

Addendum 1 (cont'd)

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**ING 911- Bacterial reverse mutation test
(Plate incorporation and Preincubation methods)**

2.2.2. NEGATIVE CONTROL

The negative control article will be the vehicle used for the formulation of the test article.

2.2.3. POSITIVE CONTROL ARTICLES

The following positive control articles are used :

Chemical	Strains	Concentration ($\mu\text{g}/\text{plate}$)
2-Nitrofluorene (2-NF)	TA98	5
Sodium azide (NaA)	TA100 and TA1535	10
9-Aminoacridine (9-AA)	TA1537	50
Methyl methanesulfonate (MMS)	WP2 <i>uvrA</i> pKM101	1000
2-Aminoanthracene (2-A)	all the strains with metabolic activation	5

2.2.4. METABOLIC ACTIVATION SYSTEM : S9 MIX

In many cases a test article is not itself mutagenic, but possesses a mutagenic activity through one or several metabolic derivatives. To take this phenomenon into account the test article is placed in presence of liver enzymes to mimic the normal process of metabolism (metabolic activation system). This solution is used at the rate of 0.5 ml per plate containing the test chemical or controls. For the plates tested without metabolic activation, phosphate buffer is used instead of S9 Mix, at the same rate.

PREPARATION OF ENZYME S9 FRACTION :

Commercially available freeze-dried S₉ fraction (IFFA CREDO, L'Arbresle, FRANCE) is kept between -80°C and -70°C. Each batch is checked by the manufacturer for sterility, protein content and enzymatic activities. This S₉ fraction is obtained from the liver of rats treated with a mixture of phenobarbital and methylcholantrene.

The S₉ fraction will be reconstituted using water for injection and will be used on the day of formulation.

PREPARATION OF S9 Mix :

The mixture of metabolic activation S9 Mix will be prepared immediately prior to the test and kept on ice during the test. Its composition will be as follows (for a 5 ml preparation) :

- MgCl₂ (0.4 M) + KCl (1.65 M) _____ 0.1 ml
- Glucose 6 Phosphate (1 M) _____ 0.025 ml
- NADP (0.1 M) _____ 0.2 ml
- Phosphate buffer (pH 7.4 - 0.2 M) _____ 2.5 ml
- S₉ fraction (batch n° : will be indicated in the report) _____ 0.5 ml
- Water for injection _____ 1.675 ml



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**ING 911- Bacterial reverse mutation test
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- Preparation : the test article will be prepared as a solution in the vehicle at different concentrations.
- Frequency of preparation : the formulations will be performed on every treatment day.
- Storage : at room temperature, protected from light.

2.4. ANALYSES

The determination of the stability of the formulated test article is the responsibility of the Sponsor.

3. METHODS AND EXPERIMENTAL DESIGN**3.1. TEST SYSTEM**

- Identification :
 - Mutants of *Salmonella typhimurium* LT₂ strains TA98, TA100, TA1535 and TA1537.
 - Mutant of *Escherichia coli* : WP2 *uvrA* pKM101.
- Origin :
 - *Salmonella typhimurium* : B. Ames Laboratory, University of California, Berkeley - USA.
 - *Escherichia coli* : MRC Cell Mutation Unit, University of Sussex, Brighton - UK.
- Genetic characteristics :

Strains	histidine biosynthesis (his ⁻ or tryp ⁻)	U.V. sensitive (uvr ⁻)	Crystal Violet sensitive (rfa ⁻)	Presence of R-factor plasmid(s)
TA98	his D 3052 (frameshift)	yes	yes	yes [*]
TA100	his G 46 (base-pair substitution)	yes	yes	yes [*]
TA1535	his G 46 (base-pair substitution)	yes	yes	no
TA1537	his C 3076 (frameshift)	yes	yes	no
WP2 <i>uvrA</i> pKM101	tryp E (base-pair substitution)	yes	-	yes [*]

* : Ampicillin-resistance



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These genetic characteristics are checked for each frozen stock culture preparation.

The number of colonies obtained in the absence of any treatment (spontaneous revertants) are compared versus historical data for each culture.

- Storage : Stock strains are maintained deep frozen in liquid nitrogen.

