Receipt No. T96-2503
Report No. T-4663

STUDY CODE : K01-1815

(写)

FINAL REPORT

BACTERIAL REVERSE MUTATION TEST
OF
υ -1

October, 1996

Hita Research Laboratories
Chemical Biotesting Center
Chemicals Inspection & Testing Institute
Japan

QUALITY ASSURANCE STATEMENT

Hita Research Laboratories, Chemical Biotesting Center
Chemicals Inspection & Testing Institute, Japan

Sponsor: SUMITOMO 3M LIMITED
Title: Bacterial reverse mutation test of v-1
Study code: K01-1815

This report was audited by the Quality Assurance Section.
I, the undersigned, hereby declare that this report reflects
the original Japanese report.

(Date) December 17, 1996

(Signature)

Section Chief, Quality Assurance

Keiji Shiraishi, B.S.

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report.
(Study code No. K01-1815 issued on October 30, 1996)

(date) December 17, 1996

(signature) Shozo Ogura

Shozo Ogura
Hita Research Laboratories
Chemical Biotesting Center
Chemicals Inspection &
Testing Institute, Japan

GLP STATEMENT

Hita Research Laboratories, Chemical Biotesting Center
Chemicals Inspection & Testing Institute, Japan

Sponsor: SUMITOMO 3M LIMITED
Title: Bacterial reverse mutation test of υ-1
Study Code No.: K01-1815

I, the undersigned, hereby declare that this study was conducted in compliances with "Standards to be observed by Testing Institutions for Toxicity Investigations" (Japan's MOL, No.76, September 1, 1988).

Management: Signed in original October 30, 1996
Shigetaka Yamane, Ph. D.

QUALITY ASSURANCE STATEMENT

Hita Research Laboratories, Chemical Biotesting Center
Chemicals Inspection & Testing Institute, Japan

Sponsor: SUMITOMO 3M LIMITED
Title: Bacterial reverse mutation test of υ-1
Study Code No.: K01-1815

This study was audited by the Quality Assurance Section and the study procedures were inspected on the following dates.

Dates of Inspections and Audits	Dates of Reports to Study Director	Dates of Reports to Management
September 12, 1996	September 13, 1996	September 17, 1996
October 1, 1996	October 1, 1996	October 1, 1996
October 30, 1996	October 30, 1996	October 30, 1996

I, the undersigned, hereby declare that this report provides an accurate description of the methods and procedures used in this study and that the reported results accurately reflect the raw data obtained.

Section Chief, Quality Assurance: Signed in original October 30, 1996
Keiji Shiraishi, B.S.

Study code: K01-1815
Test substance code: HR3291
Sponsor code: S-030

TITLE

Bacterial reverse mutation test of v-1

SPONSOR

SUMITOMO 3M LIMITED
8-8, Minami-Hashimoto 3-chome Sagamihara-shi, Kanagawa, 229 Japan

TESTING FACILITY

Hita Research Laboratories, Chemical Biotesting Center
Chemicals Inspection & Testing Institute, Japan
822, 3-chome, Ishii-machi, Hita, Oita 877, Japan

PURPOSE OF STUDY

The purpose of this study was to determine the mutagenic potential of the test substance using *Salmonella typhimurium* and *Escherichia coli*.

TESTING METHOD

This study was conducted in accordance with the following guidelines: "Standards for Toxicity Investigations" (Japan's MOL, No.77, September 1, 1988).

GLP COMPLIANCE

This study was carried out in compliance with the following GLP requirement: "Standards to be observed by Testing Institutions for Toxicity Investigations" (Japan's MOL, No.76, September 1, 1988).

PERIOD OF STUDY

Commencement of test:	September 17, 1996
Dose finding test:	September 25, 1996
Completion of observation:	October 14, 1996
Presentation of final report:	October 30, 1996

LOCATION AND PERIOD FOR RETENTION OF RAW DATA

Data and test substance are retained in the archives and the test substance storage room of Hita Research Laboratories for 10 years following the date of the notification specified under Item 1 of Article 57-2 of Industrial Safety & Health Law, respectively.

After termination of the retention period, any measures taken are done so with the approval of the sponsor.

PERSON CONCERNED WITH STUDY

Study Director:

Signed in original October 30, 1996
Shozo Ogura
Hita Research Laboratories
Mutagenicity Section

Study Staff: Tsunehiko Inai, B.S.

Person in charge of Storage: Shizuka Kouda

ANY UNEXPECTED SITUATIONS AND DEVIATIONS FROM PROTOCOL

There were no unexpected situations and deviations from protocol which might have affected the test results.

CONTENTS

SUMMARY

The reverse mutation test of υ-1 was performed on *Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537 and a *Escherichia coli* strain WP2 *uvrA* using the pre-incubation method with and without metabolic activation.

The results showed that the numbers of their revertant colonies for all strains in groups which were treated with the test substance were less than twice that of each negative control with and without S9 Mix.

The numbers of the revertant colonies in the negative control and the positive controls were within the background data in our laboratories.

Based upon the above results, υ-1 was judged to have no reverse mutagenic potential under the present test conditions.

MATERIALS AND METHODS

1. TEST SUBSTANCE AND POSITIVE CONTROLS

1.1 Test substance (Information provided by the sponsor)

1) Name

Potassium salt of N-ethyl-N-perfluorobutylsulfonylglycine

Other name: υ -1

CAS No.: 67584-51-4

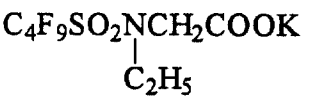
2) Lot No.

Lot 1

3) Supplier

SUMITOMO 3M LIMITED

4) Structural formula or rational formula (Outline of manufacturing method, in case both were unknown)



(molecular formula $C_8H_7F_9KNO_4S$)

5) Purity

97.3 w/w%

6) Impurities

KCl 2.7 w/w%

7) Physicochemical properties

Appearance at ordinary temperature:	light gray powder	
Molecular weight:	423.30	
Stability:	stable	
Melting point:	—	
Boiling point:	—	
Vapor pressure:	—	
Partition coefficient:	—	
Solubility:	—	
Degree of solubility:	Water:	≧5 w/v%*
	DMSO:	≧5 w/v%*
	Acetone:	< 10 w/v%*
	Others:	—

* Examined in our laboratories

8) Storage conditions

room temperature

9) Care on handling

Gloves, a mask, a head cap and a lab coat were worn when handling.

