert them to a non-histidine-requiring state, and they can then grow without exogenous histidine. Different tester strains are used because each strain is mutated by particular chemical classes of compound. A compound that is mutagenic in one strain need not be so in another.

OBJECTIVE

To evaluate the mutagenic potential of the test item by the bacterial reverse mutation test using *Salmonella typhimurium* tester strains.

STUDY DATES

Study Start Date	21 March 2022
Experiment Start Date	16 April 2022
Experiment Completion Date	22 April 2022

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

TEST AND CONTROL ITEM DETAILS

The test item, Taglus Premium Thermoforming Foils was received at GLR Laboratories Private Limited, 02 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 at GLR Laboratories Private Limited, to confirm it.

Test Item	Taglus Premium Thermoforming Foils
Batch / Lot No.	22022080-1
Manufacture Date	02 February 2022
Expiry Date	02 February 2025
Appearance	Transparent circular disk
Ingredients	PETG (Polyethelene Tertamethylene Glycol)
Temperature Stability	37 °C
Sterility	Non-sterile
Handling Procedure	The test item was handled with all necessary protective clothing and all recommended safety and sterile measures were followed.

Description of the test item

The test item, Taglus Premium Thermoforming Foils is a transparent circular disk with a diameter, 125 mm and thickness, 0.8 mm (as stated by sponsor). 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).

Negative (solvent) control

Negative controls comprised of the treatments with the polar solvent (physiological saline) and non-polar solvent (DMSO - dimethyl sulfoxide) at the same volume per plate (0.1 mL) as the test item extracts. The details of the solvents are as follows.

Physiological saline (0.9% w/v sodium chloride solution)

Manufacturer	Eurolife Healthcare Private Limited
Batch no.	10210671B
Expiry date	September 2024
Appearance	Clear colourless solution

Dimethyl sulfoxide (DMSO)

Manufacturer	Sigma Aldrich
Lot no.	SHBM0179
Expiry date	18 November 2025
Appearance	Clear colourless solution

Positive control

The positive control chemicals as per table (mutagens) was used as shown in the following table (0.1 mL per plate):

Assay	Mutagen	Lot/Batch no.	Expiry/Retest Date	Solvent	Conc. (µg)	Strains
Without metabolic activation system (-S9 mix)	Sodium azide	MKCB6155	10 Aug 2022	Sterile distilled water	1.0	TA100, TA1535
	2- Nitrofluorene	S43858	10 Aug 2022	DMSO	10.0	TA98
	9-Aminoacridine	BCBK1177V	10 Aug 2022	Ethanol	50.0	TA1537
	Mitomycin-C	SLCB4710	May 2023	Sterile distilled water	0.5	TA102
With metabolic activation system (+S9 mix)	Benzo(a)pyrene	BCBX0204	July 2022	DMSO	5.0	TA100, TA98
					10.0	TA102, TA 1535 & TA1537

Conc. : Concentration

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

TEST SYSTEM

Bacteria

Species	*Salmonella typhimurium*
Strains	TA98, TA100, TA1535, TA1537 and TA102
Source	Molecular Toxicology Incorporated, USA.
Growth medium	Oxoid nutrient broth no. 2
Growth condition	37 ± 1 °C (10 h in shaker water bath at 120 rpm)
Number of cells	10^9 cells/culture

The phenotypic characteristics of tester strains were checked for the frozen stock culture. The bacterial cultures were revived from frozen stocks preserved in liquid nitrogen. The test system was suitably labelled for clear identification.

Medium

A minimal agar containing Vogel-Bonner minimal medium E and glucose; and an overlay agar containing histidine-biotin solution were used.

Metabolic activation system (S9 mix)

Treatment was carried out both in the absence and presence of a 10% mutazyme (Make: Moltox, Lot No.: 4474, Expiry Date: 15 July 2023) (pre-mixed) 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. Cultures treated in the absence of S9 mix had received an equivalent volume of sodium phosphate buffer solution. (Make: HiMedia, Lot No.: 0000455755, Expiry Date: November 2022). The quality control and production certificate of 10% mutazyme used, is included in the report (annexure 2).

