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RE: TSCA Section 8(e) Submission for C. I. Solvent Yellow 33 (CAS # 8003-22-3)

ATTN: TSCA Section 8(e) Coordinator

This submission is made in accordance with TSCA Section 8(e) requirements and discharges any TSCA Section 8(e) responsibilities that exist for our Company regarding the information described herein. We do not believe the data described in this submission reasonably support the conclusion that the subject material presents a substantial risk of injury to human health or the environment.

This submission provides results from a Clonal Transformation Assay on C. I. Solvent Yellow No. 33 as tests substance RO429.01 using Syrian Golden Hamster Embryo (SHE) Cells which shows that the test substance induced morphological changes during this in vitro evaluation. Corroborative, published data exist which shows C. I. Solvent Yellow No. 33 to be positive in several types of in vitro assays (see Moore, M. M., et. al., "Mutagenic Screening of Marker Grenade Dyes by the Salmonella Reversion Assay, L5178Y/TK +/- Mouse Lymphoma Assay, and In Vitro Sister Chromatid Exchange Analysis in Mice", Environmental and Molecular Mutagenesis 12:219-233 (1988).

We have handled and will continue to handle this material with appropriate caution in keeping with our standard practice for handling all chemical substances. We will use our procedure for communicating appropriate hazard information for the test substance by both labels and MSDS.

If you wish further information, please contact me.

Very truly yours,

THE PROCTER AND GAMBLE COMPANY



W. E. Bishop, Ph. D.
Manager Risk, Policy & Regulatory Sciences
Telephone: 513/627-6145



8ENQ-95-13463
INIT 06/14/95

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CHV STUDY NO.:16744-0.C45

**CHEMICAL IDENTIFICATION: R0429.01 is C. I. Solvent Yellow 33 (CAS No. 8003-22-3)
also known as D & C Yellow No.11**

CLONAL TRANSFORMATION ASSAY ON
RO429.01 DRD:HESE410
USING SYRIAN GOLDEN HAMSTER EMBRYO (SHE) CELLS

DRAFT REPORT

AUTHOR

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PERFORMING LABORATORY

CORNING Hazleton. (CHV)
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LABORATORY PROJECT ID

CHV STUDY NO.:16744-0.C45

SUBMITTED TO

THE PROCTER & GAMBLE COMPANY
P.O. BOX 398707
CINCINNATI, OH. 45239-8707

STUDY COMPLETION DATE

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NOTE: As of April 1, 1995, the company name, Hazleton Washington, Incorporated, was legally changed to Corning Hazleton Incorporated. Modifications are currently underway to reflect the company name change. Both designations for the company (CHV and CHV) may appear in this report.

SUMMARY

RO429.01 was tested for its potential to induce morphological transformation in the Syrian hamster embryo cell transformation assay using a 24 hr / refeed exposure protocol. Based on results of preliminary cytotoxicity assay, the doses selected for the transformation assay were 0.1 µg/ml, 1.0 µg/ml, 2.5 µg/ml, 5.0 µg/ml, and 7.5 µg/ml with cell adjustment for the upper two dose levels.

Pooled data from two independent assays at these dose levels were analyzed by Fisher's Exact Test for significant treatment related effects. Analysis of these results indicated that all of the dose groups tested showed a statistically significantly greater increase in morphological transformation frequency compared to the concurrent controls. Therefore RO429.01 is considered to be positive for its potential to induce morphological transformation when tested as described in this report.

I. INTRODUCTION

Syrian hamster embryo (SHE) cells have been used extensively to study the process of *in vitro* cell transformation and have been proposed as a useful model system to assess the potential carcinogenicity of diverse chemicals^{1,2,3,4}. Among the advantages of the SHE transformation system are the following: 1) The assay can be performed using cryopreserved cells³. 2) The SHE target cells are capable of maintaining a whole range of metabolic activities and 3) The morphological endpoint can be determined in 7-9 days^{5,6,7,8,9,10}.

II. STUDY INFORMATION

- A. Title: CLONAL TRANSFORMATION ASSAY USING SYRIAN GOLDEN HAMSTER EMBRYO (SHE) CELLS
- B. CHV Study Number: 16744-0.C45
- C. Test Article: RO429.01 was received at CORNING Hazleton on February 2, 1995. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.
- D. Control Articles:
1. Negative (untreated) Control and Solvent for Test Article:

LeBoeuf's modification of Dulbecco's Modified Eagle's Medium containing 20% fetal bovine serum and 4 Mm L-glutamine.
 2. Solvent Control for Positive Control:

0.2% dimethylsulfoxide (DMSO)
Source: Aldrich Chemical Co., Lot# 04801JF
 2. Positive Control:

Benzo(a)pyrene 2.5 µg/ml
Source: Sigma, Lot #13F-9007
- E. Test System:
- Syrian golden hamster embryo cells
Source: Genetic and Cellular Toxicology Division
Hazleton Washington, Inc.
Freeze Date: June 3, 1994; Set XXIII

F. Sponsor: The Procter & Gamble Company
P.O. Box 398707
Cincinnati, OH 45239-8707

Authorized Representative: Gary A. Kerckaert

G. Testing Facility: CORNING Hazleton.
9200 Leesburg Pike
Vienna, Virginia 22182

H. Personnel:

1. Study Director: Roger M. Brauninger, M.S.
2. Laboratory Supervisor: Hussain S. Shaffi

I. Schedule:

1. Experimental Start Date: 2/16/95
2. Experimental Termination Date: 4/12/95

J. Records to be maintained:

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived in the storage facilities of CORNING Hazleton for at least one year following submission of the final report to the sponsor. After the one year period, the sponsor may elect to have the aforementioned materials retained in the storage facilities of CORNING Hazleton for an additional period of time or sent to a storage facility designated by the sponsor. Any remaining test article sample will be discarded according to CHV safety procedures after the final report is mailed.

III. PROCEDURES

A. Objective:

The objective of this assay is to determine the potential of the test article to induce a statistically significantly greater increase in morphological transformation frequency compared to controls in the Syrian hamster embryo cell transformation system.

B. Methods:

1. Preliminary Cytotoxicity Assay

Approximately 70-100 target SHE cells (in 2 ml complete medium) were added to each 60mm petri dish each containing about 4×10^4 X-irradiated (~5,000 rads) feeder SHE cells in 2 ml complete medium seeded 24 hours earlier. The cultures were then incubated at $37 \pm 1^\circ\text{C}$ in $10 \pm 0.5\%$ CO_2 in humidified air for approximately 24 hours. Test article stock solutions were prepared fresh in the appropriate solvent. Dosing solutions at twice the final desired concentration were then prepared by diluting the chemical directly in complete medium. Treatment consisted of delivering 4 ml of these dosing solutions to each of 15 dishes per treatment group (each containing 4 ml of complete medium, feeder cells and target cells). The cultures were incubated in the presence of the test substance for approximately 24 hours after which the dishes were refed with 8 ml of complete medium and then returned to the incubator for a period of seven to eight days.

After the incubation period, 10 of the dishes in each treatment group were washed once with Hank's balanced salt solution (HBSS), fixed with methano., stained with about 10% buffered aqueous Giemsa, washed in tap water and air dried. The number of colonies per dish was then determined. The remaining 5 dishes in each treatment group were washed once with Calcium and Magnesium free HBSS (HBSS-CMF), treated with 0.05% trypsin-EDTA to detach the cells, pooled, and the average cell density per dish was determined by counting the cells with either a Counter Model F electronic particle counter or hemocytometer using the trypan blue exclusion method. Colony size was determined by dividing the number of cells/dish by the number of colonies/dish.

The relative cytotoxicity for each treatment group (measured by the reduction in plating efficiency and cell number of the treated SHE cells compared with the controls) was then evaluated by the Study Director for determining doses to be used in the SHE transformation Assay.