1.2 Positive controls

1) 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2)

Manufacturer: Wako Pure Chemical Industries, Ltd.
Lot No.: LEN0571
Properties: reddish-orange crystalline powder
Purity: 99.5%
Grade: special grade

2) Sodium azide (NaN_3)

Manufacturer: Wako Pure Chemical Industries, Ltd.
Lot No.: DLP2438
Properties: white crystalline
Purity: 99.4%
Grade: special grade

3) 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine · 2HCl (ICR-191)

Manufacturer: Polysciences, Inc.
Lot No.: 412795
Properties: yellow crystalline powder
Purity: —
Grade: —

4) 2-Aminoanthracene (2AA)

Manufacturer: Wako Pure Chemical Industries, Ltd.
Lot No.: DLR7869
Properties: yellowish-green-brown powder
Purity: 95.7%
Grade: —

5) Storage conditions

A cold and dark place

6) Care on handling

Gloves, a mask, a head cap and a lab coat were worn when handling.

2. BACTERIAL STRAINS

2.1 Strains selected

Salmonella typhimurium strains TA100, TA98, TA1535 and TA1537 were obtained from Dr. B.N. Ames, University of California, U.S.A. ,on June 20, 1990.

A *Escherichia coli* strain WP2 *uvrA* was obtained from Japan Bioassay Laboratories, on April 6, 1995.

S. typhimurium strains TA100, TA1535 and a *E. coli* strain WP2 *uvrA* were used for the detection of base-pair substitution mutation, while *S. typhimurium* strains TA98 and TA1537 were for the detection of frameshift mutation.

2.2 Storage

The test strains were stored as frozen stock cultures (0.045 ml of dimethyl sulfoxide (DMSO)*/0.5 ml of broth culture) at –80℃ (ultra-deep freezer MDF-291, Sanyo).

* Purity ≧ 99.0%, Lot No. CF103, Dojindo Laboratories

2.3 Characterization of strains

1) Characteristics of strains

Strains	Mutation on synthesis of amino acid	Mutation on excision repair	Membrane mutation (LPS)	R-factor (pKM101)
Salmonella typhimurium				
TA1535	*hisG46*	△*uvrB*	*rfa*	–
TA1537	*hisC3076*	△*uvrB*	*rfa*	–
TA98	*hisD3052*	△*uvrB*	*rfa*	+
TA100	*hisG46*	△*uvrB*	*rfa*	+
Escherichia coli				
WP2 *uvrA*	*trp*	△*uvrA*	+	–

The amino acid requirement for growth was demonstrated by using histidine for *S. typhimurium* strains and tryptophan for *E. coli* strain. The presence of R-factor, membrane mutation and mutation on the ability to repair DNA lesions were confirmed by ampicillin resistance, sensitivity to crystal violet and UV sensitivity, respectively.

2) Date of characterization

Salmonella typhimurium	TA1535	July 18, 1996
	TA1537	June 7, 1996
	TA98	June 7, 1996
	TA100	March 6, 1996
Escherichia coli	WP2 *uvrA*	April 18, 1996

3. MEDIUM AND S9 MIX

3.1 Medium

1) Minimal glucose agar plate (prepared in our Laboratories)

The medium was prepared as follows, and poured 30 ml into a petri dish.

Components	Amount included in one litre
20 × Vogel-Bonner E	50 ml
40 w/v% Glucose	50 ml
Agar	15 g

(1) Agar: Bacto-Agar (Lot No. 71892AJB or 90800JA, Difco Laboratories)

(2) Manufacturing date: dose finding test on September 11, 1996
main test on October 3, 1996

2) Soft agar

The solution containing 0.5 mM histidine and 0.5 mM biotin for *S. typhimurium* strains or 0.5 mM tryptophan for *E. coli* strain was added to the soft agar solution containing 0.6 w/v% agar (Bacto-Agar, Lot No. 71892AJB, Difco Laboratories) and 0.5 w/v% NaCl in a ratio of 1 : 10.

3.2 S9 Mix

1) Rat liver S9 (Kikkoman Co., Ltd.)

Induction method: SD male rats, 7-week-old (203-254 g), were intraperitoneally administrated phenobarbital (30 mg/kg × 1 time, 60 mg/kg × 3 times) and 5,6-benzoflavone (80 mg/kg × 1 time).

Lot No.: RAA-350 (manufactured on August 23, 1996, purchased on September 4, 1996)

Storage: -80℃ (ultra-deep freezer MDF-291, Sanyo)

2) Cofactor for S9 Mix (Oriental Yeast Industries, Ltd.)

Lot No.: 999602

Storage: -20℃ (bio-freezer GS-2603, Nippon Freezer Ltd.)

3) Composition of S9 Mix

One ml of S9 Mix contained 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4μmol NADH, 100 μmol of 0.2 M sodium-phosphate buffer (pH 7.4) and 0.1 ml S9.

4. PRE-CULTURES

From the stock cultures, 20 μl of the bacterial suspension was inoculated to L-tube containing 10 ml nutrient broth No.2 (Lot No. 194 56443, OXOID Ltd.) and the bacterial culture was incubated at 37 ± 0.5℃ for 8 h with shaking at 50 times/min by the Monod shaker (MONOSIN- II A, Taitec Co., Ltd.) The viable cell counts calculated from the values which were determined at 660 nm by spectrophotometry (Novaspec, LKB Japan) at the end of incubation are shown below.

		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
No. of viable cells ($\times 10^9$/ml)	Dose finding test	2.1	2.1	4.4	2.4	2.1
	Main test	2.1	2.1	4.0	2.3	2.0

5. PREPARATION OF TEST SUBSTANCE AND POSITIVE CONTROLS

5.1 Test substance

1) Preparation

The test substance was dissolved in distilled water (distilled water for injection, Lot No. K6B74, Otsuka Pharmaceutical Factory) to make 5 w/v% concentration and diluted with the same solvent to give appropriate concentrations.