Preparation of media and reagents

Histidine and biotin preparation

About 5 mL L-Histidine (Make: Sigma-Aldrich, Lot No.: SLCB4332, Expiry Date: November 2025) and 5 mL D-Biotin (Sigma-Aldrich, Lot No: SLCF3557, Expiry Date: January 2024) solutions were prepared. About 1.5 mL of L-Histidine and

1.83 mL of D-Biotin solution was added to both sodium phosphate buffer solution and 10% Mutazyme.

Overlay (top) agar

Top agar (0.5% of Bacto agar, 0.5% of NaCl) was prepared in 350 mL of double distilled water and autoclaved for 15 minutes at 121 °C.

Minimal glucose agar

About 51 g of bacto agar was added to 3162 mL of distilled water and autoclaved for 15 minutes at 121 °C. The solution was cooled slightly, 68 mL of sterile 50 x VB (Vogel-Bonner) salts and 170 mL of sterile 40% glucose solution was added. All the ingredients were mixed thoroughly and poured into each petri plate at the volume of approximately 20 mL per plate. The 50 x VB (Vogel-Bonner) salts and 40% glucose solution were autoclaved separately.

Vogel-Bonner medium E (50xVB):

Ingredients	Per 80 mL	
Magnesium Sulphate ($MgSO_4.7H_2O$)	00.80 g	This solution was autoclaved for 15 mins at 121 °C.
Citric Acid Monohydrate	08.00 g	
Potassium Phosphate, Dibasic (anhydrous) (K_2HPO_4)	40.00 g	
Sodium Ammonium Phosphate ($NaNH_4HPO_4.4H_2O$)	14.00 g	

TEST ITEM PREPARATION

The test item was extracted at a ratio of 3 cm^2/ mL (as the thickness of the test item was greater than 0.5 mm) in the polar (physiological saline) and non-polar (DMSO - dimethyl sulfoxide) solvents, respectively at 37 ± 1 °C for 71 h and 15 minutes. The total surface area of the test item is 245 cm^2 (as calculated in our laboratory). Solvent controls were also subjected to similar extraction conditions. This fulfilled the requirement of ISO 10993-12:2012 and ISO 10993-12:2021. The details of extracts preparation are as follows:

Extract	Extraction vehicle	Surface of the test item (cm^2)	Volume of vehicle (mL)	Extract preparation start time	Extract preparation end time	Appearance of extracts
Negative control (polar solvent)	Physiological saline	NA	5	11:15 a.m. on 16 April 2022	10:30 a.m. on 19 April 2022	Colourless clear solution; no particulates
Test item extract in polar solvent (polar extract)	Physiological saline	245#	81.67			Colourless clear solution no particulates*
Negative control (non-polar solvent)	DMSO	NA	5			Colourless clear solution; no particulates
Test item extract in non-polar solvent (non-polar extract)	DMSO	245#	81.67			Colourless clear solution; no particulates*

One test item used for each extraction.; NA-Not applicable
* No change in colour of the extract, compared to extraction vehicle alone

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 change was observed in retrieved test items. After extraction, all extracts were transferred to sterile containers and added to the test system within 3 h.

TEST METHOD

The experiment was performed by direct plate incorporation method in the presence and absence of metabolic activation system with triplicate plating. Suspensions of bacterial cells in late exponential growth or early stationary phase of growth (approximately 10^9 cells/mL) was exposed to neat test item extracts (100%). Negative (solvent) and positive controls were included without and with S9 mix. In this plate incorporation method, the following suspensions were added to 2 mL of overlay agar and plated on to the minimal agar: 0.1 mL of fresh bacterial culture, 0.1 mL of test item extracts or control, 0.5 mL of 10% S9 mix or sodium phosphate buffer solution.

