



STUDY TO DETERMINE THE ABILITY OF
UVINUL T 150; BATCH 08-0083 (ZHT No. 93/246)
TO INDUCE MUTATION IN THE PRESENCE OF ULTRA
VIOLET LIGHT IN ONE TRYPTOPHAN-REQUIRING
STRAIN OF ESCHERICHIA COLI

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Sponsor project number: 44M0246/939007

Hazleton Europe study number: 729/185

Hazleton Microtest study number: BLG 9/EU

Hazleton Microtest report reference: EU1REBLG.009

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IN ONE TRYPTOPHAN-REQUIRING STRAIN OF ESCHERICHIA COLI

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DATE OF START OF EXPERIMENTAL WORK:

8 February 1994

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21 February 1994

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HE study number: 729/185

Sponsor project number: 44M0246/939007

STUDY DIRECTOR'S STATEMENT

I, the undersigned, declare that this study was carried out according to the principles of Good Laboratory Practice set out by the Department of Health (London), United Kingdom Compliance Programme, 1989.

I further declare that this report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study.

STUDY DIRECTOR: .. *M. Bell*

DATE: .. 2.6.94

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QUALITY ASSURANCE STATEMENT

I hereby confirm that this report is a full and accurate representation of the study data. The procedures involved in this type of study are regularly inspected. Dates of inspections of critical phases/procedures relevant to this study and dates of the findings observed during the inspections and reported to the Study Director are listed. All findings are reported to Management within 3 weeks of inspection. The date of the report audit is also stated.

Hazleton Europe will be responsible for archiving (at Otley Road, Harrogate, North Yorkshire) raw data, reports and relevant specimens connected with the study, unless otherwise requested by the Sponsor, for a maximum of 10 years. Following this period, the situation regarding continued archive facilities will be discussed with the Sponsor.

QUALITY ASSURANCE OFFICER: *Clare Waers*.....

DATE: *2 June 1994*

Date(s) of inspections:

15 February 1994

Date(s) of findings

reported to Study Director:

15 February 1994

Date of report audit:

24 May 1994

HM study number: BLG 9/EU

HE study number: 729/185

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1 SUMMARY

Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was assayed for an ability to induce mutation in *Escherichia coli* strain WP2, following exposure to a range of doses of UV light, in 2 separate experiments.

An initial range-finder experiment was carried out in which Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was assayed for toxicity in *E. coli* strain WP2 (in the absence of UV irradiation) using final concentrations of the test chemical at 8, 40, 200, 1000 and 5000 µg/plate plus a solvent and positive control. No toxicity was seen following any of these treatments (as would normally be indicated by thinning of the background bacterial lawn or marked reductions in revertant numbers), but precipitation of the test agent was observed on all plates treated at 1000 or 5000 µg/plate. To avoid multiple precipitating doses with the treatments in the presence of UV light, a maximum test dose of 1000 µg/plate was used in Experiment 1. Results of Experiment 1 treatments also showed no evidence of toxicity, but precipitation was observed on all plates treated at 1000 µg/plate, and so this dose was retained for Experiment 2 treatments, as an estimate of the limit of solubility in this assay system. A narrowed dose range was used for Experiment 2 treatments, in order to examine more closely those concentrations of Uvinul T 150; batch 08-0083 (ZHT No. 93/246) most likely to exhibit a mutagenic effect.

Negative (solvent) and positive control treatments were included with all treatments. Mean numbers of revertant colonies on negative control plates fell within acceptable ranges, and were significantly elevated by positive control treatments. The inclusion of a known photomutagen (8-methoxypsoralen) as a control in each experiment also demonstrated the ability of the method employed to detect the mutagenicity of a compound requiring photoactivation.

2 Following exposure to 4 different doses of UV light, Uvinul T 150; batch (05-2729) (ZHT No. 93/246) treatments of *E. coli* strain WP2 failed to result in any significant increases in revertant numbers. The data obtained therefore gave no indication of an ability of the test chemical to induce mutation following photoactivation.

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It was concluded that Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was not photomutagenic under the conditions used in this assay, when tested at concentrations up to a maximum of 1000 $\mu\text{g}/\text{plate}$ (a precipitating dose).

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2 INTRODUCTION

The European Scientific Committee for Cosmetology (SCC) has recommended the *in vitro* testing of sunscreens for photomutagenic potential (1). These requirements have been explained and summarized by Loprieno (2). In response to these suggestions published by the SCC, Molecular Toxicology (Microtest) have developed protocols for the testing of sunscreens for photomutagenicity (3,4). This report describes the bacterial assay.

Several *Escherichia coli* strains have been used for mutagen screening (5). Prominent among these are WP2 and its repair-deficient derivatives. WP2 is a tryptophan-requiring strain in which agents causing base substitution mutations can increase the frequency of Trp+ revertants (6). The following bacterial strain was used in this study:

| <u>Strain</u> | <u>Type of mutation</u> | <u>Mutant gene</u> |
|--------------------|-------------------------|--------------------|
| <i>E. coli</i> WP2 | base-pair substitution | tryptophan |

This *E. coli* strain is reverted to prototrophy by base change either at the site of the original alteration or elsewhere in the genome so that the original defect is suppressed (7).

When exposed to a mutagen, some of the bacteria in the treated population undergo genetic changes which revert them to a non-tryptophan-requiring state and they can then grow in the absence of exogenous tryptophan.

The objective of this study was therefore to evaluate the mutagenic activity of Uvinul T 150; batch 08-0083 (ZHT No. 93/246) in the presence of UV-irradiation, both unfiltered and filtered through glass, by examining the reversion of a strain of *E. coli*.

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3 MATERIALS

3.1 Test chemical

Uvinul T 150; batch 08-0083 (ZHT No. 93/246), an off-white powder, was received on 3 February 1994, and stored desiccated and refrigerated in the dark. Prior to the study, the test substance was characterized analytically by the Sponsor. The stability of the test chemical during the study period will be determined by the Sponsor at a later date, by reanalysis. The stability of the test chemical in DMSO (the vehicle used) and in water will also be determined analytically by the Sponsor. All analytical data may be requested from the Sponsor.