2) Stability of the test solution

No denaturation of the test solution was observed for the color and the exothermic reaction until 2 hours after preparation.

3) Preparation time

Prepared immediately before use and used within 0.5 h at room temperature.

5.2 Positive controls

1) Preparation

NaN_3 was dissolved in distilled water (Lot No. K6B74). AF-2, ICR-191 and 2AA were dissolved in DMSO (Lot No. CD069).

2) Preparation time and storage condition

Prepared on every 3 months and stored at -80℃ (ultra-deep freezer MDF-291, Sanyo).

6. METHODS

The test was carried out for *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and a *E. coli* strain WP2 *uvrA* using the pre-incubation method both with and without metabolic activation system. The plating was done in triplicate for the negative control and in duplicate for the test substance and positive controls.

6.1 Procedures

After 0.1 ml of the test substance solution, 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) or S9 Mix, and 0.1 ml of the bacterial culture were added to a tube, the mixtures were incubated for 20 min at 37 ± 0.5℃. Two ml of the soft agar was then added to each tube and poured onto a minimal glucose agar plate.

After incubation for 48 h at 37 ± 0.5℃, the number of revertant colonies were counted.

As the sterility test, each 0.1 ml of each bacterial suspension, test substance solution, S9 Mix or 0.1 M sodium phosphate buffer (pH 7.4) were smeared on a minimal glucose agar plate and incubated at 37 ± 0.5℃ for 48 h, and then checked the bacterial contamination. Distilled water was used as a negative control, and the following positive controls were used for each bacterial strains.

	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
S9 Mix (–)	AF-2	NaN_3	AF-2	AF-2	ICR-191
	0.01	0.5	0.01	0.1	1
S9 Mix (+)	2AA	2AA	2AA	2AA	2AA
	1	2	10	0.5	2

(μg/plate)

6.2 Dose selection

1) Dose finding test

The test was carried out at the highest dose of 5,000 μg/plate and 6 doses of 1,000, 500, 100, 50, 10 and 5 μg/plate.

As a result, growth inhibition was observed at 5,000 μg/plate both with and without S9 Mix.

2) Main test

Based on the results of the dose finding test, a main test was performed at the highest dose of 5,000 μg/plate and 5 lower doses diluted with a geometric progression of 2.

7. MICROSCOPIC OBSERVATION AND COLONY COUNTING

7.1 Microscopic observation

The state of revertant colonies (size and number of colonies), deposition of the test substance and the growth inhibition were examined with a stereo microscope.

7.2 Colony counting

The number of colonies were counted with a manual counter or a colony analyzer (CA-7 or CA-9, Toyo-sokki Co., Ltd). Correction for counting errors was made for measurements with the colony analyzer. Each plate was measured three times, and the average of these three measurements was adopted as the number of revertant colonies on the plate. The average for each dose was calculated from the values of the plates used. Decimals of the average figures were rounded off.

8. INTERPRETATION OF RESULTS

The test substance was judged to be positive, when the number of revertant colonies was twice or more of the negative control, and when the dose-relationship and the reproducibility were obtained. Any statistical procedures were not used.

RESULTS

The numbers of their revertant colonies for all strains in groups which were treated with the test substance were less than twice that of each negative control with and without S9 Mix.

The positive controls showed the distinct increase of revertant colonies, and the positive controls and the negative control were within a range of the background data in our laboratories.

The growth inhibition was observed at more than 2,500 μg/plate both with and without S9 Mix.

There were no fluctuations which affected the test results since the sterility test confirmed the absence of any micro-organisms.

CONCLUSION

In conclusion, υ -1 was judged to have no reverse mutagenic potential under the present test conditions.

REFERENCES

1. Ministry of Labor (1991) Guidebook on Mutagenicity Tests using Micro-organisms, New Edition (in Japanese) published by Japan Industrial Safety and Health Association,

2. Green M.H.L. and W.J. Muriel (1976) Mutagen testing using Trp^+ reversion in *Escherichia coli*, Mutation Res., 38: 3-32.

3. Maron, D.M., and B.N. Ames (1983) Revised methods for the *Salmonella* mutagenicity test, Mutation Res., 113: 173-215.

Dose finding test

Test substance: $\upsilon - 1$

With(+) or without(-) S9 Mix	Test substance concentration (μg/plate)	Number of revertants (number of colonies/plate)				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2 *uvrA*	TA 98	TA 1537
S9 Mix (-)	negative control	90 101 (101) 111	13 10 (12) 14	33 41 (37) 37	30 33 (31) 31	9 6 (7) 7
	5	118 109 (114)	11 14 (13)	34 45 (40)	33 25 (29)	8 9 (9)
	10	99 126 (113)	11 12 (12)	39 44 (42)	19 27 (23)	9 5 (7)
	50	101 107 (104)	18 8 (13)	39 32 (36)	23 26 (25)	12 9 (11)
	100	98 116 (107)	12 16 (14)	33 34 (34)	31 36 (34)	10 10 (10)
	500	109 118 (114)	16 7 (12)	48 38 (43)	23 25 (24)	8 9 (9)
	1000	104 95 (100)	8 15 (12)	30 38 (34)	27 27 (27)	7 10 (9)
	5000	80* 76* (78*)	3* 5* (4*)	33* 20* (27*)	10* 15* (13*)	5* 8* (7*)
S9 Mix (+)	negative control	110 85 (95) 91	10 15 (12) 12	41 40 (38) 32	34 36 (34) 33	23 18 (19) 16
	5	91 96 (94)	8 12 (10)	37 39 (38)	34 34 (34)	15 17 (16)
	10	104 99 (102)	11 14 (13)	35 40 (38)	30 34 (32)	25 23 (24)
	50	101 90 (96)	12 8 (10)	35 39 (37)	31 23 (27)	17 14 (16)
	100	105 91 (98)	15 8 (12)	33 35 (34)	43 45 (44)	20 23 (22)
	500	100 96 (98)	12 7 (10)	36 41 (39)	30 43 (37)	16 21 (19)
	1000	93 110 (102)	9 12 (11)	32 32 (32)	31 39 (35)	19 20 (20)
	5000	120* 102* (111*)	6* 10* (8*)	25* 28* (27*)	25* 23* (24*)	0* 14* (7*)
Positive control not requiring S9 Mix	Name	AF-2	NaN_3	AF-2	AF-2	ICR-191
	Concentration (μg/plate)	0.01	0.5	0.01	0.1	1
	Number of colonies/plate	351 410 (381)	382 385 (384)	179 188 (184)	487 566 (527)	1734 1968 (1851)
Positive control requiring S9 Mix	Name	2AA	2AA	2AA	2AA	2AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Number of colonies/plate	727 621 (674)	133 158 (146)	650 644 (647)	304 274 (289)	149 163 (156)

Notes Parenthesis shows the mean of each plate.
* : Observed bacterial growth inhibition.

·AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

·NaN_3: Sodium azide

·ICR-191: 2-Methoxy-6-chloro-9-(3-(2-chloroethyl)-aminopropylamino) acridine·2HCl

·2AA: 2-Aminoanthracene

Main test

Test substance: $\upsilon - 1$

With(+) or without(-) S9 Mix	Test substance concentration (μg/plate)	Number of revertants (number of colonies/plate)				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2 *uvrA*	TA 98	TA 1537
S9 Mix (-)	negative control	113 112 (110) 106	18 10 (15) 17	39 36 (37) 36	28 25 (30) 38	13 10 (14) 19
	156	100 117 (109)	12 13 (13)	27 33 (30)	28 34 (31)	8 14 (11)
	313	132 100 (116)	11 15 (13)	24 33 (29)	30 34 (32)	20 8 (14)
	625	119 114 (117)	10 11 (11)	30 32 (31)	33 34 (34)	18 24 (21)
	1250	104 103 (104)	12 12 (12)	36 50 (43)	30 29 (30)	18 27 (23)
	2500	82* 91*(87*)	7* 8*(8*)	35* 30*(33*)	21* 23*(22*)	3* 9*(6*)
	5000	79* 88*(84*)	0* 0*(0*)	36* 33*(35*)	18* 24*(21*)	0* 6*(3*)
S9 Mix (+)	negative control	108 117 (115) 120	9 9 (11) 14	38 29 (34) 34	42 46 (42) 39	24 28 (26) 26
	156	103 116 (110)	6 8 (7)	49 38 (44)	35 40 (38)	23 35 (29)
	313	97 89 (93)	9 10 (10)	52 42 (47)	35 46 (41)	31 26 (29)
	625	109 115 (112)	11 13 (12)	35 36 (36)	31 37 (34)	26 26 (26)
	1250	110 112 (111)	13 7 (10)	38 53 (46)	41 45 (43)	26 32 (29)
	2500	106* 136*(121*)	8* 6*(7*)	33* 38*(36*)	37* 31*(34*)	7* 14*(11*)
	5000	103* 118*(111*)	3* 1*(2*)	41* 33*(37*)	24* 24*(24*)	0* 7*(4*)
Positive control not requiring S9 Mix	Name	AF-2	NaN_3	AF-2	AF-2	ICR-191
	Concentration (μg/plate)	0.01	0.5	0.01	0.1	1
	Number of colonies/plate	356 337 (347)	328 295 (312)	133 113 (123)	456 470 (463)	2055 2081 (2068)
Positive control requiring S9 Mix	Name	2AA	2AA	2AA	2AA	2AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Number of colonies/plate	804 874 (839)	191 163 (177)	582 561 (572)	276 255 (266)	171 159 (165)

Notes Parenthesis shows the mean of each plate.
* : Observed bacterial growth inhibition.

·AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

·NaN_3: Sodium azide

·ICR-191: 2-Methoxy-6-chloro-9-(3-(2-chloroethyl)-aminopropylamino) acridine·2HCl

