BACTERIAL REVERSE MUTATION TEST OF GEN F -20 PLUS SPRAY

STUDY No.: 110702/DM/PC

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STATEMENT OF COMPLIANCE

To the best of our knowledge and belief, this Study entitled "BACTERIAL REVERSE MUTATION TEST OF GEN F -20 PLUS SPRAY" was performed under my supervision". The objectives laid down in the study protocol were achieved.

No unforeseen circumstances were observed which might have affected the quality or integrity of the study.

Jayesh Chaudhary	Vijay Gokarn
MD, Vedic Lifesciences Pvt. Ltd.	Assistant project manager- Technical

CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled, ‘‘Bacterial Reverse Mutation Test of Gen F-20 Plus Spray”, based on the experiment conducted in one of the partnered Toxicology Laboratory Services of VEDIC LIFESCIENCES PVT LTD (203, Morya Landmark I, Off New Link Road, Andheri (W), Mumbai - 400 053,) India. The results presented here are faithful reflection of data collected during the study.

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE 2

CERTIFICATE 3

TABLE OF CONTENTS 4

QUALITY ASSURANCE STATEMENT 7

STATEMENT OF CONFIDENTIALITY 8

STATEMENT OF GLP COMPLIANCE 8

DECLARATION 8

LIST OF COMMONLY USED SYMBOLS AND ABBREVIATIONS 9

1. STUDY DETAILS 10

2. MONITORING PERSONNEL 11

3. SUMMARY 12

4. STUDY COMPLIANCE 13

5. SAFETY PRECAUTIONS 13

6. OBJECTIVE 13

7. MATERIALS AND METHODS 13

7.1 TEST ITEM INFORMATION 13

7.2 Test System 14

7.3 Source of the test system 14

7.4 Storage of the test system 14

7.5 Genetic Characterization of Tester Strains 14

7.6 Test Medium, Solutions and other Chemicals 14

7.7 Positive controls 15

8. METHODS 15

8.1 Preparation of S9 homogenate and activation mixture 15

8.2 Solubility TEST FOR selection of Solvent/ Vehicle 16

8.3 Precipitation TEST 16
8.4 Initial Cytotoxicity test for selection of test doses 16
8.5 MUTATION Assay 17
8.5.1 Tester Strain cultures 17
8.5.2 Controls 17
8.5.3 Test Concentrations 17
8.5.4 Performance of the assay 17
8.5.5 Plate Incorporation Method 18
8.5.6 Pre Incubation Method 18
8.5.7 Viable Counts 18
9. OBSERVATIONS 18
9.1 Effect of Bacterial lawn intensity 18
9.2 Number of revertants 19
10. STATISTICAL ANALYSIS 19
11. AMMENDMENTS AND DEVIATIONS 19
12. REPORT DISTRIBUTION 19
13. ARCHIVING 19
14. RESULTS AND DISCUSSSION 20
14.1 SOLUBILITY TEST 20
14.2 PRECIPITATION TEST 20
14.3 INITIAL CYTOTOXICITY TEST 20
14.4 MUTATION ASSAY 20
14.4.1 Plate Incorporation Method: Trial I 20
14.4.2 Pre Incubation Method: Trial II 20
14.4.3 Viable count 21
14.5 Quality control plates 21
15. INTERPRETATION 21
16. CONCLUSION 21
17. TABLES 22
TABLE 1. SUMMARY OF INITIAL CYTOTOXICITY TEST 23

TABLE 2. SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL -I 24

Plate Incorporation Method 24

TABLE 3. SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL -II 25

Pre Incubation method 25

18. APPENDICES 26

APPENDIX 1. INDIVIDUAL PLATE COUNT OF INITIAL CYTOTOXICITY TEST 27

APPENDIX 2. INDIVIDUAL PLATE COLONY COUNT OF REVERTANTS OF TRIAL-I 28

Plate Incorporation Method 28

APPENDIX 3. INDIVIDUAL PLATE COLONY COUNT OF REVERTANTS OF TRIAL-II 29

Pre Incubation Method.....29

APPENDIX 4. INDIVIDUAL COLONY COUNT OF VIABLE COUNT.....30

APPENDIX 5. QUALITY CONTROL PLATES DATA 31

QUALITY ASSURANCE STATEMENT

The Study No.: 110702/DM/PC, entitled "Bacterial Reverse Mutation Test of Gen F-20 Plus Spray" has been inspected according to the OECD Principles of Good Laboratory Practice {ENV/MC/CHEM (98) 17:1997}.

This study was inspected and findings reported to the Management and Study Director on the dates given below.

