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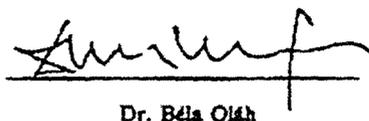
FINAL REPORT  
THE TESTING OF "HUMET" WITH  
SALMONELLA TYPHI-MURIUM REVERSE  
MUTATION ASSAY  
(AMES TEST)

1992

DECLARATION OF THE STUDY DIRECTOR

This study was performed in accordance with the Protocol agreed upon by Sponsor, the O.E.C.D. Guideline for Testing of Chemicals, number of study: 471 (1981 and continuing series) OECD: Paris and the Principles of Good Laboratory Practice (O.E.C.D., 12th May 1981). I declare that this report constitutes a true record of the actions undertaken and the results obtained in this study.

Date: 22.10.1992.



Dr. Béla Oláh  
Study Director

According to the research and development Assignment between HORIZON-MULTIPLAN PRODUCTIONS Ltd. and TOXICOLOGICAL RESEARCH CENTRE Ltd. The testing of HUMET test substance with the Salmonella typhimurium reverse mutation assay has been performed.

To the best of our knowledge and belief this study was carried out in accordance to the conditions of the contract between HORIZON MULTIPLAN PRODUCTIONS Ltd. (as Sponsor) and TOXICOLOGICAL RESEARCH CENTRE Ltd (as Testing Facility) insisting on the GLP requirements laid down therein.

So far as can be reasonably established the methods described and the results given in this report accurately reflect the data produced during the study.

The report has been checked up with raw data and confirmed as being in conformity with O.E.C.D. Principles of Good Laboratory Practice (O.E.C.D., 12th May 1981).

Date: 12.12.1992



Dr. Istvan Kiss  
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ANALYTICAL CERTIFICATION  
OF TEST SUBSTANCE : Responsibility of Sponsor

START OF EXAMINATION : 18.09.1992

END OF EXAMINATION : 01.10.1992

BASIS OF STUDY : OECD GUIDELINE FOR TESTING OF CHEMI-  
CALS (1981 and continuing series)  
OECD: PARIS

OECD NUMBER OF STUDY : 471

## QUALITY ASSURANCE STATEMENT

Study Number: 92/134-007M  
 Subject Title: "The testing of "HUMET" with Salmonella typhi-murium reverse mutation assay (AMES TEST)"  
 Test Substance: HUMET

In compliance with the Principles of Good Laboratory Practice this study has been inspected, and this report audited by the Quality Assurance Unit. As far as can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study. All inspections, data reviews and the report audit were reported in writing to the study director and to management. The dates of such inspections and of the report audit are given below:

Date	Inspections/audit	Date of Report to	
		Management and	Study Director
11.09.1992		11.09.1992	11.09.1992
29.09.1992		30.09.1992	29.09.1992
19.10.1992		19.10.1992	19.10.1992

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Date: 22.10.1992

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## 1. SUMMARY

The test substance "HUMET" (lyophilized form of the original substance) was studied for mutagenic activity by AMES' method. Experiments have been carried out twice with Salmonella typhi-murium strains TA98, TA100, TA1535, TA1537, TA1538 in presence and absence of rat liver fraction activated by Arochlor 1254 using appropriate positive and negative controls. Concentrations of the test substance were 7500, 3750, 1875, 937.5 468.75 microgram/plate.

The second study was performed under similar conditions as the first study, but in this case beside the using of 30% of S9 fraction preincubation was carried out.

Colony number in control plates and test plates was practically the same indicating that "HUMET" has no mutagenic activity in the used concentrations (<7500 microgram test substance/plate), by the used methods.

## 2. INTRODUCTION

Chemicals are examined with a bacterial system for mutagenic activity in the AMES-Test. The test has been adapted for use in detecting chemicals which are potential human carcinogens or mutagens. The Salmonella mutagenicity test along with other short-term assay is being used extensively to survey a variety of substances in our environment for mutagenic activity.

## 3. MATERIALS AND METHODS

### 3.1 TEST SUBSTANCES AND SOLVENT SELECTION

#### 3.1.1 Test Substance :

name : "HUMET"  
batch number : 2K

The test compound was labelled and stored in dark at 4°C.