·2AA: 2-Aminoanthracene

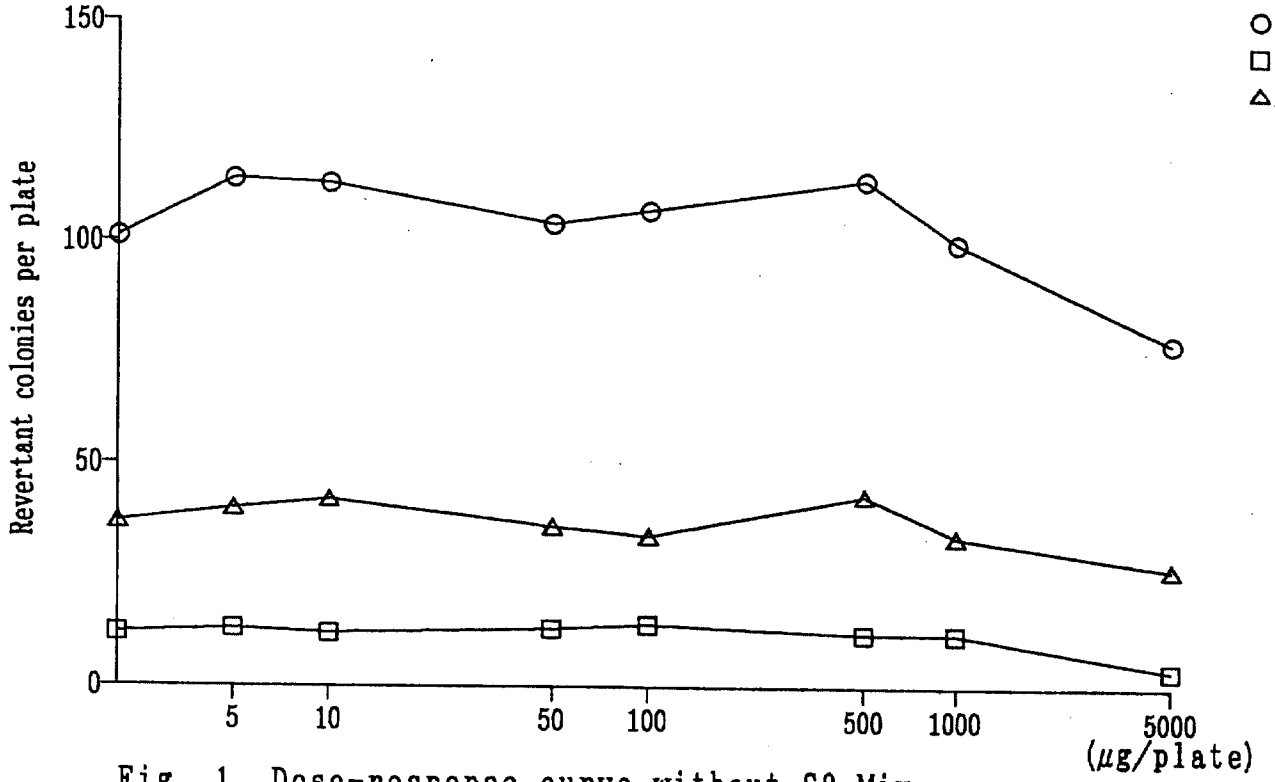


Fig. 1 Dose-response curve without S9 Mix

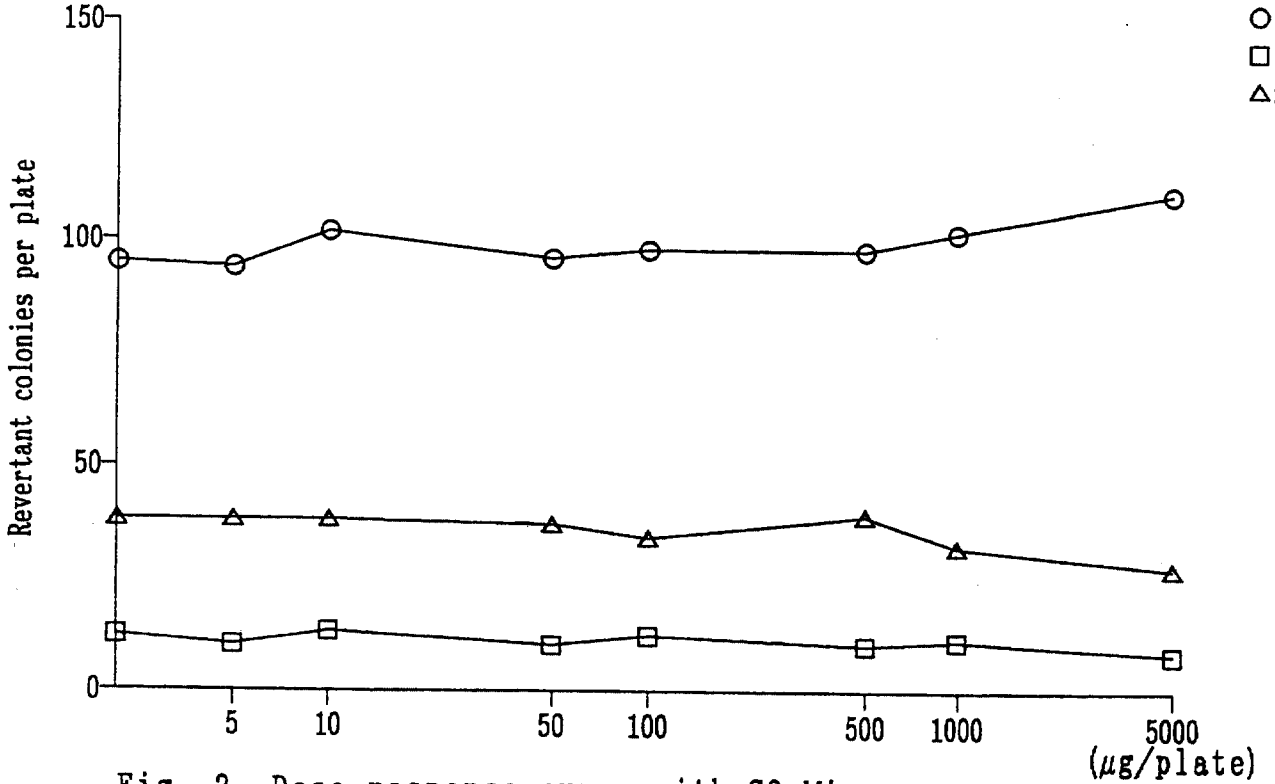


Fig. 2 Dose-response curve with S9 Mix