Followed by rapid mixing and pouring on to minimal agar plates, the overlay agar was allowed to solidify and incubated at 37 °C for 70 h and 45 minutes (19 April 2022, 01:45 p.m. to 22 April 2022, 12:30 p.m.).

Minimal agar plates were suitably labelled to clearly identify the study number, with/without S9 mix, test concentration/control details and replicate number.

Colony counting

After incubation period, the plates were examined for signs of toxicity (annexure 1) and number of revertant colonies per plate was counted manually.

ACCEPTANCE CRITERIA

The assay was considered valid based on the following criteria are met:

1. The negative (solvent) control counts fell within the historical control ranges.
2. The positive control chemicals induced increases in revertant colony numbers confirming discrimination between different tester strains, and an active S9 preparation.
3. No plates were lost through contamination or some other unforeseen event.

Acceptance under any other criteria was discussed in the report.

DATA EVALUATION

Individual plate counts from all the experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment was determined. Mean number of revertant colonies on control plates, were compared with the historical control ranges. If data from our laboratory are consistent with ranges of revertant colonies per plate, then it is considered acceptable elsewhere.

EVALUATION CRITERIA

1. The test item is considered to be mutagenic in this assay if:
   - The assay is valid.
   - It causes a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results was considered first.
2. A test item for which the results do not meet the above criteria is considered non- mutagenic in this test.

Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions or frameshifts in the genome of *Salmonella typhimurium*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

RESULTS

The mean number of revertant colonies in negative (solvent) control were similar to historical data of the laboratory. The positive control induced an increase in the number of revertant colonies when compared to negative (solvent) control; the average revertant colonies were similar to historical data of the laboratory.

No cytotoxicity (clearing or diminution of the background lawn or reduction in revertant numbers) was observed in plates treated with the neat extract of the test item in polar and non-polar solvents, with and without S9 mix, in any of the tester strains. The mean number of revertant colonies observed in these plates were comparable to the negative (solvent) control and historical data of the laboratory.

Individual plate counts, the mean number of revertant colonies per plate, the standard deviation and bacterial background lawn are given in Tables 1-5. Summary of mean colony counts are given in Table 6. Historical negative (solvent) and positive control data is given in Table 7.

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 Premium Thermoforming Foils supplied by Vedia Solutions Div. of Laxmidental Export Pvt. Ltd. Is non-mutagenic in the bacterial reverse mutation (AMES) test.

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. ISO/IEC 17025:2017, General requirements for the competence of testing and calibration laboratories.

7. 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.

8. OECD Guideline for Testing of Chemicals (No. 471, Bacterial reverse mutation test, Adopted 26 June 2020).

9. 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.

10. Ames B N, McCann J and Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31, 347-364.

11. Gatehouse D G, Wilcox P, Forster R, Rowland I R and Callander R D (1990) Bacterial mutation assays. In "Basic Mutagenicity Tests UKEMS Recommended Procedures". Report of the UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Part I Revised. Ed D J Kirkland. Cambridge University Press, pp 13-61.

12. Maron D M and Ames B N (1983) Revised methods for the *Salmonella* mutagenicity test. Mutation Research 113, 173-215.

13. Garner R C (1979) Carcinogen prediction in the laboratory: a personal view. Proc Roy Soc Ser B 205, 121-134.

TABLE 1– INDIVIDUAL PLATE COUNT TA 98

Control/Test Item Extract	Volume/ Concentration	S9 Mix	Histidine Revertant Colonies (CFU)						Bacterial Background Lawn Code
			1	2	3	Mean	±	SD	
Negative control (polar solvent) (Physiological saline)	100 µL	×	38	31	33	34	±	4	1
		✓	31	35	29	32	±	3	1
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	28	30	23	27	±	4	1
		✓	31	29	25	28	±	3	1
Negative control (non - polar solvent) (DMSO)	100 µL	×	30	41	29	33	±	7	1
		✓	34	39	28	34	±	6	1
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	24	29	30	28	±	3	1
		✓	34	38	35	36	±	2	1
Positive control	2- Nitrofluorene 10 µg/plate	×	263	259	268	263	±	5	1
	Benzo (a) pyrene 5 µg/plate	✓	298	313	308	306	±	8	1

