

ACB Wasabi Extract PF Toxicology Data

Code: 20351PF
INCI Name: Lactobacillus/Wasabia Japonica Root Ferment Filtrate
CAS #: N/A
EINECS #: N/A

Name of Study	Type of Study	Results
Dermal & Ocular Irritation Tests	<i>In-vitro</i>	Both the dermal and ocular assays reveal that ACB Wasabi Extract PF is non-irritating and should not cause any of the aforementioned conditions.
AMES Test	<i>In-vitro</i>	The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, that ACB Wasabi Extract PF was considered to be Non-Mutagenic to Salmonella typhimurium testerstrains TA98, TA100, TA1537, TA1535 and Escherichia coli WP2uvrA



Dermal and Ocular Irritation Tests

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Tradename: ACB Wasabi Extract PF

Code: 20351PF

CAS #: N/A

Test Request Form #: 18

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)

EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ACB Wasabi Extract PF** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO₂, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(3-4,5-dimethyl thiazole 2-yl)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

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Dermal and Ocular Irritation Tests

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II. Materials

- A. Incubation Conditions:** 37°C at 5% CO₂ and 95% relative humidity
- B. Equipment:** Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
- C. Media/Buffers:** DMEM based medium; DPBS; sterile deionized H₂O
- D. Preparation:** Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
- E. Tissue Culture Plates:** Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
- F. Reagents:** MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
- G. Other:** Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO₂ and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO₂ and 95% relative humidity for an additional 18 to 21 hours.

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B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO₂, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO₂, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO₂, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD₅₇₀) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is $\leq 20\%$.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is $< 60\%$ of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be $< 18\%$ for EpiDerm™ and $< 20\%$ EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability $\leq 50\%$ for EpiDerm™ or $\leq 60\%$ for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

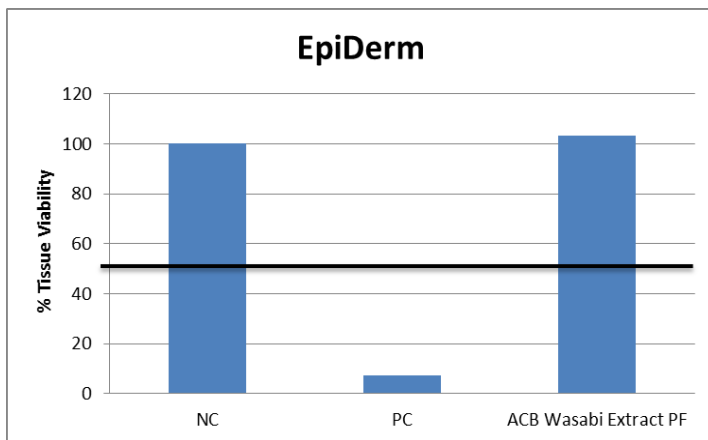


Figure 1: EpiDerm tissue viability

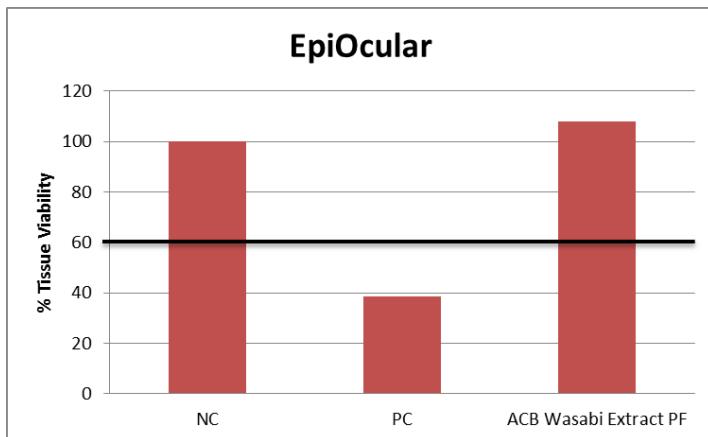


Figure 2: EpiOcular tissue viability

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Bacterial Reverse Mutation Test

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Test Article: ACB Wasabi Extract
Code Number: 20351
CAS #: N/A

Sponsor:
Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: Erica Segura
Principle Investigator: Monica Beltran

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 1623

SUMMARY

A *Salmonella typhimurium*/*Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **ACB Wasabi Extract** would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2uvrA in the presence and absence of Aroclor-induced rat liver S9. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2uvrA after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45°C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37°C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2uvrA. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

I. Introduction

A. Purpose

A *Salmonella typhimurium*/*Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

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II. Materials

- A. **Storage Conditions:** Room temperature (23-25C).
- B. **Vehicle:** Sterile DI Water.
- C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
- D. **Solubility/Stability:** 100% Soluble and Stable.
- E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System

Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa*), the deletion of *uvrB* gene and the deletion in the *uvrA* gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvrA*) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

<u>Tester strain</u>	<u>Mutations/Genotypic Relevance</u>
TA98	hisD3052, Dgal chlD bio <i>uvrB rfa</i> pKM101
TA100	hisG46, Dgal chlD BIO <i>uvrB rfa</i> pKM101
TA1537	hisC3076, <i>rfa</i> , Dgal chlD bio <i>uvrB</i>
TA 1535	hisG46, Dgal chlD bio <i>uvrB rfa</i>
WP2 <i>uvrA</i>	trpE, <i>uvrA</i>

- rfa* = causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
- uvrB* = deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
- pKM101 = plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
- uvrA* = All possible transitions and transversions, small deletions.