3.2. CULTURE CONDITIONS

- Fresh cultures in Nutrient Broth (Oxoid medium CM 67) are prepared from frozen stock for each experiment.
- The test is performed in Petri dishes (plates) containing Vogel-Bonner (VB) medium, composed as follows :
 - Stock saline solution⁽¹⁾ _____ 20 ml
 - 40 % glucose solution _____ 50 ml
 - Oxoid agar _____ 15 g
 - Distilled water _____ up to 1 litre.

⁽¹⁾stock saline solution in distilled water (g/litre):

• Mg SO ₄ . 7H ₂ O	10
• C ₆ H ₄ O ₇ . H ₂ O	100
• K ₂ H PO ₄ anhydrous	500
• Na NH ₄ HPO ₄ . 4H ₂ O	175

- The mixture containing test or control article, bacterial strain and S₉ Mix or sterile buffer as appropriate, is poured onto the VB medium using molten overlay agar composed as follows :

<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
• NaCl _____ 5 g	• NaCl _____ 5 g
• Biotin and L. Histidine (0.5 mM) _____ 100 ml	• Tryptophan (0.1 mg/ml) _____ 10 ml
• Oxoid agar _____ 6 g	• Oxoid agar _____ 6 g
• Distilled water _____ 900 ml	• Distilled water _____ 990 ml

3.3. PRE-TREATMENT PROCEDURES

- Test culture inocula . for each experiment, the test strain cultures are grown in nutrient broth (see § 3.2.) for 6-16 hours (in a 37 ± 1°C gyratory incubator) which is known to allow the cultures to grow up to the late exponential stationary or early stationary phase of growth (approximately 10⁸-10⁹ cells per ml) [1, 6]. The optical density of each overnight culture will be used to check the cell density.
- Identification of the culture the culture tubes will be labelled with the strain, the code of the stock inocula used and the date of the culture initiation.



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3.4. EXPERIMENTAL DESIGN**3.4.1. PRELIMINARY EXPERIMENT**

A preliminary study is performed using the plate incorporation method on one bacterial strain TA100, without and with metabolic activation, in order to evaluate the toxicity of the test article to the bacteria and to select the range of dose levels for the main study.

The highest dose level required is 5 000 µg/plate if the chemical and physical properties of the test article allow this dose level to be reached.

At least five different amounts of the test substance are tested with approximately half log intervals between test points, for example : 52 - 164 - 512 - 1 600 - 5 000 µg/plate.

Each experimental point is tested in triplicate. Concurrent negative (vehicle) and positive controls are included. After at least two days of incubation, the plates are scored as described in § 4 or placed at refrigerator temperature if the evaluations cannot be performed on the last day of incubation.

If the plates from at least 3 concentrations can be scored, the results constitute the actual mutagenicity data for TA100 for the first experiment.

3.4.2. MAIN STUDY

Two independent experiments are conducted to evaluate the mutagenicity of the test article. Each experimental point in the study is tested in triplicate.

The first experiment is conducted using the plate incorporation method at dose levels selected on the basis of the results obtained in the preliminary experiment.

The second experiment is performed to confirm or to complement the results of the first one (especially when equivocal or negative results are obtained) with modification of the experimental conditions using the preincubation method with a closer range of dose levels near to the top dose limit if necessary. If a clear positive response is observed in the first experiment, the requirement for a verification in a second experiment will be discussed with the Sponsor on a case-by-case basis.



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3.4.3. TREATMENT**• PLATE INCORPORATION METHOD**

After treatment each tube of molten overlay agar contains :

Without metabolic activation (- S9)	With metabolic activation (+ S9)
◆ 2 ml of molten agar	◆ 2 ml of molten agar
◆ 100 µl of bacterial culture	◆ 100 µl of bacterial culture
◆ 100 or 50 µl of the test article formulated, depending on the vehicle used	◆ 100 or 50 µl of the test article formulated, depending on the vehicle used
◆ 500 µl of sterile buffer	◆ 500 µl of S9 Mix

This mixture is poured immediately onto VB medium and allowed to solidify at room temperature.

The Petri dishes are incubated at $37 \pm 1^\circ\text{C}$ for 48 hours minimum.

