



**MUTAGENIC REVERSION ASSAY:  
Salmonella typhimurium HIS**

**Test substance: FR-91**

**Submitted by: F. Chacon**

**Laboratory: Interlab Group**



I.	INTRODUCTION .....	3
II.	ASSAY .....	4
III.	MATERIAL AND METHODS .....	4
	a) Strains .....	4
	b) Metabolic activation .....	5
	c) Cultures .....	5
	d) Positive and negative controls .....	6
	e) Test substance .....	6
	f) Assay .....	6
IV.	RESULTS AND DISCUSSION .....	7



**MUTAGENESIS STUDIES: Test substance FR-91**  
**Ames Test with Salmonella typhimurium**

**I. INTRODUCTION**

A study has been carried out on the substance submitted to our laboratory by F. Chacon with the denomination FR-91. The samples received were assigned the reference number 5187 A. Aliquots of these samples have been deposited in our reference library. The Salmonella/microsome test has been, in recent years, of considerable help in the identification of mutagens and carcinogens (Ames et al. 1975, Purchase 1982).

The molecular changes induced in the bacterial genome should be considered as evidence of genotoxic activity. Given that DNA is the universal carrier of genetic information in mammals and bacteria, analogous alterations in the nucleotide sequence can have mutagenic, carcinogenic or cytotoxic effects in mammals.

For mutagenic studies in bacterial genetic models, different indicator organisms have been used to be able to detect the varied spectrum of molecular gene mutations (base substitutions, reading frame shifts due to insertions or deletions, etc.).

The specificity of the bacterial strains for different types of preparations has been exhaustively demonstrated by the use of appropriate reference substances.

The sensitivity of the assay is increased by the use of hepatic enzymes prepared in the laboratory from mammals pretreated with Aroclor 1254.

These in vitro systems can be used to determine whether or not:

- The substance being tested has mutagenic properties per se
- Those possible metabolites generated by biotransformation --mixed function oxidases-- are genotoxically active.





-A direct mutagenic agent is detoxified by hepatic microsomal enzymes.

This assay has been carried out according to the norms of the EU, including Directive 75/831, which establishes toxicological analytic methods (84/449 and 88/302/EEC) part B, Toxicological Methods for Annex VIII and the method detailed in the Official Bulletin of the European Communities N° L251/143, 19-9-84 B.14 -Other effects: Mutagenesis -- Reverse mutation assay on Salmonella typhimurium, in accordance with the provisions of Directive 83/571/EEC for pharmaceutical specialties.

## II. ASSAY

The system of histidine reversion in Salmonella typhimurium is a microbial assay which permits the measurement of his<sup>-</sup>-his<sup>+</sup> reversion induced by chemical substances responsible for producing mutations by base substitutions or mutations which cause a reading frame shift at the level of the organism's genome.

## III. MATERIAL AND METHODS

### a) Strains

The following bacterial strains, whose genetic characteristics are described below, were used:

	His gene	Additional mutations			Mutation type
		LPS	Repair	R factor	
TA 1535	His G 46	rfa	uvr B	-	substitution
TA 1538	His D 3052	rfa	uvr B	-	displacement
TA 98	His D 3052	rfa	uvr B	pKM 101	displacement
TA 100	His G 46	rfa	uvr B	pKM 101	substitution

More detailed genetic characteristics are described in Maron and Ames (1983).





## b) Metabolic activation

Each of the experiments was carried out using metabolic fractions from rat livers. These S9 metabolic fractions were prepared according to the following protocol:

Five days before the hepatic extraction, four adult Wistar rats were injected intraperitoneally with Aroclor 1254 diluted in sesame oil in doses of 500 mg/Kg. The livers were extracted, homogenized mechanically in 0.15 M KCl at a w/v ratio of 1:3.

The homogenates were centrifuged at 9000 x g for 15 minutes. The supernatants were filtered in Millipore filters and stored in liquid nitrogen (-196°C) until use. They were never stored in liquid nitrogen for over one month.

In accordance with Ames et al. (1975), the S9 mixture was the following:

- 10% S9
- 100 mM phosphate buffer, pH 7.4
- 5 mM glucose-6-phosphate
- 4 mM NADP
- 8 mM MgCl<sub>2</sub>
- 33 mM KCl

## c) Cultures

The cultures were incubated at 37°C in the dark with agitation until the end of the logarithmic growth phase or the beginning of the stationary phase. The cell density was always between  $1 \times 10^8$  and  $1 \times 10^9$  cells/ml.