Test chemical solutions were prepared by dissolving Uvinul T 150; batch 08-0083 (ZHT No. 93/246) in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) to give the required maximum concentration treatment solution. This solution was filter-sterilized (Sartorius Minisart SRP filter, 0.2 μ m pore size) and further dilutions were made using sterile DMSO. Test chemical solutions were protected from light and used within 2½ hours of the initial formulation. Solutions were used as follows:

| Experiment | Concentration of treatment solution (mg/ml) | Final concentration (μ g/plate) |
|--------------|---|--------------------------------------|
| Range-finder | 0.08 | 8 |
| | 0.40 | 40 |
| | 2.00 | 200 |
| | 10.00 | 1000 |
| | 50.00 | 5000 |
| Experiment 1 | 0.016 | 1.6 |
| | 0.08 | 8 |
| | 0.40 | 40 |
| | 2.00 | 200 |
| | 10.00 | 1000 |
| Experiment 2 | 0.625 | 62.5 |
| | 1.25 | 125 |
| | 2.50 | 250 |
| | 5.00 | 500 |
| | 10.00 | 1000 |

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3.2 Controls

Negative controls comprised treatments with sterile DMSO, either unirradiated or exposed to the chosen doses of unfiltered or glass-filtered UV light. Further negative controls comprised the photoactivating positive control, 8-methoxypsoralen (8-MOP), but in the absence of light treatment. The positive control chemicals were supplied and used as tabulated below:

| Chemical | Source | Stock* concentration ($\mu\text{g/ml}$) | Final concentration ($\mu\text{g/plate}$) | Light |
|-----------------------------------|--|---|---|-------|
| 4-nitroquinoline 1-oxide (NQO) | Fluka Chemicals, Glossop, Derbys, UK | 200 | 20 | - |
| 8-methoxypsoralen (8-MOP) | Sigma Chemical Co, Poole, Dorset, UK | 5000 | 500 | + |

* Stock solutions were prepared in DMSO. Solutions of NQO were stored in aliquots at -80°C in the dark. 8-MOP was freshly prepared on the day of each experiment.

3.3 Preparation of buffer solution

Quantities were prepared as follows (per 100 ml):

| <u>Ingredient</u> | <u>Concentration</u> | <u>Quantity (ml)</u> |
|----------------------------|----------------------|----------------------|
| Sodium phosphate pH 7.4 | 500 mM | 20 |
| DL-tryptophan | 1 mg/ml | 2.58 |
| Casamino acids | 20% (w/v) | 0.62 |
| Sterile purified water | - | to volume |

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3.4 Bacteria

One strain of *Escherichia coli* (WP2) was used in this study. Bacteria were cultured in nutrient broth for 10 hours at 37°C in a shaking water bath. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics (tryptophan dependence and absence of the pKM101 ampicillin resistance factor). Checks were carried out according to De Serres and Shelby (8). For all experiments, treatments commenced within 2 hours of the end of the period of incubation.

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4 METHODS

4.1 Toxicity range-finder experiment

All treatments were performed under reduced laboratory lighting. Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was tested for toxicity in strain WP2, at the concentrations detailed in 3.1. Triplicate plates were used. Negative (solvent) and positive (NQO) controls were included in quintuplicate and triplicate respectively. These platings were achieved by the following sequence of additions to 2.5 ml of molten agar at 46°C:

- 0.1 ml of bacterial culture
- 0.1 ml of test agent solution or control
- 0.5 ml of buffer solution

followed by rapid mixing and pouring on to Minimal Davis agar plates. When set, the plates were inverted and incubated at 37°C in the dark for 3 days. Revertant colonies were then counted (4.4).

4.2 Mutation experiments

Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was tested for mutation in *E. coli* strain WP2 in the presence of unfiltered and glass-filtered UV light at the concentrations detailed in 3.1 and with the light fluences shown in 4.3. Platings were achieved as described in 4.1 (solvent controls in quintuplicate, all other treatments or controls in triplicate). Plates were allowed to stand for at least 10 minutes before irradiation, to allow equilibration of chemicals into the bacteria, and for the soft agar to set. Plates requiring irradiation were irradiated in groups (maximum of 3 plates per group), and were arranged such that no 2 replicate plates from the same treatment were irradiated together. All plates were kept under reduced lighting in the laboratory until the final irradiation was completed. Additional negative controls treated with solvent, 8-MOP and a set of test compound-treated plates were also kept in the laboratory in the dark and were not irradiated. All plates were then transferred to an incubator at 37°C, the plates inverted, and incubated for 3 days prior to counting.

4.3 UV irradiation

Irradiation of plates was performed using the apparatus described in Figure 1 (see Appendix 1). Petri dishes were exposed, without their lids, either directly or through a sheet of 3 mm glass, the absorbance spectrum of which is shown in Appendix 2. Plates were shielded from the light source whilst being positioned or removed. The light source was calibrated using an Osram Centra UV meter before and after each experiment and the results used to calculate the doses delivered (Appendix 1).

The doses used for irradiation were as follows:

| Experiment | Duration of treatment | mJ/cm ² | | |
|------------|-----------------------|--------------------|-----|-------------|
| | | UVA | UVB | UVA + glass |
| 1 | 11 sec | 5.3 | 1.8 | - |
| | 23 sec | 11.1 | 3.8 | - |
| | 8 min 31 sec | - | - | 230 |
| | 17 min 2 sec | - | - | 460 |
| 2 | 11 sec | 5.3 | 1.8 | - |
| | 23 sec | 11.1 | 3.8 | - |
| | 8 min 13 sec | - | - | 230 |
| | 16 min 26 sec | - | - | 460 |

4.4 Colony counting

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc), or manually for treatments of 5000 µg/plate, where precipitation of test agent prevented accurate automatic counting. For all plates, the background lawn was inspected for signs of toxicity.

4.5 Analysis of results

4.5.1 Treatment of data

Individual plate counts from all experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal range for mean numbers of spontaneous revertants on solvent control plates, for our laboratory, is presented in Appendix 7. The range quoted is based on historical control data accumulated from experiments where the response of the test strain to positive control compounds was considered satisfactory. The range presented is for negative control treatments of the test strain in the absence of UV irradiation only (UV irradiation has a tendency to increase the numbers of revertants observed).