The Sponsor put at our disposal a lyophilized form (as a test substance) of the original substance (78.50 g/l of dry remnant content) corresponding to the analytical certificate.

The analytical certificate of the test substance is shown in the APPENDIX.

#### Solubility Test

The solubility of the test substance was examined in water, Dimethyl Sulphoxide (DMSO), Dimethyl Formamide, 96% of ethanol, acetone and finally in aqueous potassium-pyrophosphate solution proposed by the Sponsor.

#### 3.1.2 Identification, Receipt and Storage

Test substance is provided by the Sponsor. We did not receive guidelines for handling and storage of the test substance. Storage circumstances were insured in accordance with the paragraph 3.1.1.

#### 3.1.3 Determination of Substance Concentrations

For determining concentrations to be used the guidelines of U.S. Environmental Protection Agency Gene-Tox Program were accepted. A 150 mg/ml basal solution was prepared from the test substance with steril water solution of Potassium pyrophosphate (1%) which was diluted in 4 steps, with log<sub>5</sub> dilution rate. For the main studies we used the same basal solution of test substance, but that was diluted in 4 steps with log<sub>2</sub> dilution rate. Test solutions were freshly prepared at the beginning and repeating of each test.

Since the test substance was not well soluble in the solvents as usual (water, DMSO, 96% of ethanol, acetone), therefore aqueous potassium-pyrophosphate solution was used as the most appropriate solvent of the test substance proposed by the Sponsor.

#### 3.1.4 Determination of Bactericid Effect of Test Substance

If the test substance has a toxicity effect, the highest concentration must reduce the survival of the cells of a minimum of 50 % or show a significant decrease of the spontaneous number of his<sup>+</sup> cells and/or of the background lawn of his<sup>+</sup> cells.

#### 3.2 TEST SYSTEM

Salmonella typhi-murium strains provided by Prof.B.N. Ames (Biochemistry Department, University of California, Berkeley USA).

## 3.2.1 Genotypes of the Strains Used for Mutagenesis Testing

TA Strain	his Mutation	Other markers	Plasmid
1535	G46	rfa uvrB	-
1538	D3052	rfa uvrB	-
1537	C3076	rfa uvrB	-
100	G46	rfa uvrB	pRM101
98	D3052	rfa uvrB	pRM101

There are several standard bacterial tester strains of different histidin mutants. TA1535 and TA100 strains can be used to detect mutagens causing base-pair substitutions, TA98, TA1537, and TA1538 to detect various kinds of frameshift mutations. In addition to the histidin mutation each strain has two additional mutations greatly increasing their sensitivity to mutagens: in one (uvrB) the repair system has been excised and in the other one (rfa) the bacterial lipopolysaccharide wall is permeable for larger molecules. The TA98 and TA100 carry an ampicilline resistance transfer factor (R-factor). Besides on this plasmid lies a gene (muc+) which in RecA+/lexA+ genotypes participates in "SOS" DNA repair.

The auxotroph strains are unable to grow on a minimal medium - containing inorganic salts and glucose as carbon source - except of spontaneous revertants, but in case of the presence of a mutagenic agent, after backmutations some of them are converted to prototroph. These can grow and form colonies even on a minimal medium. The increasing number of colonies indicate mutagenic activity.

## 3.2.2 Storage of the Test Strains

Frozen permanent copies of the tester strains are stored at -80°C. They were prepared from fresh overnight cultures to which DMSO was added as a cryoprotective agent. The frozen cultures was thawed at room temperature and a measured amount was used for inoculating the overnight cultures in the assay. A 20 µl inoculum was recommended for each 5 ml of broth.

## 3.2.3. Checking of the Tester Strains

Fresh broth cultures were used for these tests. All reagents, glassware, petri dishes inoculating loops etc. were sterile.

### 3.2.3.1. Histidine Requirement

The his- character of tester strains was confirmed by demonstrating the histidine requirement for growth on selective agar plates. The nutrients were added to minimal glucose agar before plates were poured. For each plate 0.1 ml of 0.1 M. L- histidine and 0.1 ml of 0.5 mM biotin are required. Control plates contained biotin but no histidine. The strains were inoculated onto the plates and incubated overnight at 37°C. No growth was observed on control plates.