Dates	**Inspection Phases**	**Reporting Dates**
	Initial Phase:	
01/08/2011	Final study plan review	01/08/2011
	In life phase:	
05/08/2011	Media preparation , plating and tester strain inoculation	05/08/2011
06/08/2011	Solubility and precipitation test	06/08/2011
08/08/2011	Colony counting of initial cytotoxicity	08/08/2011
11/08/2011	Plate incorporation method	11/08/2011
18/08/2011	Media preparation, plating and tester strain inoculation	18/08/2011
19/08/2011	Pre incubation method	19/08/2011
22/08/2011	Colony counting of pre incubation method	22/08/2011

Inspections were performed according to the Standard Operating Procedures of the Vedic Lifesciences Pvt. Ltd. Quality Assurance Unit. The report was audited against the approved study plan and pertinent raw data and accurately reflects the raw data.

STATEMENT OF CONFIDENTIALITY

This report contains **CONFIDENTIAL** and **PROPRIETARY** information of **LEADING EDGE MARKETING** will not be disclosed to anyone except the employees of this company wherever necessary or to persons authorized by law or judicial judgment without the expressed or written approval of Sponsor.

STATEMENT OF GLP COMPLIANCE

The Study No. 110702/DM/PC, "Bacterial Reverse Mutation Test of Gen F-20 Plus Spray" was performed in the spirit of OECD Principles of Good Laboratory Practices {ENV/MC/CHEM (98) 17: 1997}.

DECLARATION

The Study Director hereby declares that the work was performed under her supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

LIST OF COMMONLY USED SYMBOLS AND ABBREVIATIONS

OECD	-	Organization for Economic Co-operation and Development
DMSO	-	Dimethyl Sulphoxide
+S9	-	With Metabolic activation
-S9	-	Without Metabolic activation
G	-	Gram
Kg	-	Kilogram
Min	-	Minute
Mg	-	Milligram
mL	-	Milliliter
PBS	-	Phosphate Buffer Saline
CFU	-	Colony forming unit
No.	-	Number
NADP	-	Nicotinamaide adenine dinucleotide phosphate
R1	-	Replicate 1
R2	-	Replicate 2
R3	-	Replicate 3
Hr	-	hours
PB	-	Phosphate buffer
µl	-	Micro liter
SD	-	Standard Deviation

1. STUDY DETAILS

1.1 STUDY TITLE : Bacterial Reverse Mutation Test of Gen F-20 Plus Spray

1.2 STUDY NUMBER : 110702/DM/PC

1.3 SPONSOR DETAILS :

Sponsor : LEADING EDGE MARKETING
PO Box CR-56766, Suite 1210
NASSAU BAHAMAS

1.5 TEST FACILITY : VEDIC LIFE SCIENCES PVT. LTD.
Morya Landmark-I, Off Link Road,
Andheri (W), Mumbai – 400 053, INDIA,

1.6 STUDY SCHEDULE :

Study Initiation date		: 01.08.2011			
Solubility test		: 06.08.2011			
Precipitation Test		: 06.08.2011			
Initial Cytotoxicity test	Start	: 05.08.2011	End	:	08.08.2011
Mutation test		: 10.08.2011			
Plate Incorporation Method	Start	: 10.08.2011	End	:	13.08.2011
Pre-incubation Method	Start	: 18.08.2011	End	:	22.08.2011
Experiment end date		: 22.08.2011			

2. MONITORING PERSONNEL

The following personnel participated in the conduct of the study.

Sr. No.	Designation	Personnel	Signature with date
1.	**Assistant project manager-Technical**	VIJAY GOKARN VEDIC LIFESCIENCES PVT.LTD MUMBAI	
2.	**Managing Director**	JAYESH CHAUDHARY VEDIC LIFESCIENCES PVT.LTD MUMBAI	

3. SUMMARY

The test item Gen F-20 Plus Spray obtained from D M CONTACT was evaluated for Bacterial Reverse Mutation Test as per the OECD guideline for the testing of chemicals, "Bacterial Reverse Mutation Test", Test No. 471, adopted by the council on 21st July 1997.

The test item Gen F-20 Plus Spray was assessed for its mutagenic effects using *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535 and TA1537. The test item was tested at the concentration of 0.05, 0.1, 0.5, 1 and 5 µl/plate using dimethyl sulphoxide (DMSO) as solvent based on the initial cytotoxicity test. The study was conducted, with and without the metabolic activator (S-9 fraction) prepared from sodium phenobarbitone and beta-napthaflavone induced rat liver. Solvent control and appropriate positive controls (2-nitrofluorene, sodium azide and 9-aminoacridine, mitomycin C for trials "without metabolic activator" and 2-aminoanthracene for trials "with metabolic activator") were tested simultaneously. Two trials were carried out for this study in triplicates with Plate incorporation method (Trial 1) and Pre incubation method (Trial 2). Data were statistically analyzed and expressed as mean ± SD.