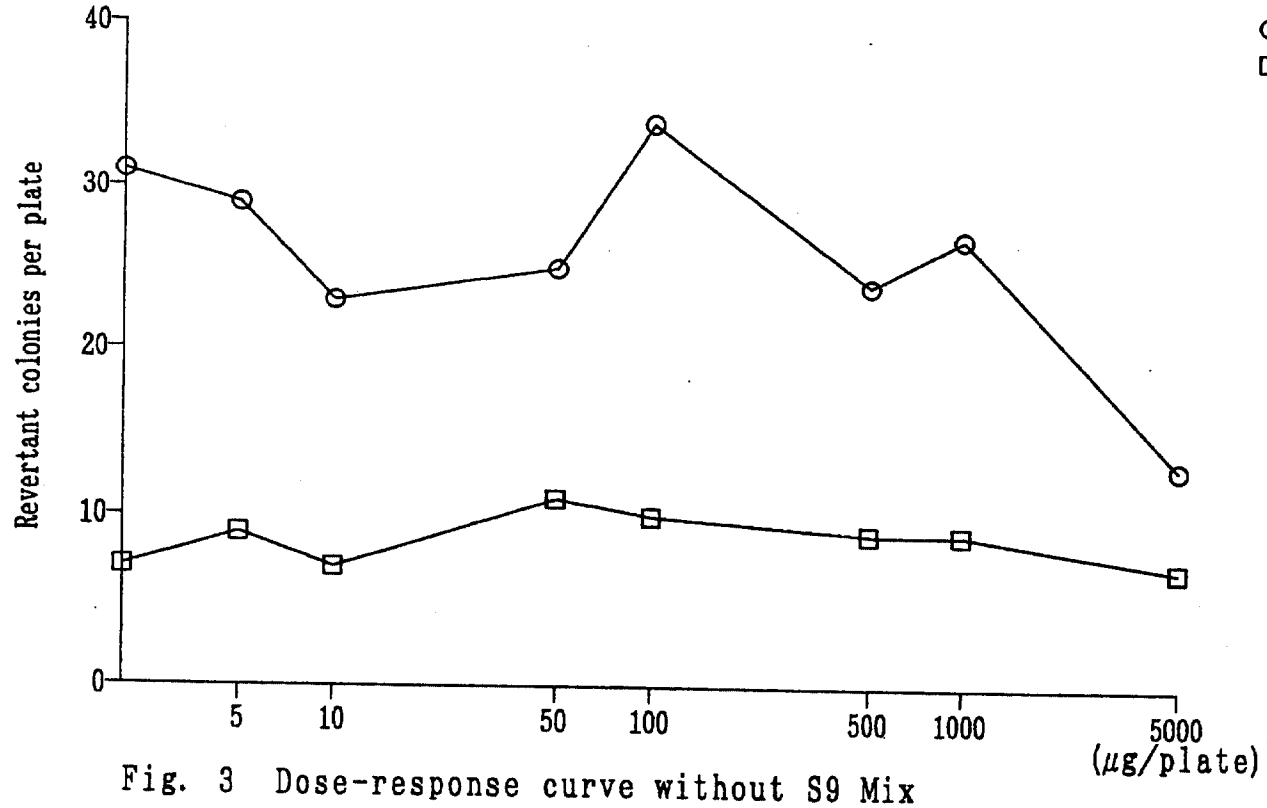


Fig. 3 Dose-response curve without S9 Mix

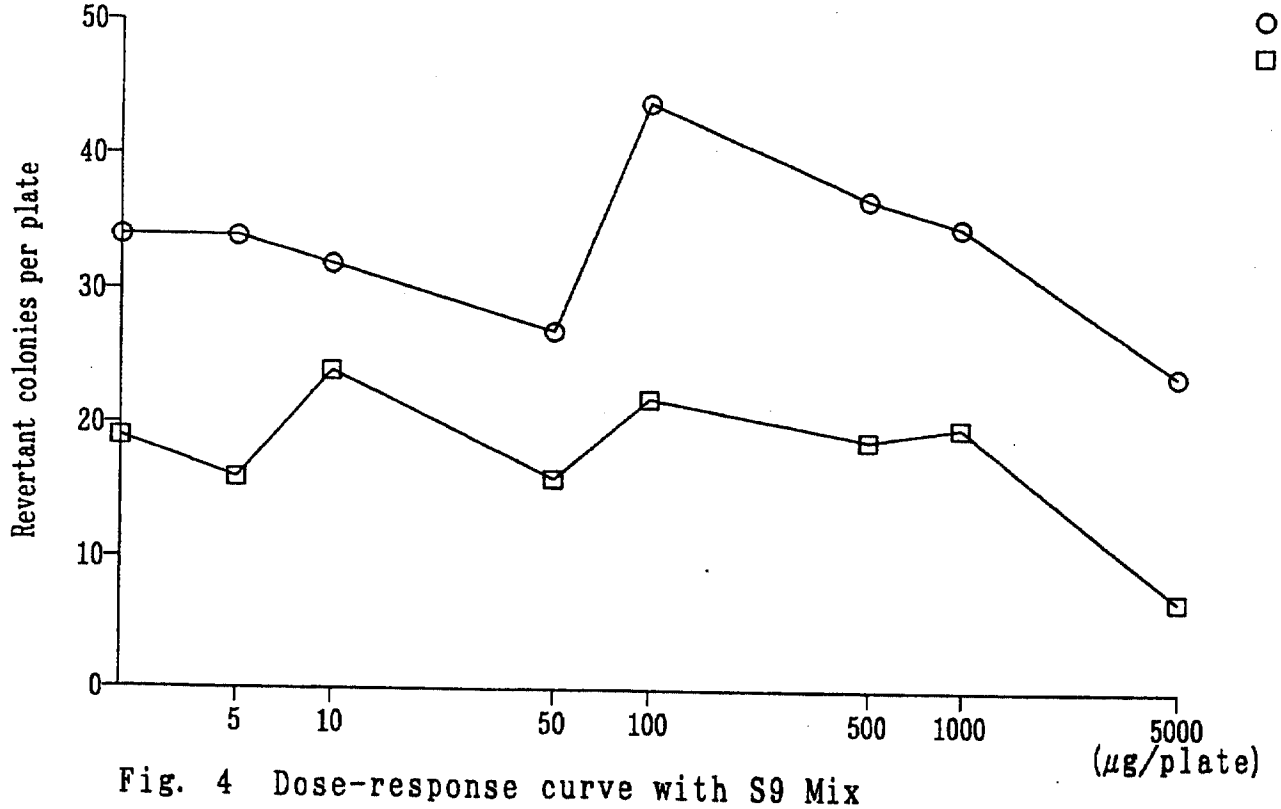


Fig. 4 Dose-response curve with S9 Mix