**d) Positive and negative controls**

The following reference substances were used to verify the response of the strain and to verify the activity of the S9 fraction used.

**-Positive**

- Sodium azide ( $\text{NaN}_3$ )
- 2-nitrofluorene (2NF)
- 2-aminofluorene (2AF)
- Cyclophosphamide (CP)

**-Negative**

- Distilled water

**e) Test substance**

The test substance, FR-91, was assayed at four serial concentrations, at none of which toxicity for the bacteria was observed.

The concentrations were:

- undiluted pure product
- dilutions 1/10, 1/100, 1/1000

**f) Assay**

Of each of the test and control solutions, 0.1 ml was added to 0.1 ml of a bacterial culture of each of the different strains, followed by 0.5 ml of the S9 mixture or buffer and 3 ml of soft agar. The contents of the tubes was mixed rapidly and tipped into petri dishes with minimal agar (Voger & Bonner 1956). For each of the dilutions tested in each of the four strains, triplicates were prepared, except in the case of the negative controls, for which sextuplicates were set up in each of the experiments (spontaneous reversion).





The plates were incubated at 37°C for 72 hours and then counted (seeded Tuesday 10.08.90, read Friday 13.08.90).

#### IV. RESULTS AND DISCUSSION

The results are presented in the tables in Annex I (individual values and means).

Survival always falls within the values of  $0.98 \times 10^8$  and  $1.57 \times 10^8$  per plate.

The values for the negative controls (distilled water) were within the normal range found in this laboratory and mentioned in the scientific literature for these strains of Salmonella typhimurium.

The values for the positive controls were, on the contrary, inducers of a substantial genotoxic response of the magnitude expected for this strain. Thus positive effects were observed for 2-Aminofluorene and cyclophosphamide after its biotransformation by microsomal enzymes and direct positive mutagenic effects were seen with for sodium azide and 2-Nitrofluorene.

In conclusion, in the presence and absence of the S9 mixture, there was no significant increase in the total number of revertants in the concentration range tested; thus the test substance submitted does not induce mutations in the bacteria exposed in vitro. We thus concluded that, under these conditions, the sample evaluated is not mutagenic.





We declare that the results emitted in this report were obtained according to the protocol described.

Madrid, 17 August 1991

LABORATORY DIRECTOR

A handwritten signature in cursive script, enclosed within a hand-drawn oval.

José Miguel Sicilia Socias

STUDY DIRECTOR

A handwritten signature in cursive script.

Luis de la Fuente Ramírez

QUALITY CONTROL TECHNICIAN

A handwritten signature in cursive script.

Angel Villanueva Rí





## ANNEX I: RESULTS TABLES



REVERSION ASSAY: Ames Test on *S. typhimurium* TA 1535

PRODUCT: FR-91

METHOD: Direct plate incorporation

EVALUATION: Visual

mg/plate	His revertants/plate				Survival x 10 <sup>6</sup>
	-S9	Mean	+S9	Mean	
Negative control	12	10.3	14	12.83	145
Dist. water	8		11		
	11		9		
	10		12		
	12		16		
	9		15		
Undiluted	12	12.7	18	19.67	146
	14		20		
	12		21		
1/10	13	12.7	21	17	139
	11		16		
	14		14		
1/100	15	13.7	19	18.67	142
	13		18		
	13		19		
1/1000	11	14	17	19	144
	16		21		
	15		19		
Positive controls	654	606	1450	1441	120
-S9: NaN <sub>3</sub> 1.5	598		1438		
+S9: CP 500	567		1435		

Date: 13/08/91

Conclusion: Assay negative





**REVERSION ASSAY: Ames Test on S. typhimurium TA 1538**

**PRODUCT: FR-91**

**METHOD: Direct plate incorporation**

**EVALUATION: Visual**

mg/plate	His revertants/plate				Survival x 10 <sup>6</sup>
	-S9	Mean	+S9	Mean	
Negative control	10	11	14	14.67	157
Dist. water	9		15		
	11		14		
	8		13		
	15		17		
	13		15		
Undiluted	7	8.67	12	13	145
	8		13		
	11		14		
1/10	8	9	12	13.67	147
	9		14		
	10		15		
1/100	9	10	16	14.33	137
	10		14		
	11		13		
1/1000	8	8.33	16	13	150
	7		11		
	10		12		
Positive controls	572	557	1423	1487	120
-S9: 2NF 5	564		1562		
+S9: 2AF 10	534		1476		