2. Target Cell Number Equalization

The number of target cells needed for those cultures requiring target cell adjustment was determined by evaluating the selected dose groups' effects on relative plating efficiency in a cytotoxicity assay. If due to cytotoxicity, the RPE of any of these dose groups fell within the range of 50-70% RPE, then adjustment of the number of target cells seeded was necessary and those dose groups then had the number of target cells seeded adjusted to yield the approximate number of colonies/dish

that of the solvent control. Conversely if the RPE of any of the selected dose levels fell outside of this range of toxicity in the cytotoxicity assay, then adjustment of the number of target cells seeded was not required.

3. pH and Osmolality Determination

Prior to performing the definitive transformation assay, an aliquot of the test material was weighed, dissolved in the appropriate solvent and diluted in complete medium to approximate the highest concentration chosen to be run in the assay. A portion of this solution was transferred to a vial and placed in the CO₂ incubator for approximately 24 hours. The remaining portion of the solution was then measured for pH using a portable pH meter and the osmolality determined using a freezing-point osmometer. The following day the pH was determined for the incubated sample.

4. SHE Transformation Assay

The transformation assay consisted of two independent experiments which were performed in an analogous manner to the preliminary cytotoxicity assay. These experiments included at least five concentrations of test substance and appropriate negative and positive controls (25 dishes per treatment group). If necessary, an additional two doses of test substance were included to evaluate the effect of adjusting the target colony number on transformation frequency. Also included were 5 feeder-cell only dishes to verify the absence of feeder cell replication.

After the incubation period, 20 of the dishes were washed once with HBSS, fixed with methanol, stained with about 10% buffered aqueous Giemsa, rinsed with tap water and allowed to air dry. After the dishes were fixed and stained, they were scored for percent plating efficiency and relative survival compared to the control dishes. The remaining 5 dishes in each treatment group were washed once with CMF-HBSS, treated with 0.05% trypsin-EDTA to detach the cells, pooled, and the average cell density per dish was determined by counting the cells with either a Coulter Model F electronic particle counter or hemocytometer using the trypan blue exclusion method.

Using a stereomicroscope the dishes were screened and individual colonies evaluated for transformed morphology. Criteria for morphological transformation are: piled up cells normally at the edge of the colony, extensive random-oriented three dimensional growth and crisscrossing cells with increased cytoplasmic basophilia at the

perimeter and in the center of the colony. The transformation assay data were analyzed for statistically significant treatment-related effects using the Fisher's Exact Test (or also if desired the Cochran-Armitage Exact trend test for a positive dose response trend increase) on pooled data from the combined experiments. The total colony number and the number of colonies with transformed morphology for each test group were recorded. Based on the raw data, plating efficiencies and transformation frequencies were then calculated.

C. Assay Acceptance Criteria:

The criteria for which an acceptable assay is judged is as follows: There must be an average of 25-45 colonies per 60 mm dish per treatment group in pooled data for the untreated and solvent controls and the plating efficiencies must be greater than 25%. The total number of colonies for the control groups when pooled must exceed 1000 per study. Transformation frequencies for the non-treated and solvent control groups for each trial must be between 0-0.6% and the number of morphologically transformed colonies induced in the B(a)P controls must be statistically significant relative to their solvent control. The top test material dose group must cause at least a 50% reduction in relative plating efficiency, unless the maximal soluble dose is run and no toxicity occurs or the number of cells/colony precludes scoring that dose level. If the results obtained are outside these parameters the study sponsor will be contacted and the deviations will be handled on a case by case basis.

D. Assay Evaluation Criteria

With pooled data from both trials, statistical tests for significant treatment-related effects will be employed in evaluating the response of the test material. A test material will be considered to be positive if it causes a statistically significant increase in morphological transformation frequency in at least two doses compared to concurrent controls or a significant increase at one dose with a statistically significant ($p < 0.05$) positive dose trend. A test material shall be considered negative if there is one or fewer doses with a statistically significant treatment-related increase and the uppermost dose of test material demonstrates a sufficient level of toxicity (as measured by either a 50% reduction in plating efficiency or colony density).

E. Statistical Analysis

The method employed for judging whether a test material causes a significant treatment related effect is a one sided Fisher's Exact Test. This method compares the relationship between the frequency of morphological

transformation of the test material dose groups and the concurrent solvent control group where a p value ≤ 0.05 is indicative of statistical significance. In addition an unstratified binomial exact permutation trend test for significant positive dose response trend will be conducted when necessary.

IV. RESULTS

A. Test Article Solubility

The test material was tested for solubility in complete medium and DMSO. Complete medium was determined to be the most appropriate solvent for the test article. Based on the test material's solubility the dose range of RO429.01 tested in the preliminary cytotoxicity assay was 0.1-100 $\mu\text{g/ml}$.

B. Preliminary Cytotoxicity Assay

Relative toxicity was determined by comparing test article treated groups with solvent treated controls. The results of cytotoxicity determination by the relative reduction in plating efficiency (Table 1 part A) showed 8% toxicity at 0.10 $\mu\text{g/ml}$, 16% toxicity at 1.0 $\mu\text{g/ml}$, 24% toxicity at 2.5 $\mu\text{g/ml}$ and 35% toxicity at 5.0 $\mu\text{g/ml}$. The relative increases in toxicity then leveled out between 7.5 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ where there was 43% toxicity at 7.5 $\mu\text{g/ml}$, 45% toxicity at 10.0 $\mu\text{g/ml}$, 53% toxicity at 25 $\mu\text{g/ml}$ and 57% toxicity at 50 $\mu\text{g/ml}$. Toxicity then increased to 82% at 75 $\mu\text{g/ml}$ and then down slightly at 100 $\mu\text{g/ml}$ to 73%. with the relative toxicity jumping between a relative enhancement and 6% toxicity. At 2500 $\mu\text{g/ml}$ the relative toxicity was 12% increasing to 44% at 5000 $\mu\text{g/ml}$. The test material however had a greater effect on the ability to the cells to proliferate than to form colonies as seen by the response measuring relative cytotoxicity by the reduction in colony density (Table 1 part B): The 0.10 $\mu\text{g/ml}$ dose level had a relative colony density showing 49% toxicity, at the 1.0 $\mu\text{g/ml}$ dose the toxicity was 51%, increasing to 65% at 2.5 $\mu\text{g/ml}$ and to 72% at 5.0 $\mu\text{g/ml}$. Except for an outlier at 75 $\mu\text{g/ml}$ where the toxicity dropped to 58%, all other dose levels had relative colony densities of less than 25%.

C. Transformation Assay

Based on the results of the preliminary cytotoxicity assay and on discussions with the sponsor, the following doses were chosen to be tested in the transformation assay: 0.1 $\mu\text{g/ml}$, 1.0 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 5.0 $\mu\text{g/ml}$, and 7.5 $\mu\text{g/ml}$. In addition both the 5.0 $\mu\text{g/ml}$, and 7.5 $\mu\text{g/ml}$ dose groups were run with and without the number of target cells seeded adjusted to control levels.

Prior to performing both of the definitive transformation assays, the osmolality and pH of the top dose (7.5 µg/ml) was determined: The test material solution had a measured osmolality of 315 mOSM/kg versus a reference of 290 mOSM/kg and the pH of the solution after 24 hours incubation was 6.73. Both of these values were considered acceptable.

As seen from the data summary (Table 2) a statistically significantly greater increase in morphological transformation frequency compared to the concurrent controls occurred every dose group tested when the data was analyzed using the Fisher's Exact test. This response occurred over the entire range of toxicity obtained in the definitive assays, however note that the maximum toxicity achieved was 38% (7.5CA µg/ml).

The results of the experiments are summarized on Table 2 with graphical representation of relative plating efficiency and transformation frequency shown in Figure 1. Individual trial data are summarized on Tables 3 and 5 as are the relative colony densities on Tables 4 and 6. The individual data for each dose in the reported trials are included as appendix A. Except for not obtaining a 50% reduction in relative plating efficiency as mentioned above, all acceptance criteria for a valid assay were met, therefore the study was considered valid.