From the experimental results obtained, the mean numbers of revertant colonies in the above mentioned concentrations were comparable to those of the solvent control, in both the trials- Plate Incorporation method and Pre incubation Method, in the presence and absence of metabolic activation. There was no significant increase in number of revertant colonies at all concentrations tested in both plate incorporation and Pre incubation method. The number of revertant colonies in the positive controls has shown 2.3 to 21.7 fold increase with statistical significance at $p<0.001$ under identical conditions.

Conclusion

From the results obtained, the test item Gen F-20 Plus Spray is found to be non-mutagenic at the highest concentration of 5 µl/plate in Bacterial Reverse Mutation Test in the tester strain *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of metabolic activator in both the Plate incorporation and Pre-incubation method under laboratory conditions applied.

4. STUDY COMPLIANCE

The study was performed in accordance with the following:

a. The study was conducted as per OECD Guideline No. 471 for testing of chemicals, "Bacterial Reverse Mutation Test", adopted: 21st July.

b. In spirit of OECD Principles of Good Laboratory Practices (1997).

c. The standard operating procedures at Vedic Lifesciences Pvt. Ltd. and as per the mutually agreed study plan with the sponsor.

5. SAFETY PRECAUTIONS

No known hazards are associated with the test material; however, general precautions were taken when working with the test material to minimize the exposure. Gloves, cap and face mask were used in addition to protective body garments and rubber slipper to ensure adequate personnel health and safety and to avoid inhalation and skin contact with the test item.

6. OBJECTIVE

The objective of Bacterial Reverse Mutation test was to assess the genotoxic potential of the test item Gen F-20 Plus Spray particularly for point mutation inducing activity by using *Salmonella typhimurium* tester strains.

7. MATERIALS AND METHODS

7.1 TEST ITEM INFORMATION

The test item information as furnished by the sponsor is as follows

Product name	: Gen F-20 Plus Spray
Test Item code by Test facility	: D109 TOX 093
Physical appearance	: Clear liquid with berry flavor
Manufactured Date	: September 2010
Expiry Date	: May 2013
Batch No.	: 111037
Storage conditions	: +18 to + 36º C
Name of the Supplier	: D M CONTACT

The Test item data sheet and certificate of analysis of test item has been presented as Annexure No. 1 and Annexure No.2.

7.2 TEST SYSTEM

Bacteria	: Histidine auxotrophic strains of *Salmonella typhimurium*
Frame shift mutation	: TA98 and TA1537
Base pair substitution	: TA100, TA102 and TA1535

7.3 SOURCE OF THE TEST SYSTEM

Tester strains used in the present study were procured from Molecular Toxicology, Inc., 157, Industrial Park, Dr. Biine, NC 28607, USA

Date of receipt : 19th April 2010

7.4 STORAGE OF THE TEST SYSTEM

Tester strain stocks were stored at -80±5°C in oxoid nutrient broth No. 2 with DMSO. Laboratory stocks were maintained as master plates of each strain on minimal glucose agar with histidine and biotin and stored at 2 to 8°C.

7.5 GENETIC CHARACTERIZATION OF TESTER STRAINS

Strains were maintained as master plates and periodically checked for viable counts and genetic characteristics as mentioned below:

a. Histidine requirement

b. rfa mutation

c. uvr B mutation

d. R-factor (pKM 101 plasmid)

e. Spontaneous reversion

Date of permanent stock preparation	: 07.04.2011
Batch No.	: BIO-001
Date of master Plate preparation	: 08.04.2011
Date of characterization	: 11.04.2011

All the tester strains fulfilled the quality check criteria. Tester strains from master plate prepared on 15.06.2011 and 03.08.2011 were used for the study.

7.6 TEST MEDIUM, SOLUTIONS AND OTHER CHEMICALS

All the chemicals used for media preparation including Vogel Bonner media, soft agar, Phosphate buffer , Phosphate buffer saline are listed in detail.

Chemical	Batch/Lot No.	Manufactured by
Agar	6815	Reachem
Sodium chloride	P007L08	Rankem
Magnesium Sulphate ($MgSO_4.7H_2O$)	71836908-1	Fisher Scientific

Potassium Chloride	L012B09	Rankem
Magnesium Chloride	J0009J09	Rankem
Citric acid monohydrate	J008E03	Rankem
Dipotassium hydrogen phosphate (K_2HPO_4)	0000099794	Himedia
Ammonium Sodium Phosphate	83230	Sigma Aldrich
D-Glucose	0000109440	Himedia
Dimethyl Sulphoxide	SJ0S600643	Merck
L-Histidine	0000060108	Himedia
D-Biotin	0000046392	Himedia
Disodium hydrogen orthophosphate	89897002-1	Fisher Scientific
Nutrient Agar	0000080109	Himedia
Nutrient Broth No. 2	0000082498	Himedia
Potassium dihydrogen phosphate(KH_2PO_4)	J123G09	Rankem
D-Glucose-6-Phosphate	0000087808	Himedia
NADP disodium salt	0000062652 0000103163	Himedia