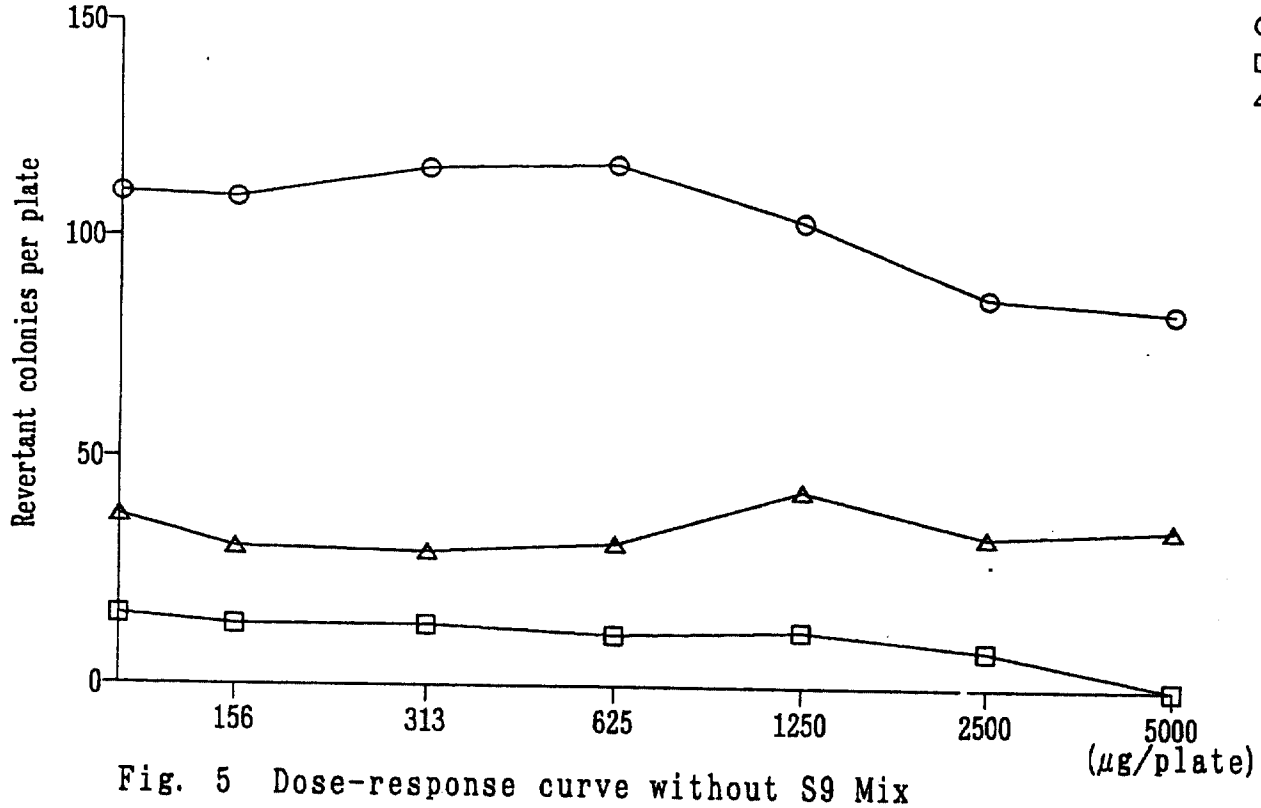


Fig. 5 Dose-response curve without S9 Mix

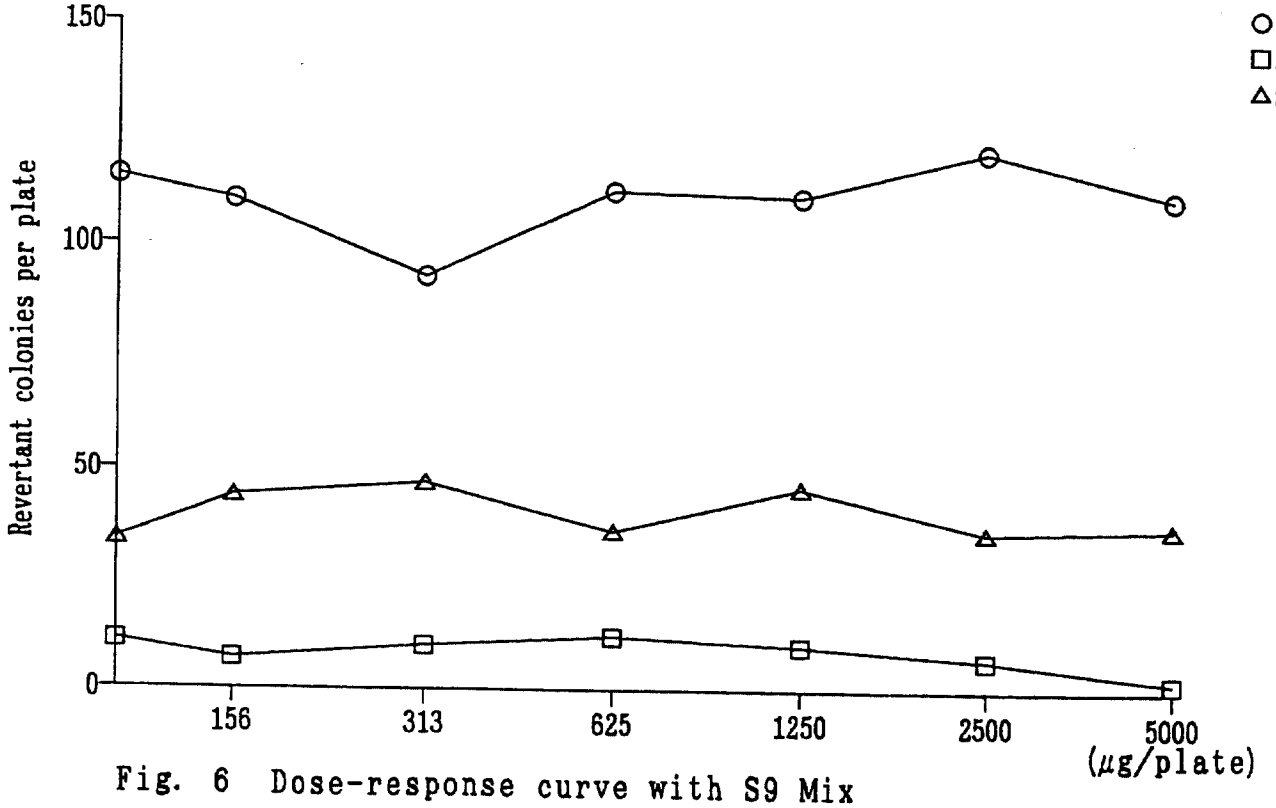


Fig. 6 Dose-response curve with S9 Mix