NA- Not Applicable
1 - Normal background lawn
× - Without S9 mix
✓ - With S9 mix
CFU- Colony forming unit

TABLE 2- INDIVIDUAL PLATE COUNT TA 100

Control/Test Item Extract	Volume/ Concentration	S9 Mix	Histidine Revertant Colonies (CFU)						Bacterial Background Lawn Code
			1	2	3	Mean	±	SD	
Negative control (polar solvent) (Physiological saline)	100 µL	×	129	132	134	132	±	3	1
		✓	138	131	141	137	±	5	1
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	129	131	123	128	±	4	1
		✓	121	124	128	124	±	4	1
Negative control (non - polar solvent) (DMSO)	100 µL	×	124	130	133	129	±	5	1
		✓	142	138	139	140	±	2	1
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	123	124	119	122	±	3	1
		✓	124	131	140	132	±	8	1
Positive control	Sodium azide 1 µg/plate	×	678	641	598	639	±	40	1
	Benzo (a) pyrene 5 µg/plate	✓	718	741	707	722	±	17	1

NA- Not Applicable
1 - Normal background lawn
× - Without S9 mix
✓ - With S9 mix
CFU- Colony forming unit

TABLE 3 - INDIVIDUAL PLATE COUNT TA 102

Control/Test Item Extract	Volume/ Concentration	S9 Mix	Histidine Revertant Colonies (CFU)						Bacterial Background Lawn Code
			1	2	3	Mean	±	SD	
Negative control (polar solvent) (Physiological saline)	100 µL	×	251	242	248	247	±	5	1
		✓	243	248	239	243	±	5	1
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	244	239	237	240	±	4	1
		✓	241	238	233	237	±	4	1
Negative control (non - polar solvent) (DMSO)	100 µL	×	261	258	255	258	±	3	1
		✓	232	227	224	228	±	4	1
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	246	250	243	246	±	4	1
		✓	221	223	218	221	±	3	1
Positive control	Mitomycin C 0.5 µg/plate	×	625	739	788	717	±	84	1
	Benzo (a) pyrene 10 µg/plate	✓	696	759	785	747	±	46	1

NA- Not Applicable
1 - Normal background lawn
× - Without S9 mix
✓ - With S9 mix
CFU- Colony forming unit

TABLE 4 - INDIVIDUAL PLATE COUNT TA 1535

Control/Test Item Extract	Volume/ Concentration	S9 Mix	Histidine Revertant Colonies (CFU)						Bacterial Background Lawn Code
			1	2	3	Mean	±	SD	
Negative control (polar solvent) (Physiological saline)	100 µL	×	5	15	12	11	±	5	1
		✓	13	8	7	9	±	3	1
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	6	9	4	6	±	3	1
		✓	9	7	5	7	±	2	1
Negative control (non - polar solvent) (DMSO)	100 µL	×	9	12	13	11	±	2	1
		✓	14	11	10	12	±	2	1
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	8	6	4	6	±	2	1
		✓	7	9	12	9	±	3	1
Positive control	Sodium azide 1 µg/plate	×	340	331	299	323	±	22	1
	Benzo (a) pyrene 10 µg/plate	✓	294	289	278	287	±	8	1

NA- Not Applicable
1 - Normal background lawn
× - Without S9 mix
✓ - With S9 mix
CFU- Colony forming unit