7.7 POSITIVE CONTROLS

Positive control	CAS No.	Batch/ Lot No.	Storage Condition	Manufactured by
2-Nitrofluorene	607-57-8	03403ED	Room temperature	Sigma Aldrich
2-Aminoanthracene	613-13-8	S43858-369	Room temperature	Sigma Aldrich
9-Aminoacridine	90-45-9	31036803-1	Room temperature	Fischer Scientific
Sodium azide	26628-22-8	106F06681	Room temperature	Sigma
Mytomycin C	50-07-7	117K1684	2-8° C	Sigma

8. METHODS

8.1 PREPARATION OF S9 HOMOGENATE AND ACTIVATION MIXTURE

Sodium phenobarbitone and β- Naphthoflavone induced rat liver S9 homogenate is used as the metabolic activation system in the present study. The S9 homogenate was prepared from male Wistar induced with intraperitoneal injection of Sodium phenobarbitone and β- Naphthoflavone at 16 mg/ml and 20 mg/ml respectively for 3

days prior to sacrifice. The S9 homogenate of batch No. S9-I/11 was prepared and stored in the test facility at -80±5°C until used. Each batch of S9 homogenate was assessed for sterility by streaking the supernatant fluid on nutrient agar plates and incubated at 37±1°C for 24 hours. It was found sterile. Protein content (modified Lowry Assay, Sword and Thomson, 1980) and for its ability to metabolize the promutagens 2-Aminoanthracene and Benzo(a)pyrene to mutagens using *Salmonella typhimurium* TA100 strain.

S9 homogenate was thawed immediately before use and mixed with the co-factor solution containing 4 mM NADP, 5 mM Glucose-6-phosphate, 8 mM $MgCl_2$ and 33 mM KCl in PBS.

Details of S9 homogenate

Date of preparation	: 09.04.2011
Batch No.	: S9-I/11
Date of characterization	:
Sterility check	: 09.04.2011
Activity check	: 15.04.2011

8.2 SOLUBILITY TEST FOR SELECTION OF SOLVENT/ VEHICLE

The solubility test of test item was carried out with distilled water and DMSO. Test substance of 500 µl was mixed with 1ml of distilled water and the volume was made up to 10 ml in volumetric flask. 500 µl of the test substance was mixed with 1ml of DMSO and the volume was made up to 10 ml in volumetric flask.

8.3 PRECIPITATION TEST

The test item prepared in solubility test was serially diluted using DMSO to get concentrations of 8,9, 10, 20, 30, 40 and 50 µl/ml and precipitation test was carried out for the same. The plates were incubated for 2 hrs at 37 ±1°C. Precipitation test was carried out with the concentrations of 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4 and 5µl/plate.

8.4 INITIAL CYTOTOXICITY TEST FOR SELECTION OF TEST DOSES

Initial toxicity test was conducted using 15 hr old culture of TA100 tester, both in the presence and the absence of metabolic activator, in triplicate, along with concurrent solvent control (DMSO).Bases on Precipitation test each concentration of test item was mixed with soft agar containing histidine and biotin, S9 mix (for presence of metabolic activation) or PBS (for absence of metabolic activation), *Salmonella typhimurium* TA 100 of cell density approximately 1.5 X 10^9 cells/ml and overlaid on to pre-labeled VB agar plates. The plates were incubated at 37 ±1°C for 48 hrs.

8.5 MUTATION ASSAY

8.5.1 Tester Strain cultures

Culture of *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537 from respective genotyped master plates of each strain were grown in nutrient broth No. 2 separately for 15 hrs 05 minutes for Plate incorporation method and 15 hr for pre incubation method at 37±1°C in the shaking water bath with shaking and cell density was adjusted to 1.5 X 10^9 cells/ml using Macfarland Nephlometer standards.

8.5.2 Controls

As the test item was soluble in DMSO, same was used to dissolve the test item and positive controls. DMSO served as vehicle control in the assay.

Positive control: Following Positive controls for respective tester strains for concerned metabolic activation was used in the study.

Strain	Metabolic Activation	Positive controls	Concentration (µg/plate)
TA 98	+	2-Aminoanthracene	4
	-	2-Nitrofluorene	2
TA 100	+	2-Aminoanthracene	4
	-	Sodium azide	1
TA 102	+	2-Aminoanthracene	4
	-	MitoMycin C	0.5
TA 1535	+	2-Aminoanthracene	4
	-	Sodium azide	1
TA 1537	+	2-Aminoanthracene	4
	-	9-Aminoacridine	50

8.5.3 Test Concentrations

Based on the initial cytotoxicity test results, the following concentrations were selected for the mutation assay (µl/plate):

a. 0.05 b. 0.1 c. 0.5 d. 1 e. 5

8.5.4 Performance of the assay

Labeling:

The petri-dishes were labeled with the study number, strain number, treatment group, plate number and activation and number of triplicates. Three replicate plates each for the controls , five treatment levels and positive control were maintained.