V. CONCLUSIONS

There was a statistically significant increase in the frequency of morphological transformation compared to concurrent controls in all of the dose groups when RO429.01 was tested using the 24 hour / refeed protocol. RO429.01 is therefore considered to be positive for its potential to induce morphological transformation when tested as described in this report.

VI. REFERENCES

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4. Pienta R.J., Poiley J.A., and Lebherz W.B. III: Further evaluation of a hamster embryo cell carcinogenesis bioassay. In Nieburgs H.E., Valli V.E.O., and Kay S.A. (eds.): Cancer Prevention and Detection Part 1, Vol. 2, Marcel Dekker, Inc. NY, pp. 1993-2011 1978.
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9. LeBoeuf R.A., and Kerckaert G.A.: Enhanced morphological transformation and early passage Syrian hamster embryo cells cultured in medium with a reduced bicarbonate concentration and ph.. Carcinogenesis 8:689-697 1987.
10. LeBoeuf R.A., Kerckaert G.A., Aardema M.J., and Gibson D.P.: Multistage neoplastic transformation of Syrian hamster embryo cells cultured at ph. 6.70. Cancer Research 50:3722-3729, 1990.

VII. APPROVALS

This study was performed in the spirit of compliance with the requirements of the U.S. Food and Drug Administration's Good Laboratory Practice Regulations found in Title 21 CFR Section 58. There was no in-process monitoring and no final report audits were conducted.

Hussain Shaffi (Supervisor) Date

Roger Brauning M.S. Date
Staff Scientist (Study Director)

16744-0-C45 SUMMARY (R0429.01)
FIGURE 1 (24 HOUR / REFEED)

—■— TF(%) -·-·-·- RPE(%) - - TF(%) -▲- TF(%) -○- RPE(%)
 NON-ADJUSTED TARGETS 2.5 ug/ml B(a)P ADJUSTED TARGETS

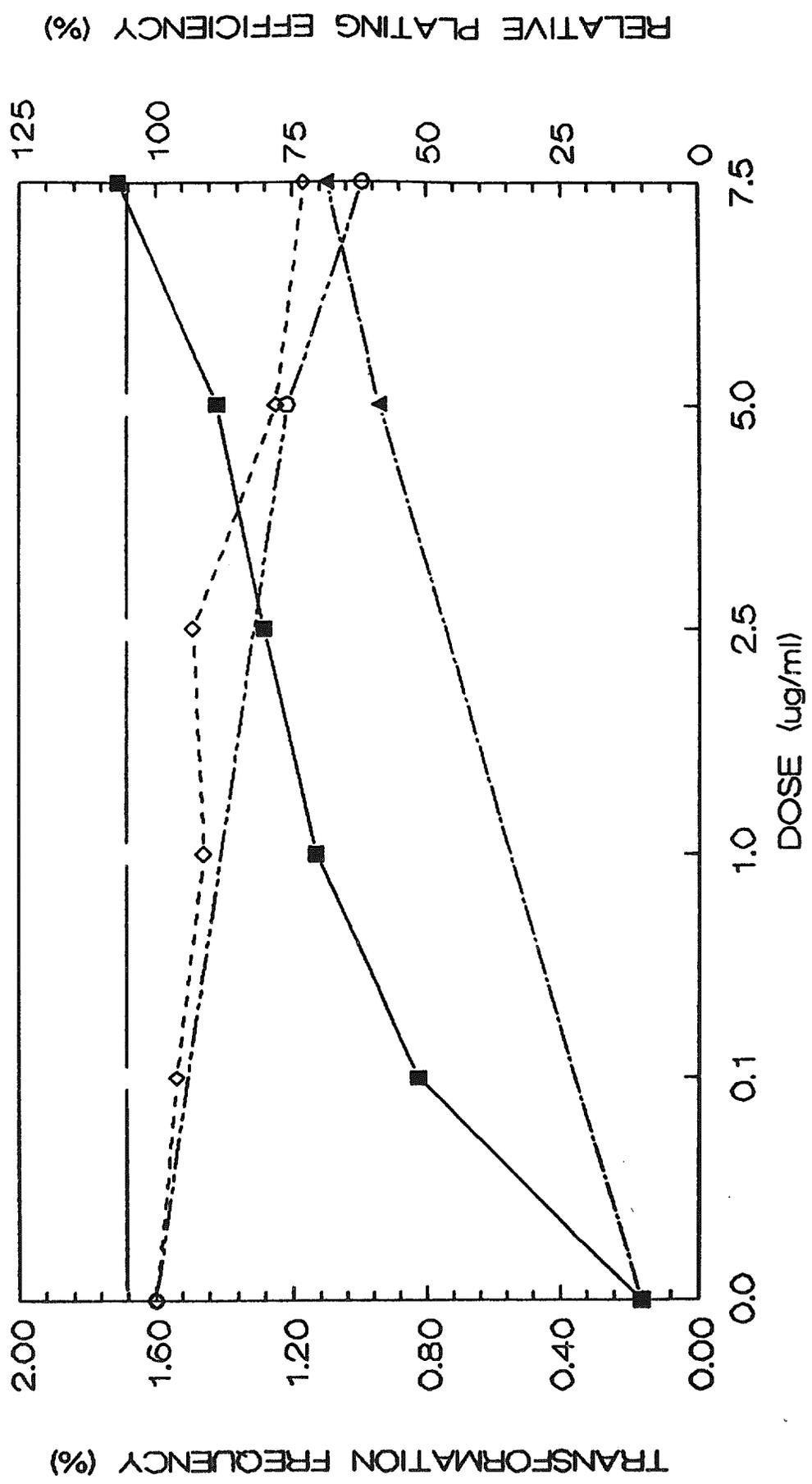


TABLE I
 24 HR / REFEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO429.01

A. Cytotoxicity by Relative Plating Efficiency
 (76 target cells seeded)

Dose	PLATE COUNTS										Average number Colonies/Dish	Average P.E. ¹ ± S.E. ²	Relative Plating Efficiency %
	1	2	3	4	5	6	7	8	9	10			
MEDIUM	48	28	30	35	29	39	30	32	32	36	34	49 ± 2.8	100
0.1 µg/ml	34	42	28	37	15	43	27	25	30	36	32	45 ± 3.8	92
1.0 µg/ml	33	35	27	23	34	38	24	25	23	25	29	41 ± 2.5	84
2.5 µg/ml	29	30	19	29	25	34	29	20	26	20	26	37 ± 2.3	76
5.0 µg/ml	22	24	27	24	22	22	24	17	21	21	22	32 ± 1.2	65
7.5 µg/ml	27	24	22	17	23	22	17	18	16	13	20	28 ± 1.9	57
10.0 µg/ml	15	24	19	16	24	20	14	23	16	15	19	27 ± 1.8	55
25.0 µg/ml	22	13	5	23	26	18	10	15	14	12	16	23 ± 2.9	47
50.0 µg/ml	13	20	10	11	14	9	12	14	20	23	15	21 ± 2.2	43
75.0 µg/ml	5	5	6	9	7	14	4	2	6	5	6	9 ± 1.5	18
100.0 µg/ml	9	4	6	5	5	9	11	14	12	12	9	13 ± 1.6	27

TABLE 1
 24 HOUR / REFEEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO429.01