For evaluation of test chemical and positive control data there are many statistical methods in use, and several are acceptable (9,10). The m-statistic was calculated to check that the data were Poisson-distributed (10), and then Dunnett's test was used to compare the counts of each dose with the control. For any positive responses, then the presence or otherwise of a dose response was checked by linear regression analysis (10).

4.5.2 Acceptance criteria

The assay was considered valid if the following criteria were met:

- 1) the mean negative control counts fell within an acceptable range (Appendix 7)
- 2) the positive control chemicals induced clear increases in revertant numbers
- 3) no more than 5% of the plates were lost through contamination or some other unforeseen event.

4.5.3 Evaluation criteria

A test compound was considered to be photomutagenic if:

- 1) the assay was valid (see 4.5.2)

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- 2) Dunnett's test gave a significant response ($p \leq 0.01$) after irradiation, and the data set(s) showed a significant dose correlation
- 3) the positive responses described in 2) were reproducible
- 4) significant responses were induced in the presence of UV light, but not in its absence
- 5) the induction of revertants occurred at significantly higher frequencies, or at lower doses, in the presence of UV light.

5 RESULTS

5.1 Toxicity, precipitation and dose selection

Range-finder experiment treatments were carried out using concentrations of Uvinul T 150; batch 08-0083 (ZHT No. 93/246) at 8, 40, 200, 1000 and 5000 $\mu\text{g}/\text{plate}$. These treatments showed no clear evidence of a toxic effect (see Appendix 4), as would normally be indicated by thinning of the background bacterial lawn or marked reductions in revertant numbers. However, precipitation of the test agent was observed following treatments of 1000 or 5000 $\mu\text{g}/\text{plate}$. To prevent treatment of multiple precipitating doses, and as an estimate of the limit of solubility in this assay system, a maximum test dose of 1000 $\mu\text{g}/\text{plate}$ was used for treatments with UV light in Experiment 1. The remaining test doses for Experiment 1 were as detailed in section 3.1.

Following Experiment 1 treatments, no clear evidence of toxicity was observed, but once again precipitation of the test agent was observed at 1000 $\mu\text{g}/\text{plate}$. Due to the lack of toxicity observed in the first experiment, but the presence of precipitation at 1000 $\mu\text{g}/\text{plate}$, this dose was retained as the maximum concentration for Experiment 2 treatments. For this experiment the dose range was narrowed (62.5 - 1000 $\mu\text{g}/\text{plate}$), in order to examine more closely those concentrations of Uvinul T 150; batch 08-0083 (ZHT No. 93/246) most likely to exhibit a mutagenic effect.

No clear evidence of toxicity was observed following Experiment 2 treatments, but precipitation of test agent was observed on all plates treated at 1000 $\mu\text{g}/\text{plate}$.

5.2 Mutation

The individual plate counts were averaged to give mean values which are presented in Appendices 5 and 6. From the data it can be seen that mean solvent control counts on unirradiated plates fell within the historical range (Appendix 7). Negative controls comprising 8-MOP treatments in the absence of UV light gave numbers of mutants corresponding to solvent control counts. Conversely, 8-MOP treatments in the presence of UV

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irradiation resulted in large increases in revertant numbers when compared with the concomitant irradiated solvent controls. The positive control chemical NQO also induced large increases in numbers of revertants, further demonstrating the capacity of the test strain to detect mutagenic activity. Finally, fewer than 5% of plates were lost and the study was therefore accepted as valid.

In both mutation experiments carried out in this study, Uvinul T 150; batch 08-0083 (ZHT No. 93/246) treatments with each of the 4 doses of UV light failed to result in a significant increase in revertant numbers, when compared with those counts observed on the solvent control plates (when the data were analysed at the 1% level using Dunnett's test, see Appendices 5 and 6). It was considered therefore, that the data obtained in this study provided no evidence of an ability of Uvinul T 150; batch 08-0083 (ZHT No. 93/246) to induce mutation, following photoactivation.

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6 CONCLUSION

It was concluded that Uvinul T 150; batch 08-0083 (ZHT No. 93/246) was not photomutagenic under the conditions used in this assay, when tested at concentrations up to a maximum of 1000 $\mu\text{g}/\text{plate}$ (a precipitating dose).

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APPENDIX 1

Calibration of the Osram Vitalux light source

Dosimetry for UVA and UVB was measured before and after each exposure of plates, in the centre and towards opposite sides of the 20 cm diameter field (see Fig 1). Duplicate readings were taken before exposure, at the three positions and a single measurement taken at position b following treatments. Exposure times to be used were calculated using the initial measurements, taking an average dose as:

$$\frac{a + 2b + c}{4}$$

where a, b and c are the doses at these detector positions.

The actual doses delivered, taking into account small drifts in light fluence over the duration of each experiment, were calculated by substituting the start and finish values for the 2b in the equation above.

Calibration measurements and calculated doses of UV light delivered are presented on the following pages.

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APPENDIX 1 (continued)

Light calibration and dose calculations for Experiment 1

| | | EXPOSURE TIME REQUIRED TO DELIVER SPECIFIED UV DOSE | | | | | | | |
|--|------|---|-----|------------|-----|------------|-----|-----------------|-----|
| | | CIRCLE (a) | | CIRCLE (b) | | CIRCLE (c) | | CIRCLE (b[end]) | |
| UVA (100 mJ/cm ²) | 1 | 3 min 33 | sec | 3 min 21 | sec | 3 min 38 | sec | 3 min 21 | sec |
| | 2 | 3 min 27 | sec | 3 min 21 | sec | 3 min 35 | sec | -- | |
| | mean | 3 min 30 | sec | 3 min 21 | sec | 3 min 36.5 | sec | 3 min 21 | sec |
| mJ/cm ² /min | | 29 | | 30 | | 28 | | 30 | |
| UVA (100 mJ/cm ² + glass) | 1 | -- | | 3 min 46 | sec | -- | | -- | |
| | 2 | -- | | 3 min 45 | sec | -- | | -- | |
| | mean | -- | | 3 min 45.5 | sec | -- | | -- | |
| mJ/cm ² /min | | -- | | 27 | | -- | | -- | |
| UVB (10 mJ/cm ²) | 1 | 1 min 06 | sec | 1 min 01 | sec | 1 min 08 | sec | 1 min 00 | sec |
| | 2 | 1 min 04 | sec | 1 min 00 | sec | 1 min 07 | sec | -- | |
| | mean | 1 min 05 | sec | 1 min 00.5 | sec | 1 min 07.5 | sec | 1 min 00 | sec |
| mJ/cm ² /min | | 9 | | 10 | | 9 | | 10 | |