TABLE 5 - INDIVIDUAL PLATE COUNT TA 1537

Control/Test Item Extract	Volume/ Concentration	S9 Mix	Histidine Revertant Colonies (CFU)						Bacterial Background Lawn Code
			1	2	3	Mean	±	SD	
Negative control (polar solvent) (Physiological saline)	100 µL	×	18	20	21	20	±	2	1
		✓	17	22	19	19	±	3	1
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	14	15	11	13	±	2	1
		✓	17	18	20	18	±	2	1
Negative control (non - polar solvent) (DMSO)	100 µL	×	23	14	15	17	±	5	1
		✓	11	13	16	13	±	3	1
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	11	12	22	15	±	6	1
		✓	16	21	16	18	±	3	1
Positive control	9-Aminoacridine 50 µg/plate	×	359	371	327	352	±	23	1
	Benzo (a) pyrene 10 µg/plate	✓	241	249	255	248	±	7	1

NA- Not Applicable
1 - Normal background lawn
× - Without S9 mix
✓ - With S9 mix
CFU- Colony forming unit

TABLE 6 - SUMMARY OF MEAN COLONY COUNT

Control/Test Item Extract	Volume/ Concentration	S9 mix	Average Histidine Revertant colonies/Plate														
			TA100			TA102			TA 1535			TA1537			TA98		
Negative control (polar solvent) (Physiological saline)	100 µL	×	132	±	3	247	±	5	11	±	5	20	±	2	34	±	4
		✓	137	±	5	243	±	5	9	±	3	19	±	3	32	±	3
Test item extract in polar solvent (Physiological saline)	100 µL (100% extract)	×	128	±	4	240	±	4	6	±	3	13	±	2	27	±	4
		✓	124	±	4	237	±	4	7	±	2	18	±	2	28	±	3
Negative control (non-polar solvent) (DMSO)	100 µL	×	129	±	5	258	±	3	11	±	2	17	±	5	33	±	7
		✓	140	±	2	228	±	4	12	±	2	13	±	3	34	±	6
Test item extract in non-polar solvent (DMSO)	100 µL (100% extract)	×	122	±	3	246	±	4	6	±	2	15	±	6	28	±	3
		✓	132	±	8	221	±	3	9	±	3	18	±	3	36	±	2
Mitomycin C	0.5 µg	×		NA		717	±	84		NA			NA			NA	
Sodium azide	1 µg	×	639	±	40		NA		323	±	22		NA			NA	
2 - Nitrofluorene	10 µg	×		NA			NA			NA			NA		263	±	5
9 - Aminoacridine	50 µg	×		NA			NA			NA		352	±	23		NA	
Benzo (a) pyrene	5 µg	✓	722	±	17		NA			NA			NA		306	±	8
	10 µg	✓		NA		747	±	46	287	±	8	248	±	7		NA	

Values are Mean ± SD of 3 plates; × - without S9 mix, ✓ - with S9 mix; NA - Not Applicable

TABLE 7 - HISTORICAL CONTROL VALUE

Negative (solvent) control- physiological saline

	Strains	TA 98	TA100	TA102	TA1535	TA1537		TA 98	TA100	TA102	TA1535	TA1537
-S9 mix	Minimum revertant frequency/plate (CFU/plate)	16	97	211	3	9	**+S9 mix**	22	105	169	2	5
	Maximum revertant frequency/plate (CFU/plate)	32	144	274	11	29		37	139	254	10	23
	Mean ± 2SD	25 ± 8	119 ± 18	241 ± 32	6 ± 4	20 ± 10		29 ± 8	123 ± 18	214 ± 46	6 ± 6	17 ± 10
	Range (CFU/plate)	16-32	97-144	211-274	3-11	9-29		22-37	105-139	169-254	2-10	5-23

-S9 mix: without metabolic activation system, +S9 mix: with metabolic activation system, SD: Standard deviation

Data obtained from the studies performed in the year 2021.