B. Cytotoxicity by Relative Colony Density

Dose	Total No. of Cells/Volume	# of Dishes Counted	Average No. Cells/Dish	Colony Density	Relative Colony Density %
MEDIUM	8.18×10^6 / 5 ml	5	1.64×10^6	4.81×10^4	100
0.1 µg/ml	3.88×10^6 / 5 ml	5	7.76×10^5	2.43×10^4	51
1.0 µg/ml	3.39×10^6 / 5 ml	5	6.78×10^5	2.34×10^4	49
2.5 µg/ml	2.21×10^6 / 5 ml	5	4.42×10^5	1.70×10^4	35
5.0 µg/ml	1.47×10^6 / 5 ml	5	2.94×10^5	1.34×10^4	28
7.5 µg/ml	1.10×10^6 / 5 ml	5	2.20×10^5	1.10×10^4	23
10.0 µg/ml	1.07×10^6 / 5 ml	5	2.14×10^5	1.13×10^4	23
25.0 µg/ml	8.50×10^5 / 5 ml	5	1.70×10^5	1.10×10^4	23
50.0 µg/ml	6.70×10^5 / 5 ml	5	1.34×10^5	8.93×10^3	19
75.0 µg/ml	6.10×10^5 / 5 ml	5	1.22×10^5	2.03×10^4	42
100.0 µg/ml	4.80×10^5 / 5 ml	5	9.60×10^4	1.07×10^4	22

TABLE 1
24 HR / REFEED
SUMMARY OF PRELIMINARY CYTOTOXICITY USING
SYRIAN HAMSTER EMBRYO CELLS
OF RO429.01

¹Average PE = Average PE of combined dishes

$$\text{Standard Error (SE)} = \frac{\text{Standard deviation of combined average PE}}{\sqrt{\text{combined total no. of dishes counted}}}$$

$$\text{²Relative Plating Efficiency (RPE)} = \frac{\text{plating efficiency}}{\text{PE of solvent control}} \times 100$$

$$\text{³Total No. of Cells (Volume)} = \text{Cell Suspension Concentration} \times \text{Suspension Volume}$$

$$\text{⁴Average No. Cells/Dish} = \frac{\text{Total no. of cells}}{\text{No. of dishes counted}}$$

$$\text{⁵Colony Density} = \frac{\text{Average No. cells/dish}}{\text{Average no. of colonies/dish}}$$

$$\text{⁶Relative Colony Density} = \frac{\text{Colony density}}{\text{Colony density of solvent control}} \times 100$$

TABLE 2
24 HR / REFEEED
SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
TRANSFORMATION ASSAYS OF
RO429.01

Dose	Total Colonies Scored	MT ¹ / MT Freq ² .	Average P.E. ± S.E. ^{3,4}	Relative P.E. (%) ⁵	MT Value 1 Tail Fisher's ⁶
MEDIUM	1245	2 / 0.161	45 ± 1.3	100	
DMSO (0.2%)	1221	3 / 0.246	44 ± 1.3	98	
B(a)P 2.5 µg/ml	1129	19 / 1.683	40 ± 0.8	91	0.0002 ⁷
Dose: 0.1 µg/ml	1214	10 / 0.824	43 ± 1.0	96	0.0171 ⁷
1.0 µg/ml	1149	13 / 1.131	41 ± 1.6	91	0.0023 ⁷
2.5 µg/ml	1170	15 / 1.282	42 ± 0.8	93	0.0007 ⁷
5.0 µg/ml	986	14 / 1.420	35 ± 1.3	78	0.0004 ⁷
5.0CA µg/ml	1484	14 / 0.943	34 ± 0.9	76	0.0057 ⁷
7.5 µg/ml	935	16 / 1.711	33 ± 1.1	73	0.0001 ⁷
7.5CA µg/ml	1366	15 / 1.098	28 ± 0.7	62	0.0021 ⁷

TABLE 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL
TRANSFORMATION ASSAYS OF
RO429.01

¹MT = Combined total no. of morphologically transformed colonies

$$^2\text{MT Freq} = \frac{\text{MT}}{\text{Total colonies scored}} \times 100$$

³Average PE = Average PE of combined trials

$$^4\text{Standard Error (SE)} = \frac{\text{Standard deviation of combined average PE}}{\sqrt{\text{combined total no. of dishes counted}}}$$

$$^5\text{Relative PE} = \frac{\text{Average PE}}{\text{Average PE of solvent control}} \times 100$$

⁶MT P value = Probability of statistically significant treatment related effects using Fishers' Exact Test compared to control groups.

⁷Statistically significant treatment related difference between the morphological transformation frequency (MTF) of the solvent control group compared to the MTF of the treatment group at $P \leq 0.05$ using a 1-tailed Fishers Exact Test.

⁸TR = Unstratified binomial exact permutation trend test for significant positive dose response trend where a $P \leq 0.05$ or less is considered statistically significant.

TABLE 3
TRIAL 1

24 HR / REFEED
SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
TRANSFORMATION ASSAY OF
RO429.01

Dose	Total Colonies Scored	MT ¹ / MT Freq. ²	Average P.E. ³ ± S.E. ⁴	Relative P.E. (%) ⁵
MEDIUM	659	1 / 0.152	47 ± 1.6	100
DMSO (0.2%)	649	2 / 0.308	46 ± 1.5	98
B(a)P 2.5 µg/ml	563	11 / 1.954	40 ± 1.4	87
Dose: 0.1 µg/ml	637	8 / 1.256	46 ± 1.3	98
1.0 µg/ml	657	12 / 1.826	47 ± 1.8	100
2.5 µg/ml	607	10 / 1.647	43 ± 1.1	91
5.0 µg/ml	568	9 / 1.585	41 ± 1.6	87
5.0CA µg/ml	745	6 / 0.805	35 ± 1.3	74
7.5 µg/ml	473	10 / 2.114	34 ± 1.1	72
7.5CA µg/ml	715	7 / 0.979	29 ± 1.0	62

TABLE 4
 TRIAL 1
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO429.01

Cytotoxicity by Relative Colony Density

Dose	Total No. of Cells/Volume ^a	Average No. Cells/Dish ^b	Average No. Colonies/Dose	Colony Density ^a	Relative Colony Density ^b
Medium	8.11x10 ⁶ / 5 ml	1.62x10 ⁶	33	4.91x10 ⁴	100
DMSO (0.2%)	9.43x10 ⁶ / 5 ml	1.89x10 ⁶	32	5.91x10 ⁴	120
B(a)P 2.5 µg/ml	7.80x10 ⁶ / 5 ml	1.56x10 ⁶	28	5.57x10 ⁴	94
Dose: 0.10 µg/ml	8.83x10 ⁶ / 5 ml	1.77x10 ⁶	32	5.53x10 ⁴	113
1.0 µg/ml	9.93x10 ⁶ / 5 ml	1.99x10 ⁶	33	6.03x10 ⁴	123
2.5 µg/ml	5.38x10 ⁶ / 5 ml	1.08x10 ⁶	30	3.60x10 ⁴	73
5.0 µg/ml	3.30x10 ⁶ / 5 ml	6.60x10 ⁵	28	2.36x10 ⁴	48
5.0CA µg/ml	4.90x10 ⁶ / 5 ml	9.80x10 ⁵	37	2.65x10 ⁴	54
7.5 µg/ml	2.75x10 ⁶ / 5 ml	5.50x10 ⁵	24	2.29x10 ⁴	47
7.5CA µg/ml	5.35x10 ⁶ / 5 ml	1.07x10 ⁶	36	2.97x10 ⁴	60

TABLE 5
 TRIAL 2
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO429.01

Dose	Total Colonies Scored	MT / MT Freq.	Average P.E. \pm S.E.	Relative P.E. (%)
MEDIUM	586	1 / 0.171	42 \pm 2.0	100
DMSO	572	1 / 0.175	41 \pm 1.9	98
B(a)P 2.5 μ g/ml	566	8 / 1.413	40 \pm 1.0	98
Dose: 0.1 μ g/ml	577	2 / 0.347	41 \pm 1.3	98
1.0 μ g/ml	492	1 / 0.203	35 \pm 2.0	83
2.5 μ g/ml	563	5 / 0.888	40 \pm 1.0	95
5.0 μ g/ml	418	5 / 1.196	30 \pm 1.2	71
5.0CA μ g/ml	739	8 / 1.083	34 \pm 1.2	81
7.5 μ g/ml	462	6 / 1.299	33 \pm 1.8	79
7.5CA μ g/ml	651	8 / 1.229	27 \pm 1.1	64