Calculation of UV doses delivered

$$\text{UVA delivered by light source} = \frac{a + b + c + b(\text{end})}{4} = 29 \text{ mJ/cm}^2/\text{min}$$

$$\text{UVB delivered by light source} = \frac{a + b + c + b(\text{end})}{4} = 10 \text{ mJ/cm}^2/\text{min}$$

$$\text{UVA delivered through glass} = 27 \text{ mJ/cm}^2/\text{min}$$

| TIME | | mJ/cm ² | | |
|------|-----|--------------------|-----|-------------|
| min | sec | UVA | UVB | UVA + GLASS |
| 0 | 11 | 5.3 | 1.8 | - |
| 0 | 23 | 11.1 | 3.8 | - |
| 8 | 31 | - | - | 230 |
| 17 | 02 | - | - | 460 |

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APPENDIX 1 (continued)

Light calibration and dose calculations for Experiment 2

| | | EXPOSURE TIME REQUIRED TO DELIVER SPECIFIED UV DOSE | | | |
|--|------|---|----------------|----------------|-----------------|
| | | CIRCLE (a) | CIRCLE (b) | CIRCLE (c) | CIRCLE (b[end]) |
| UVA (100 mJ/cm ²) | 1 | 3 min 25 sec | 3 min 21 sec | 3 min 51 sec | 3 min 15 sec |
| | 2 | 3 min 22 sec | 3 min 21 sec | 3 min 48 sec | -- |
| | mean | 3 min 23.5 sec | 3 min 21 sec | 3 min 49.5 sec | 3 min 15 sec |
| mJ/cm ² /min | | 29 | 30 | 26 | 31 |
| UVA (100 mJ/cm ² + glass) | 1 | -- | 3 min 37 sec | -- | -- |
| | 2 | -- | 3 min 38 sec | -- | -- |
| | mean | -- | 3 min 37.5 sec | -- | -- |
| mJ/cm ² /min | | -- | 28 | -- | -- |
| UVB (10 mJ/cm ²) | 1 | 1 min 05 sec | 0 min 59 sec | 1 min 06 sec | 0 min 59 sec |
| | 2 | 1 min 05 sec | 0 min 58 sec | 1 min 07 sec | -- |
| | mean | 1 min 05 sec | 0 min 58.5 sec | 1 min 06.5 sec | 0 min 59 sec |
| mJ/cm ² /min | | 9 | 10 | 9 | 10 |

Calculation of UV doses delivered

$$\text{UVA delivered by light source} = \frac{a + b + c + b(\text{end})}{4} = 29 \text{ mJ/cm}^2/\text{min}$$

$$\text{UVB delivered by light source} = \frac{a + b + c + b(\text{end})}{4} = 10 \text{ mJ/cm}^2/\text{min}$$

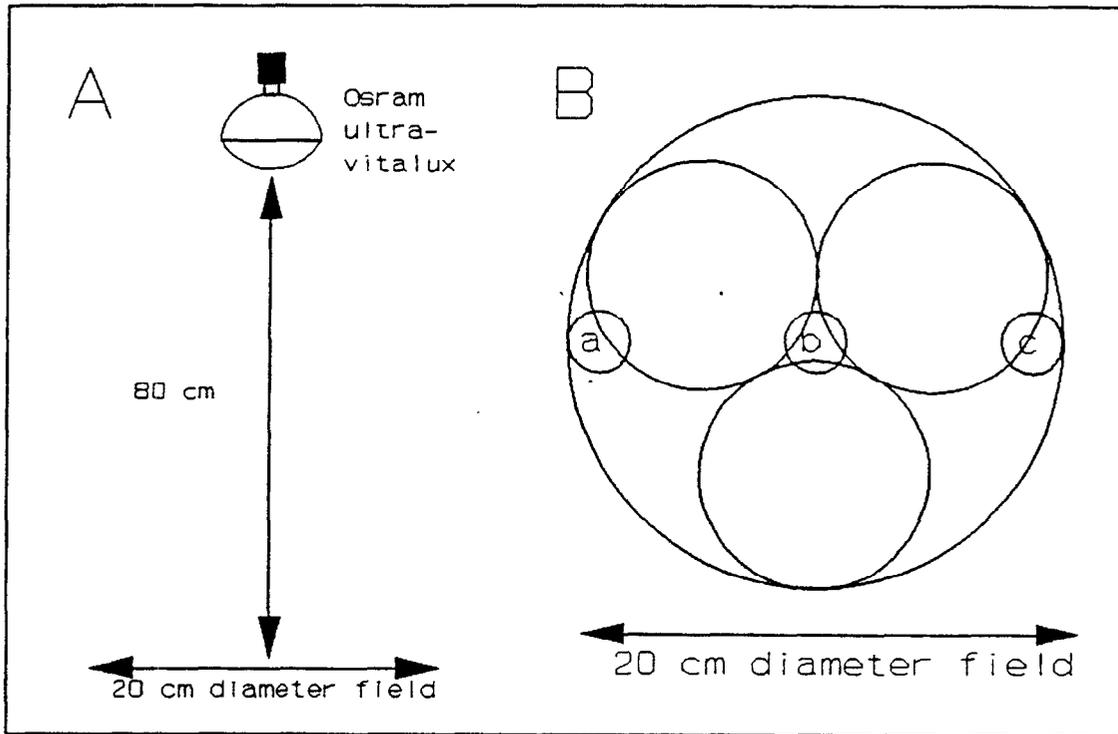
$$\text{UVA delivered through glass} = 28 \text{ mJ/cm}^2/\text{min}$$

| TIME | | mJ/cm ² | | |
|------|-----|--------------------|-----|-------------|
| min | sec | UVA | UVB | UVA + GLASS |
| 0 | 11 | 5.3 | 1.8 | - |
| 0 | 23 | 11.1 | 3.8 | - |
| 8 | 13 | - | - | 230 |
| 16 | 26 | - | - | 460 |