TABLE 7 (CONT.)- HISTORICAL CONTROL VALUE

Negative (solvent) control- dimethyl sulfoxide

	Strains	TA 98	TA100	TA102	TA1535	TA1537		TA 98	TA100	TA102	TA1535	TA1537
-S9 mix	Minimum revertant frequency/plate (CFU/plate)	20	101	168	2	10	**+S9 mix**	18	111	166	1	9
	Maximum revertant frequency/plate (CFU/plate)	38	144	309	11	28		40	145	228	15	30
	Mean ± 2SD	29 ± 10	125 ± 22	242 ± 58	6 ± 4	19 ± 10		28 ± 12	127 ± 18	201 ± 34	9 ± 6	20 ± 12
	Range (CFU/plate)	20-38	101-144	168-309	2-11	10-28		18-40	111-145	166-228	1-15	9-30

-S9 mix: without metabolic activation system, +S9 mix: with metabolic activation system, SD: Standard deviation

Data obtained from the studies performed in the year 2021.

TABLE 7 (CONT.)- HISTORICAL CONTROL VALUE

Positive control

S9 details	Chemicals	Sodium azide (1 µg/plate)		Mitomycin C (0.5 µg/plate)	2-Nitrofluorene (10 µg/plate)	9-Aminoacridine (50 µg/plate)
-S9 mix	**Strains**	**TA 100**	**TA 1535**	**TA 102**	**TA 98**	**TA 1537**
	Minimum revertant frequency/plate (CFU/plate)	502	285	527	216	217
	Maximum revertant frequency/plate (CFU/plate)	657	328	734	254	365
	Mean ± 2SD	586 ± 84	309 ± 22	648 ± 112	234 ± 20	296 ± 78
	Range (CFU/plate)	502-657	285-328	527-734	216-254	217-365

-S9 mix: without metabolic activation system, SD: Standard deviation

Data obtained from the studies performed in the year 2021.

TABLE 7 (CONT.)- HISTORICAL CONTROL VALUE

Positive control

S9 Details	Chemicals	Benzo (a) pyrene (5 µg/plate)	Benzo (a) pyrene (10 µg/plate)	Benzo (a) pyrene (10 µg/plate)	Benzo (a) pyrene (5 µg/plate)	Benzo (a) pyrene (10 µg/plate)
+S9 mix	**Strains**	**TA 100**	**TA 1535**	**TA 102**	**TA 98**	**TA 1537**
	Minimum revertant frequency/plate (CFU/plate)	606	195	610	221	108
	Maximum revertant frequency/plate (CFU/plate)	730	297	755	294	259
	Mean ± 2SD	672 ± 72	240 ± 40	691 ± 84	260 ± 36	203 ± 76
	Range (CFU/plate)	606-730	195-297	610-755	221-294	108-259

+S9 mix: with metabolic activation system, SD: Standard deviation

Data obtained from the studies performed in the year 2021.