### 3.2.3.2 rfa Mutation

Strains having the deep rough (rfa) character were tested for crystal violet sensitivity. For this test nutrient agar plates were inoculated with cultures of the strains to be tested and a sterile filter paper disc containing crystal violet (10 microlitres of a 1 mg/ml solution) was placed on the surface of each inoculated plate. After 12 h incubation a clear zone of inhibition (approximately 14 mm) appeared around the disc, indicating the presence of rfa mutants.

### 3.2.3.3 uvrB Mutation

The presence of this mutation was confirmed by demonstrating UV sensitivity in the strains. The strain cultures were plated across a nutrient agar plate then one half was irradiated by a 15/W germicid lamp at a distance of 33 cm. Non-R-factor strain (TA1537, TA1535, TA1538) were irradiated for 6 sec, and TA98 and TA100 were irradiated for 8 sec. The plates were incubated at 37°C at 12-24 h. Strains with the uvrB deletion grew only on the unirradiated side of the plate.

### 3.2.3.4 R-factor

To test ampicillin resistance, cultures were plated onto the surface of a medium containing ampicillin (10 microgram of 8 mg/ml in 0.02 N NaOH). After incubation only strains containing R-factor (TA98 and TA100) were grown.

### 3.2.4 Spontaneous Reversion

Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Spontaneous reversion of the

tester strains to histidine independence has been measured as a routine in mutagenicity experiments and expressed as the number of spontaneous revertants per plate. Our laboratory values for spontaneous revertants per plate are as follows: (-S9) TA1535: 7-30, TA98: 15-60, TA100: 75-200, TA1537: 3-30, TA1538: 15-25.

### 3.3 MEDIUMS AND CULTURE CONDITIONS

Experimental methods are essentially the same as those described by AMES et al. (1,2). *Salmonella typhi-murium* strains were kindly provided by Prof. B.N Ames (Biochemistry Department, University of California, Berkeley USA).

#### 3.3.1 Positive and negative controls

Positive and negative (untreated and solvent) controls was included in the experiment.

To prove integrity of the test system and effectiveness of the metabolic activation system known mutagenic compounds served as positive reference.

Assay	Chemical	Solvent	Responding Salmonella Strains
Nonactivation	Sodium azide (SAZ)	Water	TA1535
	4-nitro-o-pheny- lenediamine(NPD)	DMSO	TA1538,TA98
	9-aminoacridine (9AA)	Ethanol	TA1537 -
	Methyl methane sulphonate (MMS)	DMSO	TA100,
Activation	2-anthracine(2AA)	DMSO	For all

#### Solvent controls:

There was also included in each assay.

### 3.3.2 Solvent

Aqueous Potassium Pyrophosphate was used to prepare stock solutions of test material. All dilutions of test materials was made in same solvent.

### 3.3.3 Activation System

#### 3.3.3.1 Induction of Liver Enzymes

Male Wistar rats weighing around 200 g received a single i.p. injection of Aroclor 1254 (diluted in peanut oil) to a concentration of 200 mg/ml at a dosage of 500 mg/kg five days before sacrifice. The rats were given drinking water and food ad libitum until 12 h before sacrifice when food was removed.

#### 3.3.3.2 Removal of Liver from Rats

In order to obtain a clean S9 preparation, the livers must be removed aseptically using sterile surgical tools. On the fifth day of induction the rats were stunned by a blow to the head and then decapitated, and were placed on their back on an autopsy board. The fur was swabbed thoroughly with 70 % ethanol. The skin was cut and lifted from underlying layers. After swabbing and cutting the muscle layer the livers were excised.

#### 3.3.3.3 Preparation of Liver Homogenate S9 Fraction

All steps of procedure were carried out at 1-4 °C using cold sterile solutions and glassware. Freshly excised livers were placed in beakers containing 1 ml chilled 0.15 M KCl per g of wet liver and were washed several times. Washed livers were transferred to a beaker containing 0.15 M KCl /3 ml/g wet liver, and were homogenized in a Braun "Potter-S" homogenizer with a teflon pestle. Homogenates were centrifuged for 10 min at 9000 g (8000 rpm in rotor the A-50 of PRESPIN 50, MSE) and the supernatant was decanted and retained. Freshly prepared S9 fraction was distributed in 1-5 ml portions, frozen quickly and stored at -80°C. Sterility of the preparation was checked. Protein concentration was determined by the LOWRY's method (4).