APPENDIX 1 (continued)

Figure 1

Diagram showing the irradiation apparatus



A Details of the lamp and setup

B Irradiation field and arrangement of petri dishes

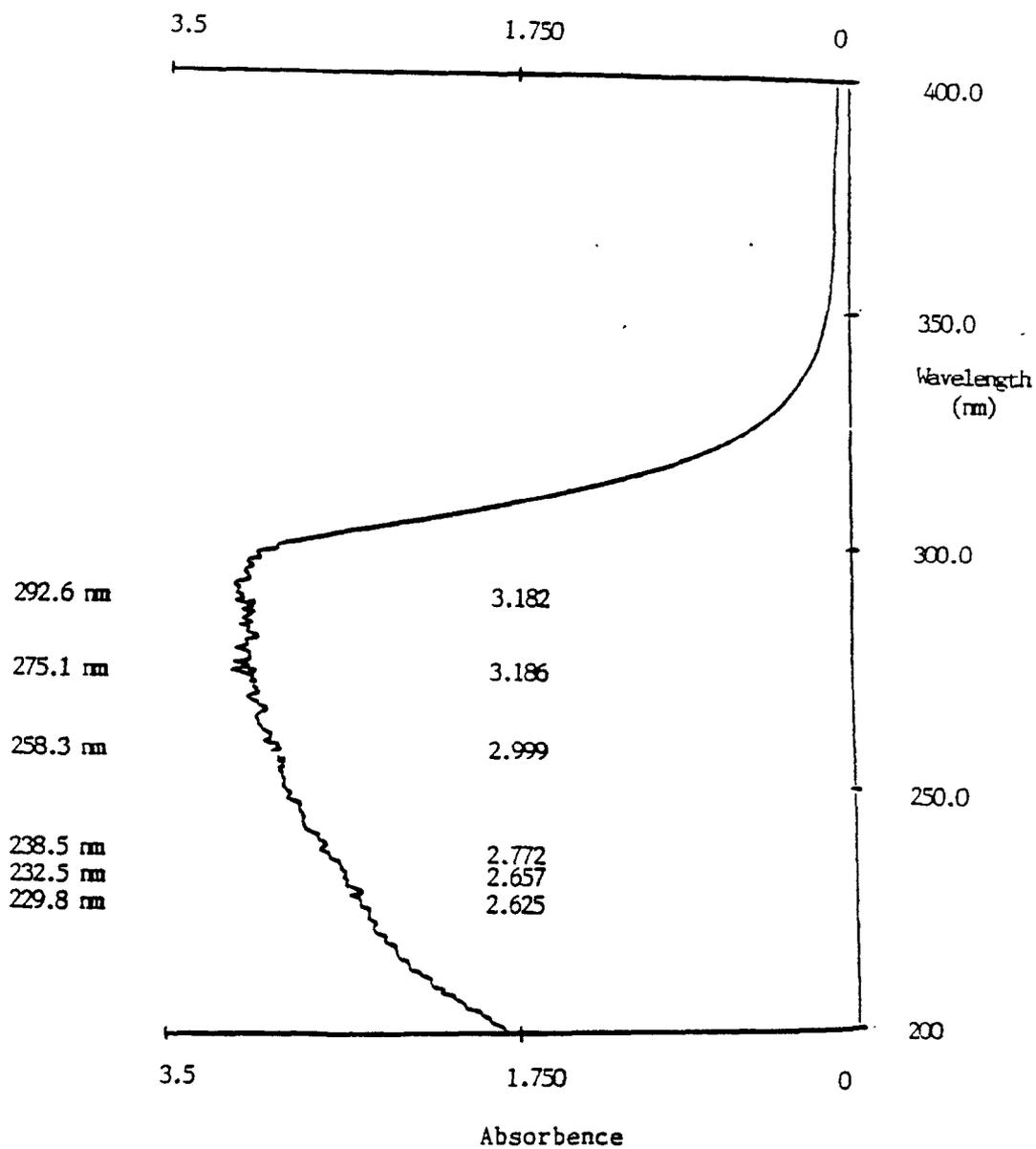
HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 2

Absorbance characteristics of the 3 mm thick sheet of glass



HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 3

Table of symbols

P = precipitation of test compound observed

C = contaminated plate

W = wet plate

L = plate lost (eg plate damage or technical error)

M = plate counted manually

S = slight toxicity ie some thinning of background lawn and/or presence of microcolonies

V = very thin background lawn

A = absence of background lawn

T = toxic, no revertant colonies observed

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 4

Range-finder : Raw plate counts and calculated mutagenicity data for Uvinul T 50; batch 08-0083 (ZHT No. 93/246), negative and positive controls (no light treatment).

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

=====

Table Number :1

| | | | |
|------------------|---------------|-------------------|-------------------------|
| HM Study Number | :BLG 9/EU | Experiment Number | :Range-finder |
| Positive Control | :NQO | S-9 Present | :NO |
| Compound Name | :Uvinul T 150 | Strain Number | :WP2 |
| Irradiation Time | :0 secs | UV Dose (UVA/UVB) | :0/0 mJ/cm ² |