TABLE 6
 TRIAL 2
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO429.01

Cytotoxicity by Relative Colony Density

Dose	Total No. of Cells/Volume	Average No. Cells/Dish	Average No. Colonies/Dose	Colony Density	Relative Colony Density%
Medium	$8.60 \times 10^6 / 5 \text{ ml}$	1.72×10^6	29	5.93×10^4	100
DMSO (0.2%)	$7.76 \times 10^6 / 5 \text{ ml}$	1.55×10^6	29	5.34×10^4	90
B(a)P 2.5 $\mu\text{g/ml}$	$8.94 \times 10^6 / 5 \text{ ml}$	1.79×10^6	28	6.39×10^4	108
Dose: 0.10 $\mu\text{g/ml}$	$1.13 \times 10^7 / 5 \text{ ml}$	2.26×10^6	29	7.79×10^4	131
1.0 $\mu\text{g/ml}$	$6.24 \times 10^6 / 5 \text{ ml}$	1.25×10^6	25	5.00×10^4	84
2.5 $\mu\text{g/ml}$	$4.92 \times 10^6 / 5 \text{ ml}$	9.84×10^5	28	3.51×10^4	59
5.0 $\mu\text{g/ml}$	$6.25 \times 10^6 / 5 \text{ ml}$	1.25×10^6	21	5.95×10^4	100
5.0CA $\mu\text{g/ml}$	$9.97 \times 10^6 / 5 \text{ ml}$	1.99×10^6	37	5.38×10^4	91
7.5 $\mu\text{g/ml}$	$4.91 \times 10^6 / 5 \text{ ml}$	9.82×10^5	23	4.27×10^4	72
7.5CA $\mu\text{g/ml}$	$9.38 \times 10^6 / 5 \text{ ml}$	1.88×10^6	33	5.70×10^4	96

TABLES 3 through 7

24 HR / REFEED
INDIVIDUAL TRIAL SUMMARY
OF
RO429.01

¹MT = Combined total no. of morphologically transformed colonies

²MT Freq = $\frac{\text{MT}}{\text{Total colonies scored}} \times 100$

³Average PE = Average PE of combined trials

⁴Standard Error (SE) = $\frac{\text{Standard deviation of combined average PE}}{\sqrt{\text{combined total no. of dishes counted}}}$

⁵Relative PE = $\frac{\text{Average PE}}{\text{Average PE of solvent control}} \times 100$

⁶Total No. of Cells (Volume) = Cell Suspension Concentration x Suspension Volume

⁷Average No. Cells/Dish = $\frac{\text{Total no. of cells}}{\text{No. of dishes counted}}$

⁸Colony Density = $\frac{\text{Average No. cells/dish}}{\text{Average no. of colonies/dish}}$

⁹Relative Colony Density = $\frac{\text{Colony density}}{\text{Colony density of solvent control}} \times 100$

VIII. APPENDIX A

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: MEDIUM				DMSO (0.2%)			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	29	41	1	1	39	56	1
2	33	47		2	30	43	1
3	34	49		3	39	56	
4	27	39		4	31	44	
5	24	34		5	33	47	
6	27	39		6	29	41	
7	30	43		7	39	56	
8	37	53		8	29	41	
9	37	53		9	31	44	
10	28	40		10	32	46	
11	38	54		11	40	57	
12	40	57		12	39	56	
13	33	47		13	31	44	
14	35	50		14	31	44	
15	41	59		15	30	43	
16	37	53		16	36	51	
17	37	53		17	30	43	
18	25	36		18	30	43	
19	31	44		19	28	40	
20	36	51		20	22	31	
TOTAL COLONIES		659		TOTAL COLONIES		649	
AVERAGE COLONIES		33		AVERAGE COLONIES		32	
PE ±S.E.:		47 ± 1.6		PE ±S.E.:		46 ± 1.5	
MT/FREQ		1 / 0.152		MT/FREQ		2 / 0.308	

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:		B(a)P		2.5 µg/ml	
Dish #	Total Colonies	PE	MT		
1	33	47	2		
2	29	41	2		
3	19	27	1		
4	29	41	1		
5	19	27	3		
6	37	53	1		
7	25	36			
8	25	36	1		
9	31	44			
10	25	36			
11	30	43			
12	29	41			
13	29	41			
14	33	47			
15	25	36			
16	30	43			
17	30	43			
18	30	43			
19	27	39			
20	28	40			
TOTAL COLONIES/DOSE:		563			
AVERAGE COLONIES/DOSE:		28			
PE ±S.E.:		40 ± 1.4			
MT/FREQ.		11 / 1.954			

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: 0.1 µg/ml				1.0 µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	31	44	1	1	33	47	2
2	34	49	1	2	45	64	1
3	25	36	1	3	28	40	1
4	27	39	1	4	47	67	1
5	27	39	1	5	35	50	2
6	28	40	1	6	29	41	1
7	41	59	2	7	30	43	2
8	35	50		8	31	44	1
9	40	57		9	29	41	1
10	29	41		10	31	44	
11	30	43		11	26	37	
12	35	50		12	36	51	
13	36	51		13	32	46	
14	29	41		14	25	36	
15	31	44		15	37	53	
16	30	43		16	30	43	
17	31	44		17	31	44	
18	30	43		18	36	51	
19	33	47		19	35	50	
20	35	50		20	31	44	
TOTAL COLONIES/DOSE:		637		TOTAL COLONIES/DOSE:		657	
AVERAGE COLONIES/DOSE:		32		AVERAGE COLONIES/DOSE:		33	
PE ±S.E.:		46 ± 1.3		PE ±S.E.:		47 ± 1.8	
MT/FREQ.		8 / 1.256		MT/FREQ.		12 / 1.826	

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: 2.5 µg/ml				5.0 µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	33	47	1	1	23	33	1
2	27	39	1	2	21	30	1
3	29	41	1	3	18	26	1
4	30	43	1	4	26	37	2
5	30	43	1	5	34	49	1
6	29	41	1	6	29	41	2
7	24	34	2	7	34	49	1
8	30	43	2	8	33	47	
9	31	44		9	24	34	
10	33	47		10	35	50	
11	30	43		11	36	51	
12	36	51		12	31	44	
13	30	43		13	27	39	
14	30	43		14	25	36	
15	33	47		15	27	39	
16	35	50		16	27	39	
17	24	34		17	30	43	
18	27	39		18	30	43	
19	30	43		19	33	47	
20	36	51		20	25	36	
TOTAL COLONIES/DOSE:		607		568			
AVERAGE COLONIES/DOSE:		30		28			
PE ±S.E.:		43 ± 1.1		41 ± 1.6			
MT/FREQ.		10 / 1.647		9 / 1.585			

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: 7.5 μ g/ml

Dish #	Total Colonies	PE	MT
1	17	24	1
2	22	31	2
3	24	34	2
4	30	43	1
5	23	33	1
6	24	34	1
7	27	39	2
8	25	36	
9	21	30	
10	24	34	
11	21	30	
12	25	36	
13	24	34	
14	26	37	
15	24	34	
16	20	29	
17	22	31	
18	32	46	
19	23	33	
20	19	27	