• PREINCUBATION METHOD

Each incubation tube receives in the order indicated below :

Without metabolic activation (- S9)	With metabolic activation (+ S9)
◆ 500 µl of sterile buffer	◆ 500 µl of S9 Mix
◆ 100 µl of bacterial culture	◆ 100 µl of bacterial culture
◆ 100 or 50 µl of the test article formulated, depending on the vehicle used	◆ 100 or 50 µl of the test article formulated, depending on the vehicle used

This mixture is incubated for approximately 25 minutes at $37 \pm 1^\circ\text{C}$ under stirring.

After the incubation period, 2 ml of molten agar are added to the incubation tubes then poured onto VB medium and allowed to solidify at room temperature. The Petri dishes are incubated at $37 \pm 1^\circ\text{C}$ for 48 hours minimum



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3.5. EXPERIMENTAL CONTROLS

Every time an experiment is conducted, the following test controls are used :

- Untreated control with and without metabolic activation.
- The vehicle is used as the negative control (see § 2.2.2.) with and without metabolic activation.
- The standard mutagenic chemicals specific to the bacterial strain are used as the positive controls (see § 2.2.3.) with and without metabolic activation.
- The sterility is checked for the S9 Mix preparation, the test article and the vehicle.

4. DATA SCORING**4.1. PRECIPITATE EVALUATION**

The precipitate will be evaluated in the final mixture described in § 3.4.3. under the actual treatment conditions and evident to the unaided eye.

4.2. CYTOTOXICITY EVALUATION

After the end of the incubation period, the signs of toxicity (reduction in bacterial lawn or reduction in the number of colonies) are noted. The intensity of the cytotoxicity on the bacterial lawn will be estimated in each plate as evident to the unaided eye.

If the scoring cannot be done on the last day of incubation, the plates could be placed at refrigerator temperature without effect on the evaluation.

4.3. SCORING OF MUTANTS

After the end of the incubation period, the colonies (mutants/revertants) are counted automatically using an image analyser or manually if necessary.

If the scoring cannot be done on the last day of incubation, the plates could be placed at refrigerator temperature without effect on the counts.

5. DATA EVALUATION**5.1. DATA PROCESSING**

Individual plate counts from each experiment are recorded separately on line using the validated software COLONY (York Electronic Research - UK) which provides for each treatment the mean and standard deviation of the plate counts and the statistical analysis of the experiment.

Dunnett's test is used to compare the counts at each dose level with the negative control. If positive responses are obtained, then the presence of a dose response will be checked by a linear regression analysis



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5.2. ACCEPTANCE CRITERIA

The assay will usually be considered valid if the following criteria are met :

1. The mean negative control counts fall within the range of historical data,
2. The positive control chemicals induce clear increases in revertant numbers confirming discrimination between different strains and an active S₉ Mix preparation,
3. No more than 5 % of the plates in the assay are lost through contamination or any other unforeseen event.

5.3. EVALUATION CRITERIA

Biological relevance of the results should be considered first. Statistical methods (Dunnett's test at $p \leq 0.05$ and linear regression analysis) may be used as an aid in evaluating the test results [8]. Statistical significance should not be the only determination of a positive response.

Therefore mutagenicity evaluation of the test article will be based on the validity of the study (see § 5.2.), statistically significant, dose related or reproducible increase(s) in the number of revertant cells with evidence of a biological effect [9].

If the test does not give clearly negative or positive responses, the results will be considered as equivocal or questionable. In this case, the performance of a confirmatory assay will be recommended. Such additional testing will be performed only with the approval of the Sponsor (additional cost). Generally, dose levels selected for the confirmatory assay will be closely spaced around that which previously produced the equivocal response in an attempt to demonstrate a dose-effect relationship.