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|------------------------|-------|------|------------------|-------|-------|------|-------|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 8 | 40 | 200 | 1000 | 5000 | 20 |
| Revertants | 8 | 5 | 5 | 5 | 5P | 3MP | 161 |
| | 6 | 5 | 10 | 2 | 6P | 3MP | 236 |
| | 6 | 5 | 8 | 6 | 3P | 5MP | 265 |
| | 2 | | | | | | |
| | 6 | | | | | | |
| Mean | 5.6 | 5.0 | 7.7 | 4.3 | 4.7 | 3.7 | 220.7 |
| Increase over Control | | 0.9 | 1.4 [^] | 0.8 | 0.8 | 0.7 | 39.4 |
| Standard Deviation | 2.2 | 0.0 | 2.5 | 2.1 | 1.5 | 1.2 | 53.7 |
| R-Coefficient Gradient | | 0.48 | 0.25 | 0.23 | 0.36 | | |
| Degrees of freedom | | 0.06 | -0.01 | -0.00 | -0.00 | | |
| Significance | | 9 | 12 | 15 | 18 | | |
| | | NS | NS | NS | NS | | |
| Dunnett's t value | -0.26 | 1.35 | -0.90 | -0.57 | -1.33 | | |
| Significance | NS | NS | NS | NS | NS | | |
| M Statistic | 0.63 | | | | | | |

=====

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
 For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 5

Experiment 1 : Raw plate counts and calculated mutagenicity data for Uvinul T 150; batch 08-0083 (ZHT No. 93/246), negative and positive controls.

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

Table Number :2

HM Study Number :BLG 9/EU Experiment Number :1
Positive Control :8-MOP Glass Present :NO
Compound Name :Uvinul T 150 Strain Number :WP2
Irradiation Time :0 secs UV Dose (UVA/UVB) :0/0 mJ/cm²

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|------------------------|-----|------|-------|------|------------------|-------|-----|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 1.6 | 8 | 40 | 200 | 1000 | 500 |
| Revertants | 3 | 4 | 5 | 4 | 9 | 3P | 5 |
| | 8 | 4 | 5 | 7 | 3 | 5P | 3 |
| | 5 | 7 | 4 | 4 | 6 | 3P | 5 |
| | 4 | | | | | | |
| | 4 | | | | | | |
| Mean | 4.8 | 5.0 | 4.7 | 5.0 | 6.0 | 3.7 | 4.3 |
| Increase over Control | | 1.0 | 1.0 | 1.0 | 1.2 [^] | 0.8 | 0.9 |
| Standard Deviation | 1.9 | 1.7 | 0.6 | 1.7 | 3.0 | 1.2 | 1.2 |
| R-Coefficient Gradient | | | 0.05 | 0.04 | 0.26 | 0.24 | |
| Degrees of freedom | | | -0.02 | 0.00 | 0.01 | -0.00 | |
| Significance | | | 9 | 12 | 15 | 18 | |
| Dunnett's t value | | 0.19 | -0.01 | 0.19 | 0.80 | -0.88 | |
| Significance | | NS | NS | NS | NS | NS | |
| M Statistic | | 0.67 | | | | | |