TOTAL COLONIES/DOSE: 473
AVERAGE COLONIES/DOSE: 24
PE \pm S.E.: 34 \pm 1.1
MT/FREQ. 10 / 2.114

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: 5.0CA µg/ml				7.5CA µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	33	31	1	1	39	32	1
2	41	38	1	2	31	25	1
3	33	31	4	3	40	33	1
4	31	29		4	33	27	1
5	45	42		5	31	25	1
6	29	27		6	48	39	2
7	48	44		7	37	30	
8	33	31		8	37	30	
9	40	37		9	33	27	
10	40	37		10	31	25	
11	36	33		11	29	24	
12	39	36		12	30	24	
13	29	27		13	29	24	
14	38	35		14	31	25	
15	36	33		15	40	33	
16	28	26		16	38	31	
17	29	27		17	38	31	
18	42	39		18	36	29	
19	49	45		19	37	30	
20	46	43		20	47	38	
TOTAL COLONIES		745		715			
AVERAGE COLONIES		37		36			
PE ±S.E.:		35 ± 1.3		29 ± 1.0			
MT/FREQ.		6 / 0.805		7 / 0.979			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:		MEDIUM			DMSO (0.2%)			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT	
1	31	44	1	1	33	47	1	
2	26	37		2	21	30		
3	30	43		3	33	47		
4	38	54		4	31	44		
5	23	33		5	24	34		
6	20	29		6	24	34		
7	29	41		7	33	47		
8	20	29		8	30	43		
9	27	39		9	28	40		
10	32	46		10	37	53		
11	32	46		11	32	46		
12	32	46		12	23	33		
13	26	37		13	26	37		
14	31	44		14	20	29		
15	30	43		15	28	40		
16	17	24		16	21	30		
17	41	59		17	21	30		
18	33	47		18	30	43		
19	28	40		19	36	51		
20	40	57		20	41	59		
TOTAL COLONIES		586			572			
AVERAGE COLONIES		29			29			
PE ±S.E.:		42 ± 2.0			41 ± 1.9			
MT/FREQ.		1 / 0.171			1 / 0.175			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:	B(a)P	2.5	µg/ml
Dish #	Total Colonies	PE	MT
1	29	41	1
2	23	33	1
3	23	33	1
4	29	41	2
5	31	44	1
6	25	36	1
7	35	50	1
8	28	40	
9	27	39	
10	26	37	
11	28	40	
12	30	43	
13	29	41	
14	30	43	
15	23	33	
16	33	47	
17	28	40	
18	30	43	
19	28	40	
20	31	44	
TOTAL COLONIES/DOSE:		566	
AVERAGE COLONIES/DOSE:		28	
PE ±S.E.:		40 ± 1.0	
MT/FREQ.		8 / 1.413	

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:		0.1 µg/ml		1. µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	24	34	1	1	21	30	1
2	25	36	1	2	27	39	
3	33	47		3	21	30	
4	36	51		4	24	34	
5	19	27		5	26	37	
6	27	39		6	21	30	
7	30	43		7	25	36	
8	23	33		8	35	50	
9	30	43		9	21	30	
10	28	40		10	18	26	
11	33	47		11	20	29	
12	25	36		12	26	37	
13	30	43		13	42	60	
14	31	44		14	20	29	
15	31	44		15	21	30	
16	33	47		16	16	23	
17	33	47		17	24	34	
18	25	36		18	34	49	
19	31	44		19	21	30	
20	30	43		20	29	41	
TOTAL COLONIES/DOSE:		577		492			
AVERAGE COLONIES/DOSE:		29		25			
PE ±S.E.:		41 ± 1.3		35 ± 2.0			
MT/FREQ.		2 / 0.347		1 / 0.203			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:		2.5 µg/ml			5.0 µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT	
1	28	40	1	1	22	31	1	
2	34	49		2	17	24	1	
3	31	44	1	3	22	31	1	
4	28	40	1	4	24	34	1	
5	31	44	1	5	18	26	1	
6	19	27	1	6	26	37		
7	33	47		7	22	31		
8	28	40		8	29	41		
9	27	39		9	19	27		
10	28	40		10	24	34		
11	25	36		11	18	26		
12	27	39		12	18	26		
13	28	40		13	20	29		
14	31	44		14	16	23		
15	28	40		15	21	30		
16	24	34		16	27	39		
17	27	39		17	14	20		
18	30	43		18	20	29		
19	28	40		19	19	27		
20	28	40		20	22	31		
TOTAL COLONIES/DOSE:		563			418			
AVERAGE COLONIES/DOSE:		28			21			
PE ±S.E.:		40 ± 1.0			30 ± 1.2			
MT/FREQ.		5 / 0.888			5 / 1.196			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose:		7.5 μ g/ml	
Dish #	Total Colonies	PE	MT
1	25	36	2
2	27	39	2
3	19	27	1
4	28	40	
5	21	30	1
6	33	47	
7	17	24	
8	20	29	
9	22	31	
10	29	41	
11	16	23	
12	14	20	
13	25	36	
14	18	26	
15	18	26	
16	25	36	
17	21	30	
18	26	37	
19	22	31	
20	36	51	
TOTAL COLONIES/DOSE:		462	
AVERAGE COLONIES/DOSE:		23	
PE \pm S.E.:		33 \pm 1.8	
MT/FREQ.		6 / 1.299	

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO429.01

Dose: 5.0CA				7.5CA			
µg/ml				µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	36	33	1	1	40	33	2
2	50	46	3	2	44	36	1
3	35	32	1	3	27	22	1
4	32	30	1	4	33	27	1
5	40	37	2	5	34	28	1
6	31	29		6	33	27	1
7	35	32		7	39	32	1
8	35	32		8	31	25	
9	34	31		9	29	24	
10	37	34		10	30	24	
11	35	32		11	27	22	
12	27	25		12	31	25	
13	45	42		13	25	20	
14	41	38		14	34	28	
15	34	31		15	27	22	
16	41	38		16	30	24	
17	34	31		17	30	24	
18	29	27		18	34	28	
19	46	43		19	45	37	
20	42	39		20	28	23	
TOTAL COLONIES		739		TOTAL COLONIES		651	
AVERAGE COLONIES		37		AVERAGE COLONIES		33	
PE ±S.E.:		34 ± 1.2		PE ±S.E.:		27 ± 1.1	
MT/FREQ.		8 / 1.083		MT/FREQ.		8 / 1.229	

Appendix A

24 HR / REFEED
INDIVIDUAL TRIAL SUMMARY
OF RO429.01

$$^1\text{Plating Efficiency (PE)} = \frac{\text{Avg. no. colonies per dish} \times 100}{\text{No. of target cells seeded}}$$

²MT = Combined total no. of morphologically transformed colonies

³Average PE = Average PE of combined trials

$$^4\text{Standard Error (SE)} = \frac{\text{Standard deviation of combined average PE}}{\sqrt{\text{combined total no. of dishes counted}}}$$

$$^5\text{MT Freq} = \frac{\text{MT}}{\text{Total colonies scored}} \times 100$$

⁶C= contaminated plate

VIII. APPENDIX B

The Procter & Gamble Company

PROTOCOL C45

Clonal Transformation Assay Using Syrian Golden Hamster Embryo (SHE) Cells

Issue Date: June 18, 1994
Supersedes Issue Dated: September 20, 1991

Test Substance Identification Number (TSIN) #

R0429.01

Divisional Request Document Number (DRD) #

410

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

Testing Facility:
(To be filled in by
Operations Section)

Study # 16744-0 C45
(To be filled in by
Testing Facility)

Purpose:

To determine the potential transforming activity of a test chemical in cryopreserved Syrian Hamster Embryo (SHE) cells.

Justification for Selection of Test System:

Syrian Golden Hamster embryo cells are the test system of choice based on the amount of background data available.

Route of Administration of Test Substance:

In Vitro. Route specified by test procedure.

Records to be Maintained:

All records that would be required to construct the study and demonstrate adherence to the protocol.

Test Substances:

<u>TSIN #</u>	<u>DRD Number</u>	<u>Description</u>		<u>Expiration Date</u>
		<u>Color</u>	<u>Physical Form</u>	

PROTOCOL C45

Clonal Transformation Assay Using Syrian Golden Hamster
Embryo (SHE) Cells

Issue Date: June 18, 1994
Supersedes Issue Dated: September 20, 1991

Storage Conditions: (Check One)

- | | |
|---|---------------------------------------|
| <input type="checkbox"/> Room Temperature | <input type="checkbox"/> Refrigerator |
| <input type="checkbox"/> Freezer | <input type="checkbox"/> Other |

Hazards: (Check One)

- None known. Take ordinary precautions in handling.
 As follows:

Special instructions: (Check One)

- None
 As follows:

Solvents:

Unless the solubility properties of the test material are provided by the Sponsor or the solubility properties are available from another source, a suitable solvent must be found for the test material, using the approved SOP of the Test Facility.