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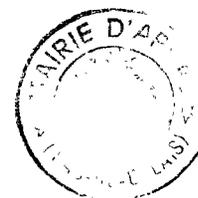
**ING 911- Bacterial reverse mutation test
(Plate incorporation and Preincubation methods)****6. QUALITY ASSURANCE**

This study will be subjected to Quality Assurance procedures in compliance with (checked as applicable) :

✓	F.D.A. : "Good Laboratory Practice" described in the U.S. Federal Register (Food and Drug Administration) dated 22 December 1978 with any applicable amendments.
✓	O.E.C.D. : "O.E.C.D. Principles of Good Laboratory Practice" concerning Mutual Acceptance of Data in the Assessment of Chemicals dated 26 November 1997 (C (97) 186 Final).
✓	E.P.A. : "Good Laboratory Practice Standards" described in the U.S. Federal Register (Environmental Protection Agency - FIFRA or TSCA) dated 29 November 1983 with any applicable amendments.
✓	M.H.W. : "Good Laboratory Practice Standards for Safety Studies on Drugs" described by the Japanese Ministry of Health and Welfare, dated 26 March 1997 (Ordinance n° 21).
✓	M.A.F.F. : "Good Laboratory Practice Standards for Toxicological Studies on Agricultural Chemicals" described by the Japanese Ministry of Agriculture, Forestry and Fisheries dated 10 August 1984 (59 Nohsan n° 3850).
✓	M.I.T.I. : "GLP Standards applied to Industrial Chemicals" described by the Ministry of International Trade and Industry (MITI) dated 31 March 1984 (Kikyoku n° 85) with any applicable amendments..

The standard protocol is inspected. Procedures similar to those used on this type of study are inspected periodically in the laboratory and data are audited periodically from a study of this type. The report will be reviewed to assure that it accurately describes the methods and procedures, and that the results accurately reflect the raw data. Reports on these activities will be made to the Study Director and to Management

Any analyses performed by the Study Sponsor or under the responsibility will not be audited by the Quality Assurance Unit.



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7. REPORT**Incidental reports**

The Study Sponsor will be informed promptly of any significant findings at any time during the study.

Draft report : no draft report will be issued.

Final report

The final report will be issued and 3 copies (2 bound and 1 unbound) sent to the Study Sponsor.

8. ARCHIVING

All raw data, supporting documents including original protocol and final report will be maintained in the archives of the testing facility for 5 years.

The test article will be kept 2 months after sending the final report, then it will be destroyed.

Duration of archiving starts after dispatch of the final report.

Once the period of archiving is over, at the request of the testing facility, the Study Sponsor can (cost not included) :

- ask to continue the archiving,
- ask for the material to be returned,
- ask for destruction of the material.

Chrysalis will not be held responsible for the safe keeping or transport of any data or samples removed or dispatched from the testing facility at the request of the Study Sponsor.



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**ING 911- Bacterial reverse mutation test
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- [2] Maron, D.M. and Ames, B.N. (1983). Revised Methods for Salmonella Mutagenicity Test. *Mutation Res.*, 113, 173-215.
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PROTOCOL : 755/001-D.

CHRYSALIS STUDY NUMBER : 755/001.

SPONSOR STUDY NUMBER : ING 911/AMTE/E COLI.

SIGNATURE PAGE

Approved by .

CHRYSALIS Preclinical Services - Europe

STUDY SPONSOR

Signature :



Name : A. FORICHON

Title : Study Director

Date : 15 February 1999.

Signature :

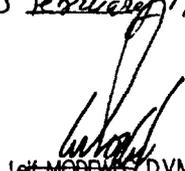


Name : B. DEMAGNY

Title : Study Monitor

Date : March 8th 1999

Signature :



Lef Mœwles D.V.M.
President
CHRYSALIS
Preclinical Services

Name :

Title : Management representative

Date : 16 Feb 99



Addendum 2 :
Quality Control Certificate of S9 fraction



Addendum 2 (cond't)

IFFA CREDO

a Charles River company

**Lyophilized S9 fraction
QUALITY CONTROL ASSAY DATA**

Lot number : SL 63
 Expiry date : July 31, 1999 (prolongated to 31/12/99)
 Volume : 1.5 ml
 Inducer Phenobarbital-Methylcholantren mixture
 Dose of induction : 50 - 20 mg/kg respectively

Species / strain : Rat / OFA Sprague Dawley
 Sex : Male
 Tissue : Liver
 Buffer : /