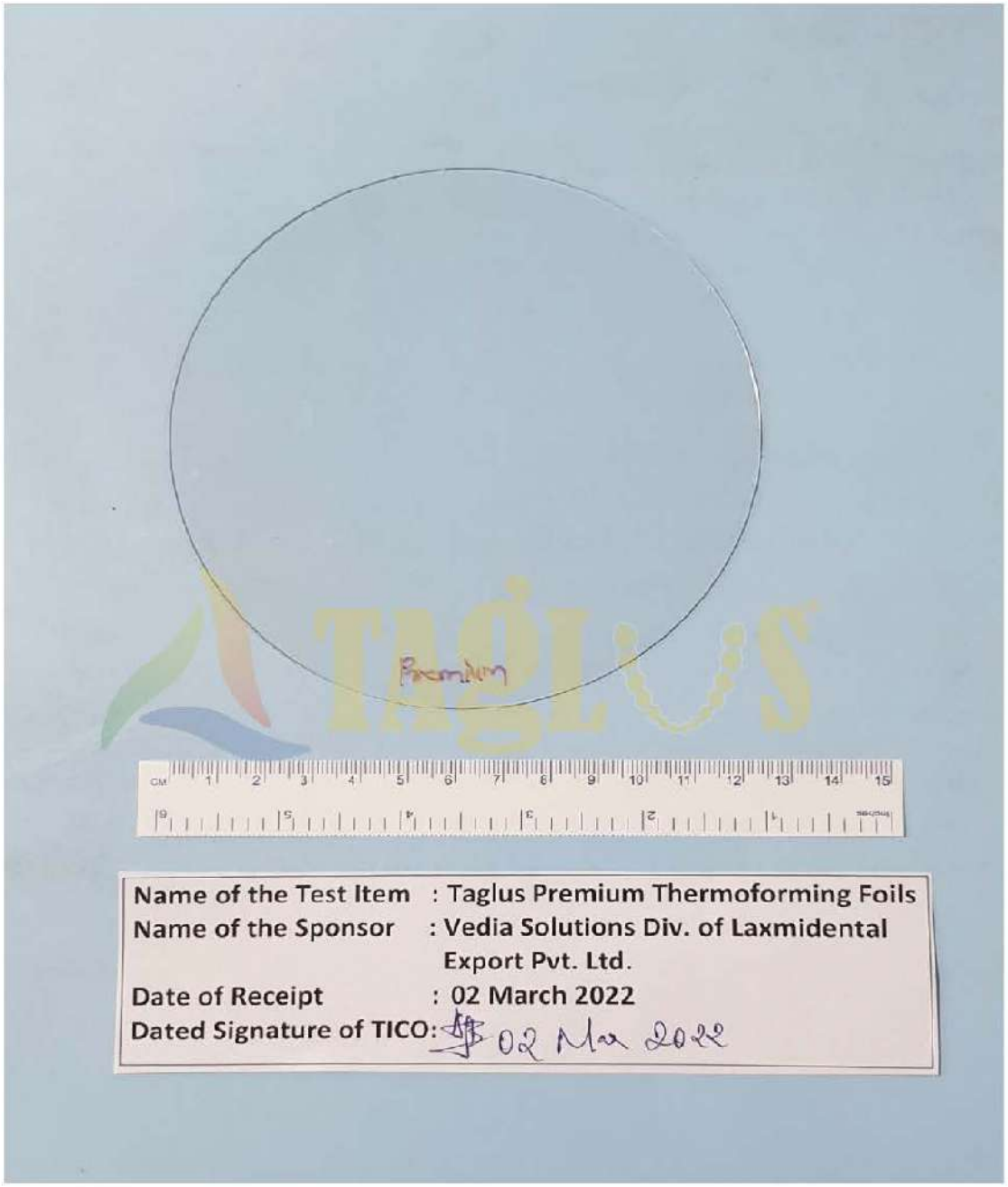
ANNEXURE 1- BACTERIAL BACKGROUND LAWN OBSERVATION CODE

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the extraction blank.
3	Moderately reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the extraction blank.
4	Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the extraction blank such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over more than or equal to 90 % of the plate.
6[a]	Obscured by particulates	The background bacterial lawn cannot be accurately evaluated due to microscopic test article particulate.
7[a]	Non-interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10 % of the revertant colony count (e.g. less than or equal to three particles on a plate with 30 revertants).
8	Interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total more than 10 % of the revertant colony count (e.g. more than three particles on a plate with 30 revertants).

[a] Nanomaterial or nanoparticles are not included in the definition of precipitates and particles.

Source: ISO 10993-33:2015 - Supplement to ISO 10993-3:2014.

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

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

No study plan amendment was made during the conduct of the study.

STUDY PLAN DEVIATION

No deviations occurred during the conduct of the study.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, a sample test item, 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 after issue of the final report. 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 2- 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 Pickett 07/19/21

ANNEXURE 3

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 4

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. In compliance with 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), GLR Laboratories has successfully completed the recertification inspection by the NGCMA during the dates 26 to 28 Mar 2022, well within the present tenure of 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 in the tenure of GLP certification.

30 MAR 2022

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

30 Mar 2022

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

Date: 30 Mar 2022