Key NS represents Not Significant
* represents p <= 0.05
** represents p <= 0.01
*** represents p <= 0.001
^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :2A
-----
HM Study Number   :BLG 9/EU           Experiment Number :1
Positive Control  :NQO                Glass Present     :NO
Compound Name     :Uvinul T 150       Strain Number     :WP2
Irradiation Time  :0 secs             UV Dose (UVA/UVB):0/0 mJ/cm2
-----
Point             -ve                                     +ve
-----
Replicates        5                                       3
Dose ug/plate     0                                       20
-----
Revertants        3                                       60
                  8                                       82
                  5                                       58
                  4
                  4
-----
Mean              4.8                                       66.7
-----
Increase over
Control                                                   13.9
-----
Standard          1.9                                       13.3
Deviation
=====
```

Key NS represents Not Significant
 * represents $p \leq 0.05$
 ** represents $p \leq 0.01$
 *** represents $p \leq 0.001$
 ^ represents maximum increase over control
For Dunnett's t value *** represents $p < 0.01$

The above treatments were performed to demonstrate the response of the bacteria to the standard positive control chemical for this strain.

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

=====
Table Number :3

| | | | |
|------------------|---------------|-------------------|-----------------------------|
| HM Study Number | :BLG 9/EU | Experiment Number | :1 |
| Positive Control | :8-MOP | Glass Present | :NO |
| Compound Name | :Uvinul T 150 | Strain Number | :WP2 |
| Irradiation Time | :11 secs | UV Dose (UVA/UVB) | :5.3/1.8 mJ/cm ² |

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|------------------------|-----|-------|-------|-------|-------|-------|------|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 1.6 | 8 | 40 | 200 | 1000 | 500 |
| Revertants | 8 | 6 | 12 | 11 | 4 | 7P | 78 |
| | 7 | 8 | 7 | 9 | 8 | 4P | 96 |
| | 6 | 8 | 6 | 2 | 7 | 7P | 66 |
| | 11 | | | | | | |
| | 11 | | | | | | |
| Mean | 8.6 | 7.3 | 8.3 | 7.3 | 6.3 | 6.0 | 80.0 |
| Increase over Control | | 0.9 | 1.0 | 0.9 | 0.7 | 0.7 | 9.3 |
| Standard Deviation | 2.3 | 1.2 | 3.2 | 4.7 | 2.1 | 1.7 | 15.1 |
| R-Coefficient Gradient | | 0.01 | 0.13 | 0.27 | 0.29 | | |
| Degrees of freedom | | -0.00 | -0.02 | -0.01 | -0.00 | | |
| Significance | | 9 | 12 | 15 | 18 | | |
| Dunnett's t value | | NS | NS | NS | NS | | |
| Significance | | -0.53 | -0.15 | -0.85 | -1.07 | -1.23 | |
| M Statistic | | NS | NS | NS | NS | NS | |
| | | 0.98 | | | | | |

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
 For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :4
-----
HM Study Number   :BLG 9/EU           Experiment Number :1
Positive Control  :8-MOP             Glass Present     :NO
Compound Name     :Uvinul T 150      Strain Number     :WP2
Irradiation Time :23 secs            UV Dose (UVA/UVB):11.1/3.8 mJ/cm2
-----
Point             -ve      2      3      4      5      6      +ve
Replicates        5      3      3      3      3      3      3
Dose ug/plate     0      1.6    8      40     200    1000   500
-----
Revertants        6      3      4      11     7      5P     184
                  13     13     9      15     10     2P     179
                  8      11     11     6      4      8P     107
                  8
                  12
-----
Mean              9.4    9.0    8.0    10.7   7.0    5.0    156.7
Increase over Control
                  1.0    0.9    1.1^   0.7    0.5    16.7
Standard Deviation
                  3.0    5.3    3.6    4.5    3.0    3.0    43.1
-----
R-Coefficient
Gradient          0.17   0.18   0.22   0.42
                  -0.17  0.04  -0.01  -0.00
Degrees of freedom
Significance       9      12     15     18
                  NS     NS     NS     NS
-----
Dunnett's t value
Significance       -0.30  -0.52  0.35  -0.86  -1.74
                  NS     NS     NS     NS     NS
-----
M Statistic       1.66
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :5
-----
HM Study Number   :BLG 9/EU           Experiment Number :1
Positive Control  :8-MOP              Glass Present     :YES
Compound Name    :Uvinul T 150       Strain Number    :WP2
Irradiation Time :8 min 31 secs      UV Dose (UVA/UVB) :230/0 mJ/cm2
-----
Point            -ve    2      3      4      5      6              +ve
-----
Replicates       5      3      3      3      3      3              3
Dose ug/plate    0      1.6    8      40     200    1000          500
-----
Revertants       5      3      3      7      5      2P            79
                  3      6      4      2      9      5P            299
                  9      6      7      2      5      6P            274
                  2
                  5
-----
Mean             4.8    5.0    4.7    3.7    6.3    4.3           217.3
Increase over Control      1.0    1.0    0.8    1.3^    0.9           45.3
Standard Deviation 2.7    1.7    2.1    2.9    2.3    2.1           120.5
-----
R-Coefficient          0.04   0.22   0.26   0.04
Gradient              -0.02  -0.03   0.01  -0.00
Degrees of freedom      9      12     15     18
Significance            NS      NS      NS      NS
-----
Dunnett's t value     0.22   0.01  -0.74   0.91  -0.23
Significance           NS      NS      NS      NS      NS
-----
M Statistic          1.23
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :6
-----
HM Study Number   :BLG 9/EU           Experiment Number :1
Positive Control  :8-MOP             Glass Present     :YES
Compound Name     :Uvinul T 150       Strain Number     :WP2
Irradiation Time  :17 min 2 secs     UV Dose (UVA/UVB):460/0 mJ/cm2
-----
Point             -ve      2      3      4      5      6              +ve
-----
Replicates        5      3      3      3      3      3              3
Dose ug/plate     0      1.6    8      40     200    1000           500
-----
Revertants        7      6      9      15     10     6P             529
                  4      9      9      8      6      7P             419
                  7      3      6      9      5      5P             484
                  7
                  13
-----
Mean              7.6    6.0    8.0    10.7   7.0    6.0            477.3
Increase over Control
                  0.8    1.1    1.4^   0.9    0.8
Standard Deviation
                  3.3    3.0    1.7    3.8    2.6    1.0            55.3
-----
R-Coefficient
Gradient          0.12   0.47   0.04   0.24
Degrees of freedom
Significance      NS      NS      NS      NS
-----
Dunnett's t value
Significance      NS      NS      NS      NS      NS
-----
M Statistic      1.03
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 6

Experiment 2 : Raw plate counts and calculated mutagenicity data for Uvinul T 150; batch 08-0083 (ZHT No. 93/246), negative and positive controls.

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

=====
Table Number :7

| | | | |
|------------------|---------------|-------------------|-------------------------|
| HM Study Number | :BLG 9/EU | Experiment Number | :2 |
| Positive Control | :8-MOP | Glass Present | :NO |
| Compound Name | :Uvinul T 150 | Strain Number | :WP2 |
| Irradiation Time | :0 secs | UV Dose (UVA/UVB) | :0/0 mJ/cm ² |

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|------------------------|------|------|------|------------------|------|-------|------|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 62.5 | 125 | 250 | 500 | 1000 | 500 |
| Revertants | 7 | 9 | 9 | 11 | 10 | 8P | 10 |
| | 7 | 17 | 12 | 14 | 9 | 8P | 12 |
| | 11 | 9 | 12 | 11 | 8 | 8P | 9 |
| | 10 | | | | | | |
| | 9 | | | | | | |
| Mean | 8.8 | 11.7 | 11.0 | 12.0 | 9.0 | 8.0 | 10.3 |
| Increase over Control | | 1.3 | 1.2 | 1.4 [^] | 1.0 | 0.9 | 1.2 |
| Standard Deviation | 1.8 | 4.6 | 1.7 | 1.7 | 1.0 | 0.0 | 1.5 |
| R-Coefficient Gradient | | | 0.38 | 0.42 | 0.02 | 0.31 | |
| Degrees of freedom | | | 9 | 12 | 15 | 18 | |
| Significance | | | NS | NS | NS | NS | |
| Dunnett's t value | | 1.74 | 1.47 | 2.09 | 0.18 | -0.52 | |
| Significance | | NS | NS | NS | NS | NS | |
| M Statistic | 0.46 | | | | | | |

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :7A
-----
HM Study Number   :BLG 9/EU           Experiment Number :2
Positive Control  :NQO                Glass Present     :NO
Compound Name     :Uvinul T 150       Strain Number     :WP2
Irradiation Time  :0 secs              UV Dose (UVA/UVB) :0/0 mJ/cm2
-----
Point             -ve                                     +ve
-----
Replicates        5                                       3
Dose ug/plate     0                                       20
-----
Revertants        7                                       70
                  7                                       63
                  11                                      60
                  10
                  9
-----
Mean              8.8                                       64.3
-----
Increase over     Control                                       7.3
-----
Standard          1.8                                       5.1
Deviation
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
 For Dunnett's t value *** represents p < 0.01

The above treatments were performed to demonstrate the response of the bacteria to the standard positive control chemical for this strain.

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :8
-----
HM Study Number   :BLG 9/EU           Experiment Number :2
Positive Control  :8-MOP             Glass Present     :NO
Compound Name     :Uvinul T 150      Strain Number     :WP2
Irradiation Time  :11 secs           UV Dose (UVA/UVB) :5.3/1.8 mJ/cm2
-----
Point             -ve      2      3      4      5      6      +ve
-----
Replicates        5       3      3      3      3      3      3
Dose ug/plate     0     62.5  125   250   500   1000   500
-----
Revertants        15     15     16     13     7     10P    115
                  11     13     10     6     14     5P    190
                  11     16     4      10     10    13P    103
                  17
                  16
-----
Mean              14.0   14.7  10.0   9.7   10.3   9.3    136.0
Increase over
Control           1.0^   0.7   0.7   0.7   0.7    9.7
Standard
Deviation         2.8    1.5   6.0   3.5   3.5   4.0    47.1
-----
R-Coefficient                0.41  0.50  0.40  0.40
Gradient                    -0.03 -0.02 -0.01 -0.00
Degrees of freedom           9     12    15    18
Significance                  NS     NS   NS     NS
-----
Dunnett's t value          0.23 -1.55 -1.51 -1.25 -1.67
Significance                 NS     NS   NS     NS
-----
M Statistic              1.30
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