#### 3.3.3.4 The S9 Mix

The S9 mix freshly prepared containing components as follows:

NADP (sodium salt) (SIGMA)	4 micromoles
D- glucose-6-phosphate (SIGMA)	5 micromoles
MgCl <sub>2</sub> (REANAL)	8 micromoles
KCl (REANAL)	33 micromoles
Sodium phosphate buffer, pH 7.4	100 micromoles
S9 homogenate from rat liver	as required

### 3.3.4 Medium

#### 3.3.4.1 Minimal Medium

Vogel-Bonner (VB) medium E	Per litre
Warm distilled water	670ml
Magnesium sulphate (7H <sub>2</sub> O)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous)	500 g
Sodium ammonium phosphate (4H <sub>2</sub> O)	175 g

Autoclave for 20 min at 121 °C with 2 % glucose and 1.5 % Bacto Agar

#### 3.3.4.2 Top Agar

Agar	6 g
Sodium chloride	5 g
Bidestilled water	1000 ml

Autoclave for 15 min at 121 °C. After cooling to 45 °C 10 ml aqueous 0.5 mM histidine-HCl, 0.5 mM biotin solution is added aseptically.

### 3.3.5 Procedure for Growing Cultures

Tester strain cultures were grown in nutrient broth No.2 (OXOID). Cultures were inoculated by thawed frozen copies and were incubated in a Gyrotony Water Bath Shaker G 76 (New Brunswick) for 12-14 h at 37°C at approximately 120 rpm.

### 3.4 DESCRIPTION OF THE TEST PROCEDURE

#### 3.4.1. Preliminary Study Method

To the accurate planning of the main study we had to carry out the examinations where we could reach wider concentration range (3.1.3).

During the preliminary study we established whether the test substance had bacteriostatic effect under the study conditions. The preliminary study was performed on TA100 and TA98 test strains under same conditions like in the main study with metabolic activity and without it.

#### 3.4.2. Main Study Method

From an overnight culture of each test strain 0.1 ml were added to separate test tubes containing 2.0 ml molten top agar supplemented with a trace of histidine and biotin.

The content of the tubes:

top agar	2 ml
test article or controls	50 microlitre
test strain culture	100 microlitre

For nonactivation tests 5 dose levels of test compound were added to the contents of appropriate tubes and poured over the surface of selective minimal agar plates.

In activation tests 5 dose levels of the test compound were added to the appropriate tubes containing bacteria. Just prior to pouring, an aliquot of S9 mix (0.5 ml) containing liver homogenate was added and the contents poured over surface of minimal agar plates and allowed to solidify.

The plates were then incubated at 37 °C for 48 hours.

The test was repeated in an independent experiment.

Since our purpose was to demonstrate mutagenetic effect and negative result was observable, therefore in the second study we increased the concentration of the S9 fraction (30%) inside the S9 MIX and preincubation was carried out.

The difference between the preincubation method and the plate incorporation method used in the main study is that the test strain, the solution of the test substance and the S9 Mix (in case of activation test) were measured into an empty tube and shaken preincubation was carried out for 30 minutes on 37 °C.

After the preincubation the appropriate amount of the top agar was added to the tubes and the content of tubes was poured over

the surface of minimal agar plates. the same amounts of the substances were measured in the second study as in the main study performed with plate incorporation method.

### 3.5 EVALUATION OF EXPERIMENTAL DATA

The colony numbers on control, positive control and the test plate were determined by a BIOTRAN III. Colony Counter (New Brunswick Co.) and were printed by a computer connected on line

to the counter. The printouts were collected, duplicated and one of them archived. Data are presented in tabular form. The printouts of actual and mean number of revertants per plate and the standard deviation can be found in Appendices.

The test material was considered to be mutagenic if the solvent control value is within the normal range, a chemical produces a positive dose response over three concentrations with the lowest increase equal to twice the solvent control value.

### 3.6 ARCHIVES

- protocol and the amendments
- all raw data
- correspondence
- study report

are stored in the archives of Toxicological Research Centers Ltd. for 5 years after the end of the study.