• **BIOCHEMICAL DATA**

	Value	Unit	Standard deviation
Proteins	38.5	mg/ml	3.40
Cytochrome P-450	23.9	nmol/ml	0.60
	0.62	nmol/mg of proteins	0.01
Cytochrome C reductase	51.8	nmol/min. x mg of proteins	9
Ethoxycoumarin deethylase	3.484	nmol/min. x mg of proteins	0.384
Ethoxyresofurin deethylase	362	pmol/min. x mg of proteins	34
Pentoxyresofurin deethylase	22	pmol/min. x mg of proteins	5
UDP Glucuronyltransferase	9.1	nmol/min. x mg of proteins	0.6

• **STORAGE AND HANDLING RECOMMENDATIONS**

Store at or below -15°C till the expiry date mentioned on the flask. Please note that the storage temperature is given exclusively for the FREEZE-DRIED S9 fraction form. The whole quantity must be reconstituted in the flask, using purified sterile water. The reconstituted S9 fraction should be maintained in ice (< +10°C), and used within few hours as the microsomal activity decrease after reconstitution.

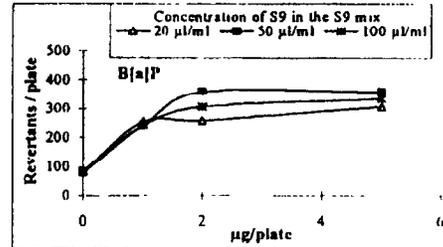
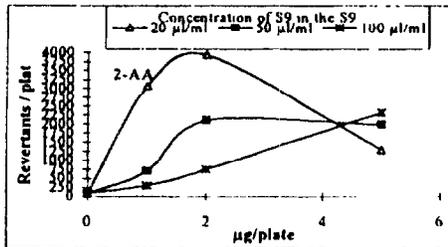
• **STERILITY TEST**

0 colonies of contaminating microflora was observed per 100 µl of product after 72 hours of incubation at 37°C on a Vogel-Bonner medium

• **PROMUTAGEN ACTIVATION DATA**

Reference : Maron, D & Ames, B, Mutation Research **113**, 173-215.

The capability of metabolic activation was tested using two promutagen compounds, 2-Aminoanthracen (2-AA) and Benzo[a]pyren (B[a]P) with 3 concentrations of S9 in the S9 mix (20 - 50 and 100 µl/ml of S9 mix). The test was conducted using triplicate plates on the strain TA 100 of Salmonella typhimurium. The mean number of revertant per plate obtained is indicated in the table below.



µl S9 per ml of S9 mix	Dose (µg/plate)	2-AA		B[a]P	
		Mean	Stand dev.	Mean	Stand dev.
20	0	84	9.54	84	9.54
	1	3070	133.1	257	7.81
	2	3938.67	373.43	259	22.61
	5	1291.33	87.23	307.33	21.08
50	0	80.33	6.43	80.33	6.43
	1	704.67	35.85	243.33	14.43
	2	2097.33	321.63	359.67	35.56
	5	1991.67	66	360	19.29
100	0	86.67	11.93	86.67	11.93
	1	297.33	15.28	242.33	16.8
	2	748.67	13.01	307	11.53
	5	2322.67	158.86	335.33	20.03

Date 03/15/97 and 16/6/99

François VEILLET
 In charge of the Laboratory

Marie-line POGGI DUFAUD
 Pharmacist

0310799 x 16/6/99



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 Boite Postale 1109 - 69592 L'Arbresle Cedex - France - Tel 04 74 61 60 60 - Telex 2206 04 74 01 60 60



Addendum 3 :
Historical data

The background data presented in this Addendum have been subject to internal checking.



Addendum 3 (cont'd)Historical data - Negative control spontaneous revertants - 1999

(Updated on 03/03/99)

Tester Strain	S9 Mix	Number of experiments	Average of the means	Standard Deviation	Range of the means	
					Lower	Upper
TA 98	-	323	21	5.0	11	40
TA 98	+	327	31	6.0	13	54
TA100	-	308	121	31.0	54	213
TA 100	+	316	118	26.0	56	210
TA 1535	-	308	20	9.0	6	63
TA 1535	+	306	16	5.0	8	50
TA 1537	-	309	6	3.0	1	20
TA 1537	+	310	8	4.0	2	32
WP2PuvrA	-	22	49	18.0	20	81
WP2PuvrA	+	20	67	21.0	28	101