```
=====
Table Number      :9
-----
HM Study Number   :BLG 9/EU           Experiment Number :2
Positive Control  :8-MOP             Glass Present     :NO
Compound Name     :Uvinul T 150      Strain Number     :WP2
Irradiation Time  :23 secs           UV Dose (UVA/UVB) :11.1/3.8 mJ/cm2
-----
Point             -ve      2      3      4      5      6      +ve
Replicates        5      3      3      3      3      3      3
Dose ug/plate     0     62.5  125  250  500  1000  500
-----
Revertants        15     16     12     17     12     15P    499
                  17     17     6      13     16     11P    422
                  16     21     13     12     7      6P     500
                  12
                  26
-----
Mean              17.2   18.0   10.3   14.0   11.7   10.7   473.7
Increase over
Control           1.0^   0.6    0.8    0.7    0.6    27.5
Standard
Deviation         5.3    2.6    3.8    2.6    4.5    4.5    44.7
-----
R-Coefficient                0.53   0.37   0.42   0.44
Gradient                    -0.05  -0.02  -0.01  -0.01
Degrees of freedom           9      12     15     18
Significance                  NS     NS     NS     NS
-----
Dunnett's t value          0.29  -2.23  -0.91  -1.76  -2.14
Significance                 NS     NS     NS     NS
-----
M Statistic              1.31
=====
```

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
 For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

=====
Table Number :10
=====

HM Study Number :BLG 9/EU Experiment Number :2
Positive Control :8-MOP Glass Present :YES
Compound Name :Uvinul T 150 Strain Number :WP2
Irradiation Time :8 min 13 secs UV Dose (UVA/UVB) :230/0 mJ/cm²

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|-----------------------|------|------|------------------|------|-----|------|-------|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 62.5 | 125 | 250 | 500 | 1000 | 500 |
| Revertants | 9 | 13 | 8 | 10 | 14 | 9P | 383 |
| | 13 | 11 | 14 | 11 | 9 | 4P | 493 |
| | 9 | 10 | 13 | 12 | 4 | 4P | 188 |
| | 9 | | | | | | |
| | 11 | | | | | | |
| Mean | 10.2 | 11.3 | 11.7 | 11.0 | 9.0 | 5.7 | 354.7 |
| Increase over Control | | 1.1 | 1.1 [^] | 1.1 | 0.9 | 0.6 | 34.8 |
| Standard Deviation | 1.8 | 1.5 | 3.2 | 1.0 | 5.0 | 2.9 | 154.5 |

R-Coefficient 0.32 0.17 0.21 0.58
Gradient 0.01 0.00 -0.00 -0.01
Degrees of freedom 9 12 15 18
Significance NS NS NS NS

Dunnett's t value 0.51 0.60 0.38 -0.78 -2.47
Significance NS NS NS NS NS

M Statistic 0.87
=====

Key NS represents Not Significant
* represents p <= 0.05
** represents p <= 0.01
*** represents p <= 0.001
^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

| Point | -ve | 2 | 3 | 4 | 5 | 6 | +ve |
|------------------------|------|-------|-------|-------|-------|-------|-------|
| Replicates | 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| Dose ug/plate | 0 | 62.5 | 125 | 250 | 500 | 1000 | 500 |
| Revertants | 13 | 6 | 12 | 6 | 10 | 7P | 475 |
| | 18 | 13 | 9 | 11 | 8 | 6P | 398 |
| | 9 | 13 | 11 | 8 | 8 | 8P | 617 |
| | 13 | | | | | | |
| | 8 | | | | | | |
| Mean | 12.2 | 10.7 | 10.7 | 8.3 | 8.7 | 7.0 | 496.7 |
| Increase over Control | | 0.9 | 0.9 | 0.7 | 0.7 | 0.6 | 40.7 |
| Standard Deviation | 4.0 | 4.0 | 1.5 | 2.5 | 1.2 | 1.0 | 111.1 |
| R-Coefficient Gradient | | | 0.22 | 0.44 | 0.43 | 0.53 | |
| Degrees of freedom | | | 9 | 12 | 15 | 18 | |
| Significance | | | NS | NS | NS | NS | |
| Dunnett's t value | | -0.72 | -0.60 | -1.81 | -1.58 | -2.50 | |
| Significance | | NS | NS | NS | NS | NS | |
| M Statistic | | 0.77 | | | | | |

Key NS represents Not Significant
 * represents p <= 0.05
 ** represents p <= 0.01
 *** represents p <= 0.001
 ^ represents maximum increase over control
For Dunnett's t value *** represents p < 0.01

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 7

Historical negative (solvent) control range for *E. coli* strain WP2

| Strain | - or + S9 | Mean number of spontaneous revertants | SD | Range* | |
|--|-----------|--|-----|--------|-------|
| | | | | lower | upper |
| WP2 | - | 10 | 5.3 | 1 | 23 |
| The above are pooled data from 21 consecutive experiments performed over the period 19.1.90 to 25.6.93 | | | | | |

* 99% confidence limits about the mean

HM study number: BLG 9/EU

HE study number: 729/185

Sponsor project number: 44M0246/939007

APPENDIX 8

Sponsor's certificate of analysis

Not provided.