8.5.5 Plate Incorporation Method

Plate incorporation assay with the same concentration as mentioned above: where the test compound and the strains along with S-9 / Phosphate buffer were mixed with 2 ml soft agar/top agar and poured on to VB agar plates. Five concentrations of the test item were plated, with each of the following tester strains: TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activator. Plates were incubated at 37±1°C for 48 hrs.

The condition of the bacterial background lawn was evaluated for evidence of the test item toxicity using the code system and revertant colonies for a given strain within the test item dilution series were counted manually.

8.5.6 Pre Incubation Method

Pre-incubation assay with the same concentration as mentioned above: where the test compound and the strains along with S-9 / Phosphate buffer were incubated with shaking at 37±1°C in the incubator for 30-45 minutes. After incubation, 2 ml soft agar/top agar was added, vortexed and poured on to VB agar plates. Plates were incubated at 37±1°C for 48 hrs. The condition of the bacterial background lawn was evaluated for evidence of the test item toxicity using the code system and revertant colonies for a given strain within the test item dilution series were counted manually.

8.5.7 Viable Counts

Viable counts of *Salmonella typhimurium* different strains was done by serially diluting each strain to 10^{-7} using Phosphate buffer saline. 100 µl of 10^{-7} dilution of each strain was mixed with 2 ml of over lay agar and poured on to nutrient agar plates in triplicates. Plates were incubated at 37±1°C for 48 hrs. Colony formed in each plate were counted manually and results were expressed as colony forming units/ml.

9. OBSERVATIONS

9.1 EFFECT OF BACTERIAL LAWN INTENSITY

The condition of the bacterial background lawn was evaluated for evidence of the test item toxicity using the code system which is as follows.

Code	Definition	Characteristics
0	No lawn	Distinguished by a complete lack (Absent) of any background lawn compared to solvent control plates.
1+	Very thin lawn	Distinguished by an extreme (Extremely thinning of the background lawn reduced) compared to solvent control plates.
2+	Thin lawn	Distinguished by a marked thinning (Moderately of the background lawn compared to reduced) solvent control plates.
3+	Slightly thin	Distinguished by a noticeable lawn (Slightly thinning of the

		background lawn reduced) compared to solvent control plates.
4+	Thick lawn	Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

9.2 NUMBER OF REVERTANTS

Revertant colonies for a given strain within the test item dilution series were counted manually.

10. STATISTICAL ANALYSIS

The data (in the form of appendix) were verified with the original raw data. The data of number of revertants were subjected to computer statistical processing wherever possible. All individual data were summarized and presented as tables. All findings were presented in the report as per the standard reporting procedure. Data was analyzed for differences among solvent control, treatment and positive control groups using ANOVA. Differences between the solvent control, treatment with different concentrations of test item and positive control groups were tested by Dunnett's test at a 5% level ($p \leq 0.05$) of significance.

11. AMMENDMENTS AND DEVIATIONS

There were no amendments or deviations during the course of the study.

12. REPORT DISTRIBUTION

Three originals of the Study Report prepared will be distributed as mentioned below

a). Copy No. 1/2-Sponsor's copy
b). Copy No. 2/2 – Archives

to QAU for final audit.

13. ARCHIVING

All materials and data generated from the experiment will be stored at archives of the test facility. The study plan, raw data, draft and final report will be maintained for 9 years from the date of completion of the study. A sample of the test item was archived at Vedic Lifesciences Pvt. Ltd. facility for a period of 5 years from the date of completion of the study or till the expiry date whichever is earlier. At the end of this period, the Sponsor's instructions will be sought to either extend the archiving period or return the archived material to the Sponsor or for the material to be disposed.

14. RESULTS AND DISCUSSSION

14.1 SOLUBILITY TEST

The test item was not soluble in water and was found to be soluble in DMSO. The volume was made up to 10 ml corresponding to 50 µl/ml stock.

Solvent : DMSO
Batch No. : SJ0S600643
Make : Merck
Storage : Room temperature

14.2 PRECIPITATION TEST

Precipicitation test was carried out with 0.5, 0.6, 0.7, 0.8, 0.9 , 1 , 2, 3, 4 and 5µl/plate. It was found that the test item did not cause any precipitation at a highest concentration of 5 µl/plate.