Date: 13/08/91

Conclusion: Assay negative





**REVERSION ASSAY: Ames Test on S. typhimurium TA 98**  
**PRODUCT: FR-91**  
**METHOD: Direct plate incorporation**  
**EVALUATION: Visual**

mg/plate	His revertants/plate				Survival x 10 <sup>6</sup>
	-S9	Mean	+S9	Mean	
Negative control	26	36.8	50	51.83	153
Dist. water	37		48		
	32		62		
	35		45		
	43		54		
	48		52		
Undiluted	31	30.7	52	47.33	126
	29		43		
	32		47		
1/10	32	30.7	45	46	139
	34		42		
	26		51		
1/100	36	35	55	58	142
	37		56		
	32		36		
1/1000	38	35.3	43	48	144
	35		51		
	33		50		
Positive controls	440	472	1475	1507	120
-S9: 2NF 5	475		1543		
+S9: 2AF 10	501		1504		

Date: 13/08/91  
 Conclusion: Assay negative





**REVERSION ASSAY: Ames Test on S. typhimurium TA 100**  
**PRODUCT: FR-91**  
**METHOD: Direct plate incorporation**  
**EVALUATION: Visual**

mg/plate	His revertants/plate				Survival x 10 <sup>6</sup>
	-S9	Mean	+S9	Mean	
Negative control	132	139	211	221.2	118
Dist. water	135		199		
	144		234		
	150		225		
	132		248		
	143		210		
Undiluted	152	156	213	220.3	107
	143		225		
	172		223		
1/10	145	144	215	216.3	112
	135		224		
	152		210		
1/100	148	148	204	205	116
	154		213		
	143		198		
1/1000	126	135	221	211.3	110
	143		205		
	135		208		
Positive controls	765	768	956	979.3	98
-S9: 2NF 5	724		980		
+S9: 2AF 10	814		1002		

Date: 13/08/91

Conclusion: Assay negative





REVERSION ASSAY: Test TA 1535

Group	With- out S9	Normal value		With S9	Normal value		Survival
	Mean	Lower limit	Upper limit	Mean	Lower limit	Upper limit	x 10 <sup>6</sup>
Neg. control	10.30	3	37	12.83	3.6	44.4	145
Undiluted	12.70	3	37	19.67	3.6	44.4	146
1/10	12.70	3	37	17.00	3.6	44.4	139
1/100	13.70	3	37	18.67	3.6	44.4	142
1/1000	14.00	3	37	19.00	3.6	44.4	144
Pos. control	606.00			1441			120





**REVERSION ASSAY: Ames Test S. Typhimurium TA 100**

Group	With- out S9	Normal value		With S9	Normal value		Survival
	Mean	Lower limit	Upper limit	Mean	Lower limit	Upper limit	x 10 <sup>6</sup>
Neg. control	139.0	75	220	221.2	90	264	118
Undiluted	156.0	75	220	220.3	90	264	107
1/10	144.0	75	220	216.3	90	264	112
1/100	148.0	75	220	205.0	90	264	116
1/1000	135.0	75	220	211.3	90	264	110
Pos. control	768.00			979.3			98





## ANNEX II: BIBLIOGRAPHY



## BIBLIOGRAPHY

AMES, BN, J. McCANN and E. YAMASAKI. *Mutation Research* 31: 347-364 (1975)  
Methods for detecting carcinogens and mutagens with the salmonella/microsome  
mutagenicity test.

MARON, DM and BN AMES. *Mutation Research* 113: 173-215 (1983) Revised  
methods for the salmonella mutagenicity test.

PURCHASE, IFH. *Mutation Research* 99: 53-71 (1982) An appraisal of a predictive  
test for carcinogenicity.

VOGEL, H and DM BONNER. *J Biol Chem* 218: 97-106 (1956) Acetylornithase of  
E. coli: partial purification and some properties.