Check which solvent(s) the test substance is soluble in if known.

- Culture medium
 DMSO
 Acetone
 Ethanol
 Other

Test System Identification:

Individual SHE cell isolates are to be identified by lot number according to the approved SOP of the Test Facility.

Test System:

Syrian Golden Hamster Embryo (SHE) cells obtained according to the procedures described in the approved SOP of the Test Facility.

The Procter & Gamble CompanyPROTOCOL C45Clonal Transformation Assay Using Syrian Golden Hamster
Embryo (SHE) Cells

Issue Date: June 18, 1994

Supersedes Issue Dated: September 20, 1991

Test System Storage:

Frozen stocks of SHE cells are prepared and maintained in a liquid nitrogen freezer according to the procedures described in the approved SOP of the Test Facility.

Dose Regimen:

7 Days 24 Hours Other
Please consult Genetic Toxicology Section.

Dose Preparation:

Dose concentrations will be determined by results of the Preliminary Cytotoxicity Test, described in the approved SOP of the Test Facility.

Using the approved SOP of the Test Facility, immediately prior to use, the test material will be diluted in an appropriate solvent to form a series of concentrations (at least five) that when diluted into culture medium will yield the appropriate set of test concentrations. The final concentration of solvent will be 0.2%.

At least one positive control at a dose ranging from 1.25 ug/ml to 10 ug/ml Benzo[a]pyrene in culture medium is employed. A 0.2% solvent control is prepared in culture medium and employed. A culture medium only (nontreated control group) will be included, only when culture medium is the solvent for the test material.

Equipment, Supplies, Biological Reagents and Solutions:

All are prepared and used according to the approved SOPs of the Test Facility.

Solubility of the Test Material: (See approved SOP of the Test Facility)

Before performing the SHE cell transformation assay, test material solubility in an appropriate solvent is established. The solvents listed on page 2 are tested in order of choice up to a test material concentration of 5 mg/ml. The solvent which dissolves the test material at this concentration is used in the SHE cell transformation assay.

The Procter & Gamble CompanyPROTOCOL C45Clonal Transformation Assay Using Syrian Golden Hamster
Embryo (SHE) Cells

Issue Date: June 18, 1994
Supersedes Issue Dated: September 20, 1991

Preliminary Cytotoxicity Assay (See approved SOP of the Test Facility).

Before performing the SHE cell transformation assay, a cytotoxicity assay is done to establish a dose range. This involves exposing SHE target cells in clonal growth for 7 days to a range of concentrations of test material. The highest dose used will be 5 mg/ml. Lower doses may be used if the solubility of the test material is limited. At least 5 concentrations of test material will be used (i.e. 1, 10, 100, 500, and 5000 ug/ml). Following exposure, SHE cell colonies in culture dishes are counted with a stereo-microscope and plating efficiencies are calculated (plating efficiency = number of colonies obtained/number of target cells seeded X 100). Also, cells in culture dishes from each dose are counted to determine the number of cells/colony. A second and if necessary, a third toxicity screen should be conducted to refine the appropriate dose range for the transformation study. The Study Director decides the doses of test material to be used in the transformation assay. The top dose will cause at least a 50% reduction in plating efficiency, compared to concurrent controls, unless the number of cells/colony is decreased due to toxicity to a point which precludes scoring that dose level. A decrease in cell number/colony should be corroborated by data generated in this toxicity screen. The low dose for the assay will be the solvent control. At least three doses equally spaced in between the top and low dose will be included in each assay.

If no toxicity is observed, the doses chosen for the transformation assay will be based on the solubility of the test material, with a maximum concentration tested of 5 mg/ml.

24 Hour Dosing Option

If the 24 hour dosing regimen is selected for the transformation assay, the Cytotoxicity Assay dosing regimen will involve 24 hour dosing rather than 7 days, to establish the test material concentrations to be used in the transformation assay.

Adjusted Target Cell Seeding: (See approved SOP of the Test Facility)

In the SHE cell transformation assay the number of colonies/dish has been observed to affect transformation frequency. Therefore a means of obtaining a constant number of colonies/dish across all dose groups is needed prior to performing the transformation assay.

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Data obtained from the preliminary cytotoxicity assay are used for adjusting the target cell seeding. Following test chemical exposure, SHE cell colonies are counted in all plates of each group. The mean number of colonies for each group is calculated. The mean plating efficiency is calculated for each group (plating efficiency = number of colonies obtained/number of target cells seeded X 100). The relative plating efficiency (RPE) for each test material group is calculated (relative PE = PE test material dose/PE solvent X 100). Target cell adjustment is done in those test material dose groups with RPE < 70%. The number of target cells needed for a test material dose group is calculated (number of target cells needed = original number of target cells seeded X 100/relative plating efficiency). In the SHE transformation assay the adjusted number of target cells for each test material dose group will be used. For the control groups (positive and nontreated) the appropriate number of target cells for this cell lot will be used to obtain 25-45 colonies/plate, with an optimum of 35 colonies/dish. (see approved SOP of the Test Facility). With target cell seeding adjustment, 25-45 colonies/dish will also be obtained for the test material dose groups, again with an optimum of 35 colonies/dish.

24 Hour Dosing Option (See description in Dosing of Cells in Transformation Assay below). If the 24 hour dosing regimen is selected for the transformation assay, the procedures for determining adjusted target cell seeding density will involve 24 hour dosing, rather than 7 days, to establish the target cell seeding densities to be used in the transformation assay.

Transformation Assay:

Feeder Cell Preparation (See approved SOP of the Test Facility).

At least two individual trials within each study will be done. Cryopreserved SHE cells from a tested and approved lot are thawed and grown to 50-90% confluency in growth flasks (2-4 days). On day one of the assay, feeder cells are detached and suspended in culture medium in a growth flask on wet ice. The cells are x-ray irradiated to a point where they are still viable, yet no longer capable of replication (~5000 rad). Confirmation of this is made by preparing 5 feeder cell only plates. Following irradiation, the cells are centrifuged to form a pellet, resuspended in culture medium and counted, using a hemacytometer. The cell concentration is adjusted to 2×10^4 cells/ml in culture medium and 2 ml of this suspension is placed into each 60 mm culture dish. Each assay will include at least 5 test material dose groups, at least 1 solvent control group, and at least 1 Benzo[a]pyrene positive control group. Each group will include at least 25 culture dishes. Dishes are incubated at $37 \pm 1^\circ \text{C}$ and $10 \pm 0.5\% \text{CO}_2$ for 24 hours.

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Target Cell Preparation (See approved SOP of the Test Facility).

On day 1 of the assay, a second vial of SHE cells from the above lot is thawed and grown to ~50% confluency in a growth flask (1 day). On day 2 of the assay, the target cells are detached, counted with a hemacytometer, and diluted with culture medium to a concentration of 30-50 viable cells/ml in culture medium for the nontreated, solvent, and BaP control groups, as well as the unadjusted test chemical dose groups. For the target cell adjusted dose groups, the required number of target cells/dish is determined from results of the cytotoxicity assay (see above). The number of target cells seeded should achieve an average of 25-45 colonies/dish in all dishes, with an optimum of 35 colonies/dish. 2 ml of the target cell suspensions are placed into each culture dish, containing 4×10^4 feeder cells. Dishes are incubated at $37 \pm 1^\circ \text{C}$ and $10 \pm 0.5\% \text{CO}_2$ for 24 hours.