14.3 INITIAL CYTOTOXICITY TEST

Based on the results of the precipitation test, the following concentrations were selected for the initial cytotoxicity test (µl/plate):

a. 0.8 b. 0.9 c. 1 d. 2 e. 3 f. 4 g. 5

The test item was found to be non cytotoxic at a maximum concentration of 5µl/plate as compared to that of the solvent control.

Refer Table 1 and Appendix 1.

14.4 MUTATION ASSAY

14.4.1 Plate Incorporation Method: Trial I

The concentrations tested at 0.05, 0.1, 0.5, 1 and 5 µl/plate showed very close resemblance to the solvent control DMSO in both with and without metabolic activation. There was no significant increase in number of revertant colonies and no change in bacterial background lawn as compared to that of the solvent control, among the tester strains. The specific positive controls tested simultaneously produced approximately 2.3 to 21.7 fold increase in mean number of revertants as compared to the solvent control.

Refer Table 2 and Appendix 2.

14.4.2 Pre Incubation Method: Trial II

Similar results were observed in the second trial, the concentrations tested at 0.05, 0.1, 0.5, 1 and 5 µl/plate showed very close resemblance to the solvent control (DMSO) in both with and without metabolic activation. There was no significant increase in number of revertant colonies and no change in bacterial background lawn

as compared to that of the solvent control, among the tester strains. The mean number of revertant colonies/plate and bacterial background lawn in the solvent control for the tested strains were comparable to that of solvent control. The specific positive controls tested simultaneously produced approximately 2.4 to 21.3 fold increase in the mean number of revertants as compared to the solvent control.

Refer Table 3 and Appendix 3.

Statistical analysis of both the trials indicated no significance in all the five concentrations tested which was compared to the respective solvent control of the five *Salmonella* strains

14.4.3 Viable count

Each tester strain diluted to 10^{-7} and plated on nutrient agar were manually counted for colony forming units (CFU).

Refer Appendix 4.

14.5 QUALITY CONTROL PLATES

Control plates with S9 mix, PBS, soft agar/top agar, highest concentration of test item and media plates incubated at 37±1°C for 48 hrs were checked for contamination.

Refer Appendix 5.

15. INTERPRETATION

The test item Gen F-20 Plus Spray was assayed for Bacterial Reverse Mutation Test at the concentration of 0.05, 0.1, 0.5, 1 and 5 µl/plate using *Salmonella typhimurium* strains; TA98, TA100, TA102, TA1535 and TA1537. In the two trials conducted, with metabolic activation (S9) and without metabolic activation, the number of revertant colonies in the five different test item concentrations did not increase significantly over the solvent control in both Plate incorporation and Pre-incubation method, while, the positive controls tested simultaneously showed a 2.4 to 21.3 fold increase in the number of revertant colonies/plate increase with statistical significance at $p<0.001$ under identical conditions. This was observed for all the five tester strains.

16. CONCLUSION

From the results obtained, the test item Gen F-20 Plus Spray is found to be non-mutagenic at the highest concentration of 5 µl/plate in Bacterial Reverse Mutation Test in the tester strain *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of metabolic activator in both the Plate incorporation and Pre-incubation method under laboratory conditions applied.

17. TABLES

TABLE 1. SUMMARY OF INITIAL CYTOTOXICITY TEST

Refer Appendix-1

Test item concentration (µl/plate)	No. of Revertants /Plate			
	With S-9	Bacterial lawn intensity	Without S-9	Bacterial lawn intensity
Vehicle control	102±0.6	4+	103±1.2	4+
0.8 µl	102±2.1	4+	103±1.5	4+
0.9 µl	102±2.1	4+	102±0.6	4+
1 µl	101±1.5	4+	102±0.6	4+
2 µl	100±1.2	4+	101±1.2	4+
3 µl	100±0.6	4+	100±0.6	4+
4 µl	99±0.6	4+	100±0.6	4+
5 µl	98±0.6	4+	99±1.0	4+

Values of Revertants are in Mean±SD

Lawn intensity: 4+= Thick lawn: Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

TABLE 2. SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL -I
Plate Incorporation Method