Samples of the test substance are stored for 5 years under the conditions given by the Sponsor.

## 4. RESULTS

### 4.1. PRELIMINARY STUDY

In the preliminary study the plate incorporation method was used as well as in the main study. The study was performed in two test strain under activation and non activation circumstances.

Experimental results were summarized in the Table I.

TABLE 1.

"HUMET" test substance  
1. Experiment 15.09.1992.  
Salmonella typhi-murium TA98

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	35 $\pm$ 2	33 $\pm$ 3
2	1500	36 $\pm$ 3	38 $\pm$ 2
3	300	35 $\pm$ 3	32 $\pm$ 4
4	60	34 $\pm$ 2	34 $\pm$ 2
5	12	37 $\pm$ 5	38 $\pm$ 2

Untreated	35 $\pm$ 3	36 $\pm$ 2
Untreated+Solvent	36 $\pm$ 5	36 $\pm$ 5
NFD (4 $\mu\text{g}$ )	2201	
2-AA (4 $\mu\text{g}$ )		3009

"HUMET" test substance  
1. Experiment 15.09.1992.  
Salmonella typhi-murium TA100

S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	130 $\pm$ 2	139 $\pm$ 4
2	1500	129 $\pm$ 2	138 $\pm$ 6
3	300	13 $\pm$ 2	136 $\pm$ 9
4	60	134 $\pm$ 3	139 $\pm$ 7
5	12	140 $\pm$ 1	138 $\pm$ 2

Untreated	140 $\pm$ 7	141 $\pm$ 3
Untreated+Solvent	135 $\pm$ 8	137 $\pm$ 3
MMS (2 $\mu\text{l}$ )	1682	
2-AA (4 $\mu\text{g}$ )		2122

## 42 MAIN STUDY

The test substance "HUMET" was studied for mutagenic activity by AMES' method. Experiments have been carried out twice with Salmonella typhi-murium strains TA98, TA100, TA1535, TA1537, TA1538 in presence and absence of rat liver fraction activated by Arochlor 1254 using appropriate positive and negative controls. Concentrations of the test substance were 7500 3750 1875 937.5 468.75 microgram/plate. In the second study S9 mix with increased S9 fraction content (30%) was used and preincubation was performed.

Experimental results were summarized in the Table 2 and 3.

Raw data are reported in the Appendix.

TABLE 2.

"HIMET" test substance  
1. Experiment 22.09.1992.  
Salmonella typhi-murium TA1535

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	13 $\pm$ 2	15 $\pm$ 1
2	3750	14 $\pm$ 0	14 $\pm$ 1
3	1875	13 $\pm$ 1	16 $\pm$ 1
4	937.50	13 $\pm$ 1	15 $\pm$ 1
5	468.75	11 $\pm$ 1	16 $\pm$ 0

Untreated 14 $\pm$  1 16 $\pm$  2  
 Untreated+Solvent 14 $\pm$  1 15 $\pm$  2  
 SAZ (4  $\mu\text{g}$ ) 589  
 2-AA (4  $\mu\text{g}$ ) 1486

"HIMET" test substance  
1. Experiment 22.09.1992  
Salmonella typhi-murium TA1538

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	14 $\pm$ 0	14 $\pm$ 2
2	3750	13 $\pm$ 3	13 $\pm$ 2
3	1875	14 $\pm$ 3	14 $\pm$ 3
4	937.50	11 $\pm$ 1	17 $\pm$ 2
5	468.75	12 $\pm$ 2	15 $\pm$ 2

Untreated 14 $\pm$  0 19 $\pm$  1  
 Untreated+Solvent 13 $\pm$  2 19 $\pm$  1  
 NPD(4  $\mu\text{g}$ ) 976  
 2-AA (4  $\mu\text{g}$ ) 415

"HIMET" test substance  
 1. Experiment 22.09.1992  
 Salmonella typhi-murium TA1537

"S9" mix

No. of concent.	concentration µg/plate	revertants mean±SD counted on 3 plates	
1	7500	21± 1	23± 2
2	3750	19± 1	24± 0
3	1875	19± 1	24± 2
4	937.50	21± 2	26± 1
5	468.75	22± 1	24± 1