Dosing of Cells

On day 3 of the assay, test material stock solutions are prepared by dissolving the test chemical in the chosen solvent at a concentration below maximum solubility. From this stock solution, at least five serial dilutions of test chemical in solvent are prepared to achieve 500X the final culture dish concentrations. Each of these test chemical solutions is diluted 1:250 with culture medium to yield a 2X desired final concentration, such that upon addition to the target cells, final concentrations attained are 1X in 0.2% solvent. Each test chemical culture dish receives 4 ml 2X test chemical in 0.4% solvent in culture medium. Each culture dish in the nontreated control group receives 4 ml of culture medium. Each culture dish in the solvent control group receives 4 ml of 0.4% solvent in culture medium. Each culture dish in the Benzo[a]pyrene positive control group receives 4 ml of 2X BaP:0.4% solvent in culture medium. For BaP, the final dish concentration will be between 1.25 ug/ml and 10 ug/ml. All culture dishes are incubated (undisturbed) at $37 \pm 1^\circ \text{C}$ and $10 \pm 0.5\% \text{CO}_2$ for 7 days.

24 Hour Dosing Option

An optional dosing regimen may be chosen by the study Sponsor. This involves removing the test material:solvent dosing solutions from dishes at a time point earlier than 7 days (usually 24 hours) and refeeding the cultures with 8 ml/dish of culture medium minus test material. Control groups (nontreated, solvent, and BaP) are also refed with culture medium at this time. Following refeeding, cultures are incubated for 7 days (8 days after initial dosing). The extra day of incubation may be necessary to overcome retarded colony growth caused by refeeding.

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Fixing and Staining of Colonies

Following incubation, the culture medium is removed from each plate, and in 20 dishes/group the SHE cell colonies are methanol fixed and Giemsa stained, using the approved SOP of the Test Facility. In the remaining 5 dishes/group, cells are detached and counted, using the approved SOP of the Test Facility.

Collection of Data:

Using a stereo-microscope, each culture dish is examined to count and record the number of colonies/dish. Each colony is evaluated and scored to be either normal or morphologically transformed (MT).

Normal Morphology

Normal colonies contain cells with an organized, often flowing, pattern of growth with minimal cell criss-crossing, particularly where the cells are at a confluent density. Normal colonies also tend to be monolayer.

Transformed Morphology

Morphologically transformed colonies contain cells arrayed in an extensive random-oriented, three dimensional, stacked growth pattern, with criss-crossing cells at the perimeter and in the interior of the colony. Cells in morphologically transformed colonies frequently are more basophilic than their normal counterparts and have increased nuclear/cytoplasmic ratios.

Calculations From Data:

For each test material dose and control group, the following are calculated and recorded:

1. Mean number of colonies/culture dish.
2. Total number of colonies/test group.
3. Mean plating efficiency (PE) \pm SEM.

$$PE = \frac{\text{number of colonies/dish}}{\text{number of cells seeded}} \times 100$$

4. Relative plating efficiency (RPE).

$$RPE = \frac{\text{test group PE} \times 100}{\text{solvent control PE}}$$

5. Number of morphologically transformed (MT) colonies.

6. MT frequency = $\frac{\text{Number of MT colonies}}{\text{Total number of colonies}} \times 100$

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7. Ave. No. of cells/dish = $\frac{\text{Total No. of cells} \times 100}{\text{Total No. of dishes counted}}$
8. Colony Density = $\frac{\text{Ave. No. cells/dish}}{\text{Ave. No. colonies/dish}}$
9. Relative Colony Density = $\frac{\text{Colony Density}}{\text{Colony Density of solvent control}} \times 100$

Statistical Analysis

With pooled data from all trials, statistical tests for significant treatment related effects on transformation frequencies are done, using a one sided Fisher's Exact Test¹. In this test the transformation frequency of the solvent control group is compared pairwise to the transformation frequencies of each test material group including the positive BaP control group. The calculated p values are recorded for each group. An unstratified binomial exact permutation trend test² (Statxact - Cytel Software) for a significant positive dose response trend is also conducted.

Criteria for Acceptable Assay:

1. With pooled data from all trials, the total number of colonies/group for the control groups (nontreated, solvent, and BaP), must be ≥ 1000 . If less than 1000 colonies/treatment group is obtained, the study sponsor should be contacted and the results handled on a case by case basis.
2. With pooled data from all trials, the plating efficiencies of the medium and solvent control groups must be $\geq 25\%$, with 25-45 colonies/dish.
3. With pooled data from all trials, the transformation frequencies of the BaP positive control groups must be statistically significantly greater ($p < 0.05$) than the transformation frequency of the solvent control group, as indicated by the Fisher's Exact Test.
4. Transformation frequencies for the non-treated and solvent control groups for each trial must be between 0-0.6%. Frequencies greater than 0.6% will be handled on a case by case basis with the study sponsor.
5. The top dose of test material must 1) cause at least a 50% reduction in plating efficiency, compared to concurrent controls, unless the reduction in the number of cells/colony due to toxicity at this top dose precludes scoring of the colonies for transformation, or 2) be a maximum testable dose based on test chemical solubility considerations or 3) a maximum of 5 mg/ml test material in the cultures.

¹Armitage, P. (1971) Statistical Methods in Medical Research, Blackwell Scientific Publications, Oxford pp 135-138.

²Statxact, Cytel Software Corp., Cambridge, MA 02139.

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Report:

A report of the results of the study will be prepared by the Contract Facility. This report will include, but not be limited to, the following:

1. The study objectives as stated in the approved protocol, and any changes to the original protocol.
2. A summary of the results as they relate to the study's objectives.
3. A detailed description of all methods used.
4. Deviations from the Test Facility's SOPs or the approved protocol.
5. The location where all data will be stored.
6. A table showing plating efficiency and cell number/dish data from the cytotoxicity test will be included. Based on these data and the criteria described for test material dose selection, a statement will be made indicating what doses of test material were chosen for the transformation study.
7. A table, from the test for adjusting target cell seeding density, showing plating efficiencies, relative plating efficiencies of various test material doses, and the adjusted number of target cells/test material dose, will be included.
8. At least two individual trials within each study will be done. A table for each trial will include for each test group:
 - a. Mean plating efficiency, SEM
 - b. Total number of colonies.
 - c. Mean number of colonies/dish.
 - d. Number of morphologically transformed (MT) colonies.
 - e. MT frequencies.
 - f. Average number of cells/dish, average number of cells/colony, and relative colony density.
9. An overall data summary will be made and included on a separate sheet. Data from all trials should be pooled for statistical analysis. This summary will include for each test group:
 - a. Mean plating efficiency, SEM.
 - b. Total number of colonies.
 - c. Mean number of colonies/dish.
 - d. Number of MT colonies.
 - e. MT frequencies.
 - f. p values comparing the solvent control to each test group.

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Criteria for Judging Test Material:

The statistical method employed to test for a significant treatment related effect will be a one sided Fisher's Exact Test. A test material will be considered positive if it causes a statistically significant increase (i.e. $p < 0.05$) in morphological transformation at at least two doses of chemical, compared to concurrent controls, with pooled data from all trials or a significant increase at one dose with a statistically significant ($p < 0.05$) positive dose-response trend. If a significant increase occurs at only one dose without a significant positive dose-response trend or a statistically significant dose-response trend without a statistically significant increase at any dose, contact the Sponsor, as additional studies may be warranted.

Protocol Changes:

If it becomes necessary to change the approved protocol, verbal agreement to make this change should be made between the Study Director and the Sponsor. As soon as practical, this change and the reasons for it should be put in writing and signed by both the Study Director and the Sponsor's Divisional Toxicologist. This document is then attached to the protocol as an amendment.

This report shall conform to all requirements outlined in Section 58.185, Subpart J, Good Laboratory Practices Regulations.

Sponsor: Mary A. Kirchweid
Divisional Toxicologist

Date Approved by Sponsor's
Divisional Toxicologist: _____

Proposed Starting Date: _____

Defined as _____

Proposed Experimental Completion Date: _____

Defined as _____

Proposed Completion Date: _____

Defined as _____

To be completed by
the Test Facility.

Study Director: _____

Date: _____

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