Refer Appendix-2

Treatment	Test concentration (µl/plate)	No. of Revertants (Mean of 3 Plates)									
		With S-9					Without S-9				
		TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Vehicle Control	100µl of DMSO	19 ±0.6	101 ±1.0	264 ±1.0	21 ±0.6	8 ±1.0	20 ±1.0	103 ±1.0	266 ±1.5	23 ±1.5	10 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Gen F-20 Plus Spray	0.05	19 ±0.6	101 ±1.0	263 ±1.0	22 ±0.6	9 ±0.6	21 ±1.0	103 ±1.0	265 ±1.0	24 ±1.0	11 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	1.1
	0.1	19 ±0.6	101 ±0.6	262 ±1.2	21 ±0.6	9 ±0.6	21 ±1.5	104 ±1.2	265 ±2.0	24 ±1.0	10 ±2.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	1.0
	0.5	19 ±0.6	101 ±1.5	263 ±1.0	21 ±0.6	8 ±1.0	21 ±1.5	103 ±2.0	266 ±1.5	23 ±1.0	9 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	0.9
	1	18 ±0.6	101 ±0.6	262 ±1.5	21 ±0.6	8 ±0.6	20 ±0.6	103 ±1.0	265 ±2.1	23 ±1.5	9 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	5	18 ±0.6	98 1.0	263 ±1.5	20 ±1.0	7 ±0.6	20 ±1.0	99 ±1.0	265 ±1.5	23 ±2.0	10 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0
Positive control	100µl of respective positive control	406† ±4.0	412† ±2.5	612† ±2.5	175† ±6.4	106† ±4.0	416† ±5.7	438† ±7.4	689† ±6.8	185† ±5.0	116† ±1.5
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	21.7	4.1	2.3	8.5	13.2	20.8	4.2	2.6	7.9	12.0

Values of Revertants are in Mean±SD

† = Statistically significant than the vehicle control group ($p < 0.001$).

TABLE 3. SUMMARY OF COLONY COUNTS OF REVERTANTS OF TRIAL -II
Pre Incubation method

Refer Appendix-3

Treatment	Test concentration (µl/plate)	No. of Revertants (Mean of 3 Plates)									
		With S-9					Without S-9				
		TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Vehicle Control	100µl of DMSO	19 ±0.6	101 ±1.0	262 ±1.5	21 ±1.0	11 ±0.6	21 ±1.0	104 ±0.6	267 ±3.2	22 ±0.6	11 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Gen F-20 Plus Spray	0.05	20 ±0.6	101 ±0.6	264 ±1.0	22 ±0.6	10 ±1.0	21 ±0.6	103 ±0.6	266 ±1.5	24 ±1.0	10 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.1	0.9	1.0	1.0	1.0	1.1	0.9
	0.1	19 ±0.6	101 ±0.6	264 ±0.6	22 ±0.6	10 ±0.6	22 ±1.0	104 ±1.0	266 ±1.0	24 ±0.6	10 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	0.5	19 ±0.6	101 ±0.6	263 ±0.6	21 ±0.6	10 ±0.6	21 ±0.6	103 ±0.6	266 ±1.0	23 ±0.6	10 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1	18 ±0.6	100 ±0.6	262 ±0.6	21 ±1.5	9 ±0.6	20 ±0.6	103 ±0.6	265 ±2.5	22 ±2.1	9 ±0.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	1.0	0.9	1.0	1.0	1.0	0.9	1.0
	5	18 ±0.6	99 ±0.6	262 ±1.0	20 ±0.6	8 ±0.6	20 ±1.2	99 ±1.2	266 ±1.0	21 ±1.0	10 ±1.0
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	0.9	1.0	1.0	0.9	0.8	1.0	1.0	1.0	1.0	0.9
Positive control	100µl of respective positive control	413† ±4.0	421† ±3.6	617† ±2.5	181† ±6.0	106† ±1.0	419† ±3.6	446† ±4.5	697† ±2.0	198† ±2.5	112† ±3.6
	Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Fold increase	21.3	4.2	2.4	8.6	9.9	20.0	4.3	2.6	9.1	10.5

Values of Revertants are in are Mean±SD

† = Statistically significant than the vehicle control group **(**$p < 0.001$).

18. APPENDICES

APPENDIX 1. INDIVIDUAL PLATE COUNT OF INITIAL CYTOTOXICITY TEST

Test item concentration (µg/plate)	No. of Revertants /Plate in triplicates									
	With S-9					Without S-9				
	R1	R2	R3	Average	Bacterial lawn intensity	R1	R2	R3	Average	Bacterial lawn intensity
Vehicle control	102	102	103	102	4+	102	102	104	103	4+
0.8 µl	104	100	103	102	4+	104	101	103	103	4+
0.9 µl	103	102	100	102	4+	102	102	101	102	4+
1 µl	102	102	100	101	4+	102	101	102	102	4+
2 µl	100	100	101	100	4+	100	102	100	101	4+
3 µl	100	100	101	100	4+	101	100	100	100	4+
4 µl	99	98	99	99	4+	101	100	100	100	4+
5 µl	97	98	98	98	4+	99	100	98	99	4+

Lawn intensity: 4+ = Thick lawn: Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