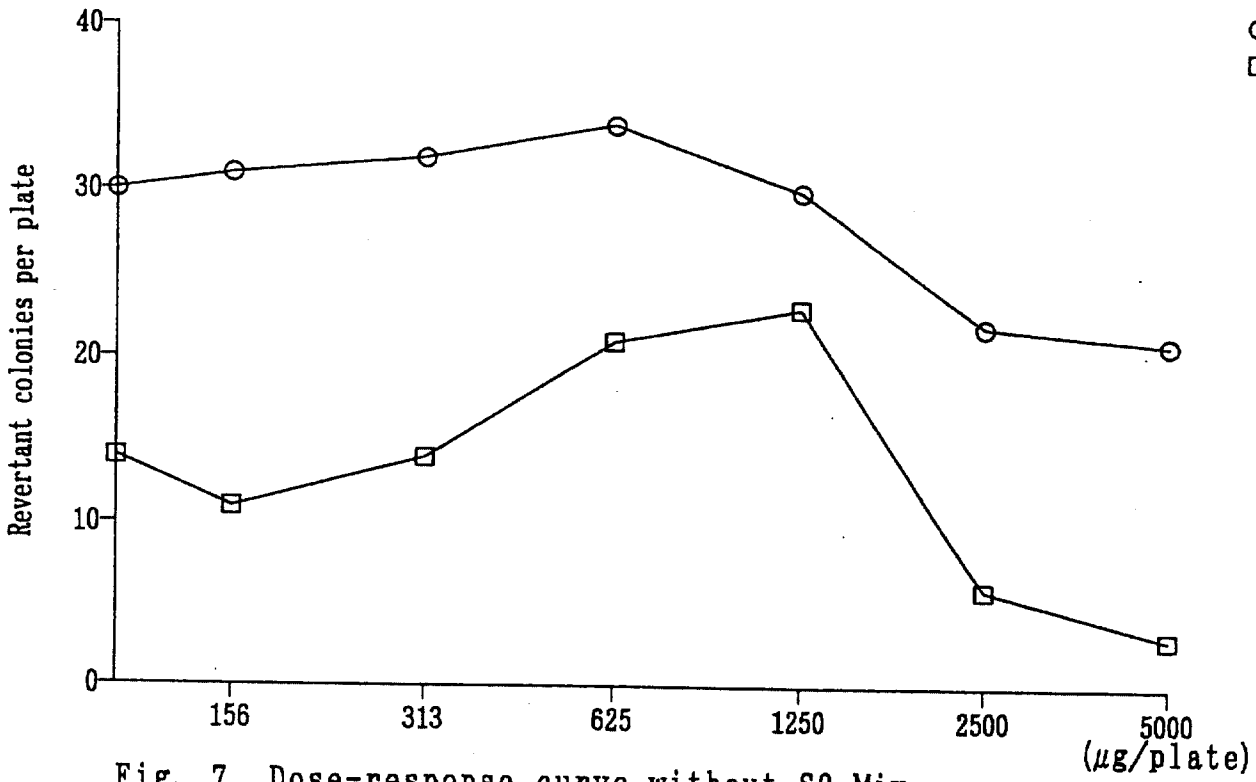


Fig. 7 Dose-response curve without S9 Mix

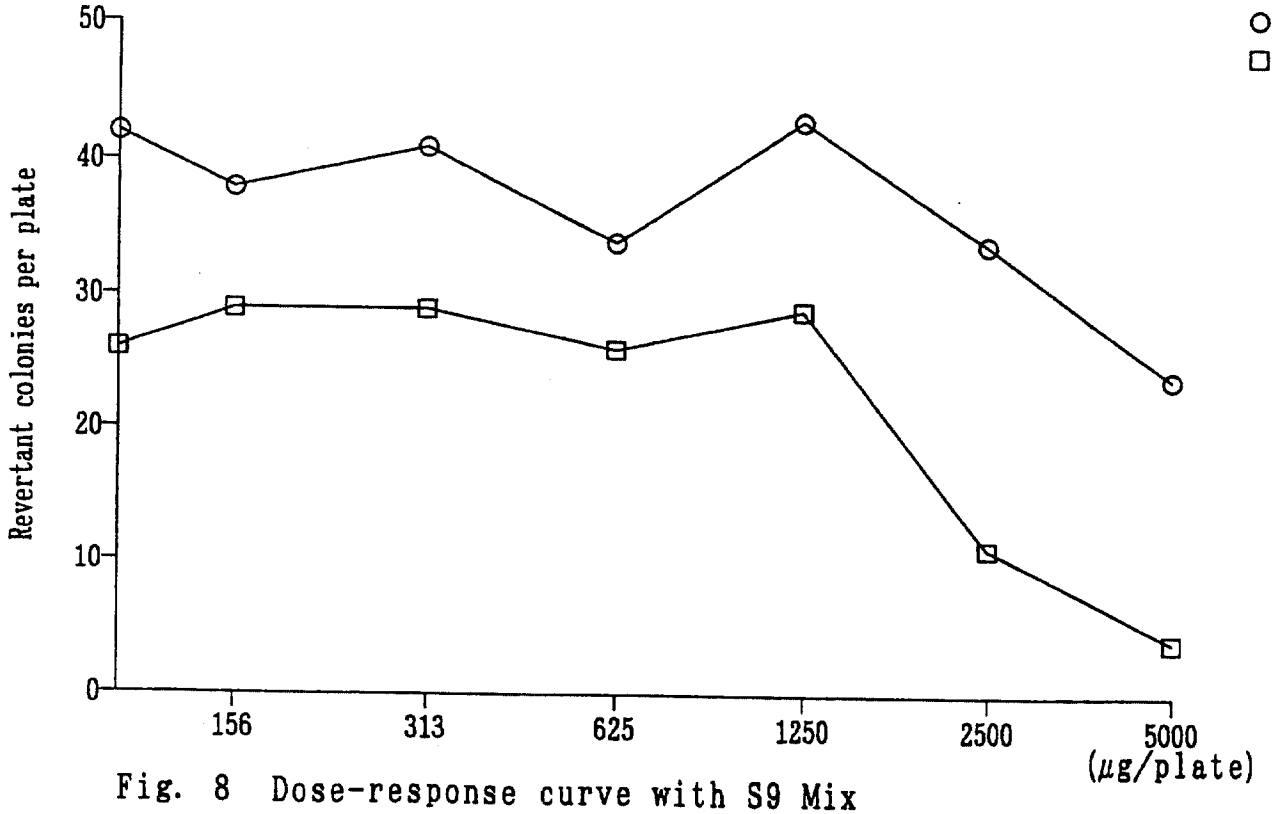


Fig. 8 Dose-response curve with S9 Mix