Untreated 20± 3 25± 2  
 Untreated+Solvent 24± 1 24± 1  
 9AA (50 µg) 1958  
 2-AA (4 µg) 1765

"HIMET" test substance  
 1. Experiment 22.09.1992  
 Salmonella typhi-murium TA98

"S9" mix

No. of concent.	concentration µg/plate	revertants mean±SD counted on 3 plates	
1	7500	34± 3	36± 1
2	3750	29± 4	37± 2
3	1875	32± 0	38± 1
4	937.50	37± 1	35± 2
5	468.75	32± 2	37± 2

Untreated 35± 3 38± 1  
 Untreated+Solvent 34± 1 39± 1  
 NPD (4 µg) 2142  
 2-AA (4 µg) 3221

"HIMET" test substance  
 1. Experiment 22.09.1992  
 Salmonella typhi-murium TA100

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	130 $\pm$ 1	145 $\pm$ 5
2	3750	127 $\pm$ 5	142 $\pm$ 13
3	1875	133 $\pm$ 8	134 $\pm$ 3
4	937.50	138 $\pm$ 5	138 $\pm$ 6
5	468.75	144 $\pm$ 1	140 $\pm$ 4

Untreated 136 $\pm$ 11 145 $\pm$ 13  
 Untreated+Solvent 133 $\pm$  9 143 $\pm$ 10  
 MMS (2  $\mu\text{l}$ ) 1454  
 2 AA ( 4  $\mu\text{g}$ ) 2028

TABLE 3.

"HIMET" test substance  
 2. Experiment 29.09.1992  
 Salmonella typhi-murium TA1535

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	12 $\pm$ 1	13 $\pm$ 1
2	3750	12 $\pm$ 2	14 $\pm$ 1
3	1875	12 $\pm$ 2	11 $\pm$ 1
4	937.50	11 $\pm$ 2	15 $\pm$ 1
5	468.75	11 $\pm$ 1	14 $\pm$ 4

Untreated 15 $\pm$  3 15 $\pm$  1  
 Untreated+Solvent 12 $\pm$  1 15 $\pm$  2  
 SAZ ( 4  $\mu\text{g}$ ) 588  
 2-AA ( 4  $\mu\text{g}$ ) 559

T R C



"HIMET" test substance  
2. Experiment 29.09.1992  
Salmonella typhi-murium TA98

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	36 $\pm$ 4	39 $\pm$ 4
2	3750	37 $\pm$ 4	37 $\pm$ 5
3	1875	36 $\pm$ 3	39 $\pm$ 4
4	937.50	40 $\pm$ 5	45 $\pm$ 3
5	468.75	33 $\pm$ 2	44 $\pm$ 7

Untreated 35 $\pm$  1 41 $\pm$  6  
 Untreated+Solvent 34 $\pm$  5 38 $\pm$  5  
 NPD ( 4  $\mu\text{g}$  ) 584  
 2-AA ( 4  $\mu\text{g}$  ) 384

"HIMET" test substance  
2. Experiment 29.09.1992  
Salmonella typhi-murium TA100

"S9" mix

No. of concent.	concentration $\mu\text{g}/\text{plate}$	revertants mean $\pm$ SD counted on 3 plates	
1	7500	144 $\pm$ 5	141 $\pm$ 11
2	3750	128 $\pm$ 9	139 $\pm$ 3
3	1875	121 $\pm$ 3	146 $\pm$ 9
4	937.50	122 $\pm$ 4	146 $\pm$ 10
5	468.75	128 $\pm$ 19	137 $\pm$ 13

Untreated 129 $\pm$  4 161 $\pm$  5  
 Untreated+Solvent 141 $\pm$ 12 147 $\pm$  5  
 MMS (2  $\mu\text{l}$  ) 1096  
 2 AA ( 4  $\mu\text{g}$  ) 799

## 5. DISCUSSION

In each completed study (preliminary study, main study 1, main study 2 complemented with preincubation and increased S9 fraction concentration) the colony number in control plates and test plates was practically the same indicating that "HUMET" has no mutagenic activity and bactericid effect, in the used concentrations by the above methods ( $\leq 7500$  micrograms test substance/plate).

## 6. REFERENCES

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APPENDICES