APPENDIX 2. INDIVIDUAL PLATE COLONY COUNT OF REVERTANTS OF TRIAL-I

Plate Incorporation Method

Treatment	Test concentration (µl/plate)	Plate No.	No. of Revertants/Plate in triplicates									
			With S-9					Without S-9				
			TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Vehicle Control	100µl of DMSO	1	19	100	264	20	7	21	102	266	22	9
		2	18	101	263	21	8	19	104	265	23	10
		3	19	102	265	21	9	20	103	268	25	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Gen F-20 Plus Spray	0.05	1	20	101	263	22	9	22	103	266	24	11
		2	19	100	264	21	9	20	102	265	23	12
		3	19	102	262	22	8	21	104	264	25	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	0.1	1	19	101	263	21	8	20	103	265	23	10
		2	20	102	263	22	9	23	105	267	25	8
		3	19	101	261	21	9	21	103	263	24	12
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	0.5	1	18	99	263	21	7	20	101	266	23	9
		2	19	101	262	20	8	21	103	265	22	8
		3	19	102	264	21	9	23	105	268	24	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	1	1	18	100	262	21	8	20	102	264	23	10
		2	19	101	264	20	8	21	104	267	21	9
		3	18	101	261	21	7	20	103	263	24	9
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	5	1	18	98	261	19	7	20	99	264	21	9
		2	18	97	264	20	7	21	98	267	23	10
		3	17	99	263	21	8	19	100	265	25	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Positive Control	100µl of respective positive control	1	402	410	610	170	102	410	432	691	190	115
		2	405	412	615	172	105	418	435	694	185	116
		3	410	415	612	182	110	421	446	681	180	118
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Lawn intensity: 4+= Thick lawn: Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

APPENDIX 3. INDIVIDUAL PLATE COLONY COUNT OF REVERTANTS OF TRIAL-II

Pre Incubation Method

Treatment	Test concentration (µl/plate)	Plate No.	No. of Revertants/Plate in triplicates									
			With S-9					Without S-9				
			TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 98	TA 100	TA 102	TA 1535	TA 1537
Vehicle Control	100µl of DMSO	1	19	101	264	20	10	22	103	263	22	11
		2	19	102	261	21	11	20	104	269	22	10
		3	20	100	262	22	11	21	104	268	21	11
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Gen F-20 Plus Spray	0.05	1	20	101	263	23	9	22	104	265	23	10
		2	20	102	265	22	10	21	103	268	24	9
		3	19	101	264	22	11	21	103	266	25	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	0.1	1	19	102	264	21	9	21	105	267	24	9
		2	20	101	264	22	10	22	103	265	25	10
		3	19	101	263	22	10	23	104	266	24	11
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	0.5	1	19	100	262	21	9	21	103	267	23	9
		2	19	101	263	22	10	22	104	265	23	10
		3	18	101	263	21	10	21	103	266	24	11
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	1	1	18	101	262	23	9	20	103	263	24	9
		2	19	100	261	20	9	21	104	265	20	9
		3	18	100	262	21	8	20	103	268	21	10
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	5	1	18	98	261	20	8	21	100	265	21	9
		2	18	99	263	19	8	21	98	266	22	10
		3	17	99	262	20	9	19	98	267	20	11
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Positive Control	100µl of respective positive control	1	409	418	615	175	105	415	450	699	195	115
		2	412	425	620	180	107	420	441	697	200	108
		3	417	420	617	187	106	422	446	695	198	113
		Lawn Intensity	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Lawn intensity: 4+= Thick lawn: Distinguished by a healthy (Normal) background lawn comparable to solvent control plates.

APPENDIX 4. INDIVIDUAL COLONY COUNT OF VIABLE COUNT

Name of the strain	Required cell counts	Trial	No. of colonies of 10^{-7} dilutions in triplicates			Average
			R1	R2	R3	
TA 98	1-2 x 10^9	Trail 1	122	125	126	124
		Trail 2	127	130	128	128
TA 100	1-2 x 10^9	Trail 1	150	152	157	153
		Trail 2	158	156	155	156
TA 102	1-2 x 10^9	Trail 1	162	165	170	166
		Trail 2	171	175	178	175
TA 1535	1-2 x 10^9	Trail 1	110	112	119	114
		Trail 2	121	123	127	124
TA 1537	1-2 x 10^9	Trail 1	100	102	105	102
		Trail 2	108	110	113	110

APPENDIX 5. QUALITY CONTROL PLATES DATA

Sl. No.	Sterility Controls	Trial I (Plate Incorporation Method)	Trial II (Pre Incubation Method)
1	Gen F-20 Plus Spray (5µl/plate)	No microbial contamination	No microbial contamination
2	DMSO	No microbial contamination	No microbial contamination
3	S-9 Mix	No microbial contamination	No microbial contamination
4	Minimal Glucose Agar Plates	No microbial contamination	No microbial contamination
5	Strain Inoculation broth	No microbial contamination	No microbial contamination
6	PBS	No microbial contamination	No microbial contamination
7	Overlay Agar	No microbial contamination	No microbial contamination