B. Metabolic Activation

Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvrA* were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control

Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of revertants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.

E. Positive Control

A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

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F. Titer of the Strain Cultures:

Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to 2×10^9 /ml.

IV. Method

A. Standard Plate Incorporation Assay:

Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for *Escherichia coli* were inoculated with 100 μ l of culture for each strain and 100 μ l of testing solution or vehicle without test material. A 500 μ l aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50 μ l aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of revertants was determined. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control of each strain used.

V. Evaluation

For the test solution to be evaluated as a test failure or "potential mutagen" there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* and *Escherichia coli* tester strain used.

VI. Results and Discussion

A. Solubility:

Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

B. Dose levels tested:

The maximum dose tested was 5000 μ g per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 μ g per plate.

C. Titer (Organisms/ml):

5×10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2×10^9 UFC/ml.

C. Standard Plate Incorporation Assay

In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2uvrA in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

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Appendix 2:

Bacterial Mutation Assay Plate Incorporation Assay Results

	Concentration µg per Plate	TA98		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	29	35	32
	1500	19	17	18
	500	21	23	22
	150	18	30	24
	50	31	22	27
	15	18	20	19
	5.0	22	21	22
	1.5	19	19	19
Test Solution w/o S9	5000	20	22	21
	1500	35	32	34
	500	17	19	18
	150	20	20	20
	50	20	23	22
	15	21	21	21
	5.0	24	21	23
	1.5	22	18	20
DI Water w/S9		34	18	20
DI Water w/o S9		4	16	10
2-aminoanthracen w/ S9		365	387	376
2-nitrofluorene w/o S9		295	211	253
Historical Count Positive w/S9		43-1893		
Historical Count Positive w/o S9		39-1871		
Historical Count Negative w/S9		4-69		
Historical Count Negative w/o S9		3-59		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration μg per Plate	TA100		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	144	60	102
	1500	124	144	134
	500	224	163	180
	150	236	240	238
	50	180	168	174
	15	160	188	174
	5.0	148	300	224
	1.5	172	136	154
Test Solution w/o S9	5000	112	125	119
	1500	100	103	102
	500	135	140	138
	150	80	48	64
	50	92	108	100
	15	132	160	146
	5.0	108	192	150
	1.5	148	176	162
DI Water w/S9		168	144	156
DI Water w/o S9		180	46	113
2-aminoanthracen w/ S9		450	437	444
Sodium azide w/o S9		520	408	464
Historical Count Positive w/S9		224-3206		
Historical Count Positive w/o S9		226-1837		
Historical Count Negative w/S9		55-268		
Historical Count Negative w/o S9		47-250		

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	Concentration μg per Plate	<i>TA1537</i>		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	11	9	10
	1500	13	12	13
	500	7	14	10
	150	11	6	9
	50	10	4	7
	15	13	8	11
	5.0	8	14	11
	1.5	13	8	11
Test Solution w/o S9	5000	30	20	25
	1500	19	10	15
	500	10	8	9
	150	4	8	6
	50	24	19	22
	15	10	12	11
	5.0	18	12	15
	1.5	16	10	13
DI Water w/S9		9	3	6
DI Water w/o S9		13	16	15
2-aminoanthracen w/ S9		314	312	313
2-aminoacridine w/o S9		320	304	312
Historical Count Positive w/S9		13-1934		
Historical Count Positive w/o S9		17-4814		
Historical Count Negative w/S9		0-41		
Historical Count Negative w/o S9		0-29		

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	Concentration μg per Plate	TA1535		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	24	21	22
	1500	13	7	10
	500	28	21	25
	150	20	15	16
	50	22	20	21
	15	24	11	18
	5.0	22	32	27
	1.5	24	13	19
Test Solution w/o S9	5000	88	82	85
	1500	72	95	84
	500	81	80	81
	150	84	80	81
	50	12	8	10
	15	23	21	22
	5.0	8	18	13
	1.5	21	16	19
DI Water w/S9		18	15	17
DI Water w/o S9		18	30	24
2-aminoanthracen w/ S9		228	217	223
Sodium azide w/o S9		408	480	444
Historical Count Positive w/S9		22-1216		
Historical Count Positive w/o S9		47-1409		
Historical Count Negative w/S9		1-50		
Historical Count Negative w/o S9		1-45		

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	Concentration μg per Plate	<i>WP2uvrA</i>		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	12	12	12
	1500	13	39	26
	500	43	40	42
	150	56	25	41
	50	38	48	43
	15	49	44	47
	5.0	57	55	56
	1.5	50	65	58
Test Solution w/o S9	5000	81	65	73
	1500	40	51	46
	500	25	32	29
	150	29	16	23
	50	33	30	32
	15	40	40	40
	5.0	50	44	47
	1.5	41	54	48
DI Water w/S9		48	41	45
DI Water w/o S9		50	51	51
2-aminoanthracen w/ S9		501	522	512
Methylmethanesulfonate w/o S9		360	230	300
Historical Count Positive w/S9		44-1118		
Historical Count Positive w/o S9		42-1796		
Historical Count Negative w/S9		8-80		
Historical Count Negative w/o S9		8-84		

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