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July 28, 1995

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RE: TSCA Section 8(e) Submission for Sodium Nitrite (CAS No. 7632-00-0)

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 Sodium Nitrite as test substance RO447.01 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 Sodium Nitrite to be positive in several types of in vitro assays.

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



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SODIUM NITRITE (24HR)

CLONAL TRANSFORMATION ASSAY ON
RO447.01 DRD:HESE410
USING SYRIAN GOLDEN HAMSTER EMBRYO (SHE) CELLS

DRAFT REPORT

AUTHOR

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PERFORMING LABORATORY

CORNING Hazleton. (CHV)
9200 LEESBURG PIKE
VIENNA, VIRGINIA 22182

LABORATORY PROJECT ID

CHV STUDY NO.:16746-0.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

RO447.01 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 375 µg/ml, 500 µg/ml, 625 µg/ml, 750 µg/ml and 875 µg/ml with cell adjustment for the upper four 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 RO447.01 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: 16746-0.C45
- C. Test Article: RO447.01 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 methanol, 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

as 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 RO447.01 tested in the preliminary cytotoxicity assay was 10-5000 $\mu\text{g/ml}$.

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 an enhancement in relative plating efficiency between the 10 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ dose groups. There was 39% toxicity at 500 $\mu\text{g/ml}$, 63% toxicity at 1000 $\mu\text{g/ml}$ and 87% toxicity at 1750 $\mu\text{g/ml}$. The relative toxicity then reached 100% for both the 2500 $\mu\text{g/ml}$ and 5000 $\mu\text{g/ml}$ dose levels. When the cytotoxicity of the test material was evaluated by the relative reduction in colony density (Table 1 part B) one saw a somewhat jumpy response: there was 9% toxicity at 10 $\mu\text{g/ml}$ and 19% toxicity at 25 $\mu\text{g/ml}$, with a slight enhancement in colony density at 50 $\mu\text{g/ml}$ before the relative toxicity increased to 18%. At 100 $\mu\text{g/ml}$ the relative toxicity increased to 18%, with the toxicity of the 175 $\mu\text{g/ml}$ dose level dropping back slightly to 13% before jumping to 18% at 250 $\mu\text{g/ml}$. The relative toxicity was 35% at 500 $\mu\text{g/ml}$ increasing to 58% for both the 1000 $\mu\text{g/ml}$ and 1750 $\mu\text{g/ml}$. Both of the other dose levels (2500 $\mu\text{g/ml}$ and 5000 $\mu\text{g/ml}$) proved to be completely toxic. An additional cytotoxicity assay was then performed to better define the relative toxicity between 750 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ in the hopes of identifying a concentration causing an approximate 50% reduction in relative plating efficiency. The results of cytotoxicity determination by the relative reduction in plating efficiency (Table 1A part A) showed an enhancement in relative plating efficiency at 250 $\mu\text{g/ml}$, 19% relative toxicity at 275 $\mu\text{g/ml}$, 32% toxicity at 500 $\mu\text{g/ml}$, 28% toxicity at 625 $\mu\text{g/ml}$ and 32% at 750 $\mu\text{g/ml}$. As measured by relative colony density (Table 1A part B) the 250 $\mu\text{g/ml}$ dose level produced 14% toxicity while at 375 $\mu\text{g/ml}$ the toxicity was 20%. The toxicity then dipped slightly to 17% at 500 $\mu\text{g/ml}$ before increasing to 31% and 41% for the 625 $\mu\text{g/ml}$ and 750 $\mu\text{g/ml}$ dose levels respectively.

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: 375 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, 625 $\mu\text{g/ml}$, 750 $\mu\text{g/ml}$, and 875 $\mu\text{g/ml}$. In addition the 500 $\mu\text{g/ml}$, 625 $\mu\text{g/ml}$, 750 $\mu\text{g/ml}$, and 875 $\mu\text{g/ml}$ dose groups were only run with 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 (875 $\mu\text{g/ml}$) was determined: The test material solution had a measured osmolality of 329 mOSM/kg versus a reference of 290 mOSM/kg and the pH of the solution after 24 hours incubation was 6.72. 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. Moreover this response occurred over the entire range of toxicity obtained in the definitive assays..

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. 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 RO447.01 was tested using the 24 hour / refeed protocol. RO447.01 is therefore considered to be positive for its potential to induce morphological transformation when tested as described in this report.

VI. REFERENCES

1. Huberman E., Salzberg S., and Sachs L.: The *in vitro* induction of an increase in cell multiplication and cellular life span by the water soluble carcinogen dimethylnitrosamine. Proc. Nat. Acad. Sci. USA, 59:77-82, 1968.

2. Kuroki T., Sato H.: Transformation and neoplastic development in vitro of hamster embryonic cells by 4-nitroquinoline-1-oxide and its derivatives. *J. Natl. Cancer Inst.* 41:53-71, 1968.
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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.
5. Huberman E., Sachs L.: Cell susceptibility to transformation and cytotoxicity by the carcinogenic hydrocarbon benzo(a)pyrene. *Proc. Nat. Acad. Sci. USA* 56:1123-1129 1966.
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7. LeBoeuf R.A., Kerckaert G.A., Poiley J.A., and Raineri R.: An interlaboratory comparison of enhanced morphological transformation of Syrian hamster embryo cells cultured under conditions of reduced bicarbonate concentration and pH.. *Mutation Res.* 222:205-218 1989.
8. LeBoeuf R.A., and Kerckaert G.A.: The induction of transformed like morphology and enhanced growth in Syrian hamster embryo cells grown at acidic pH.. *Carcinogenesis* 7:1431-1440 1986.
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)

16746-0.C45 SUMMARY (RO447.0.1)
FIGURE 1 (24 HOUR / REFEED)

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

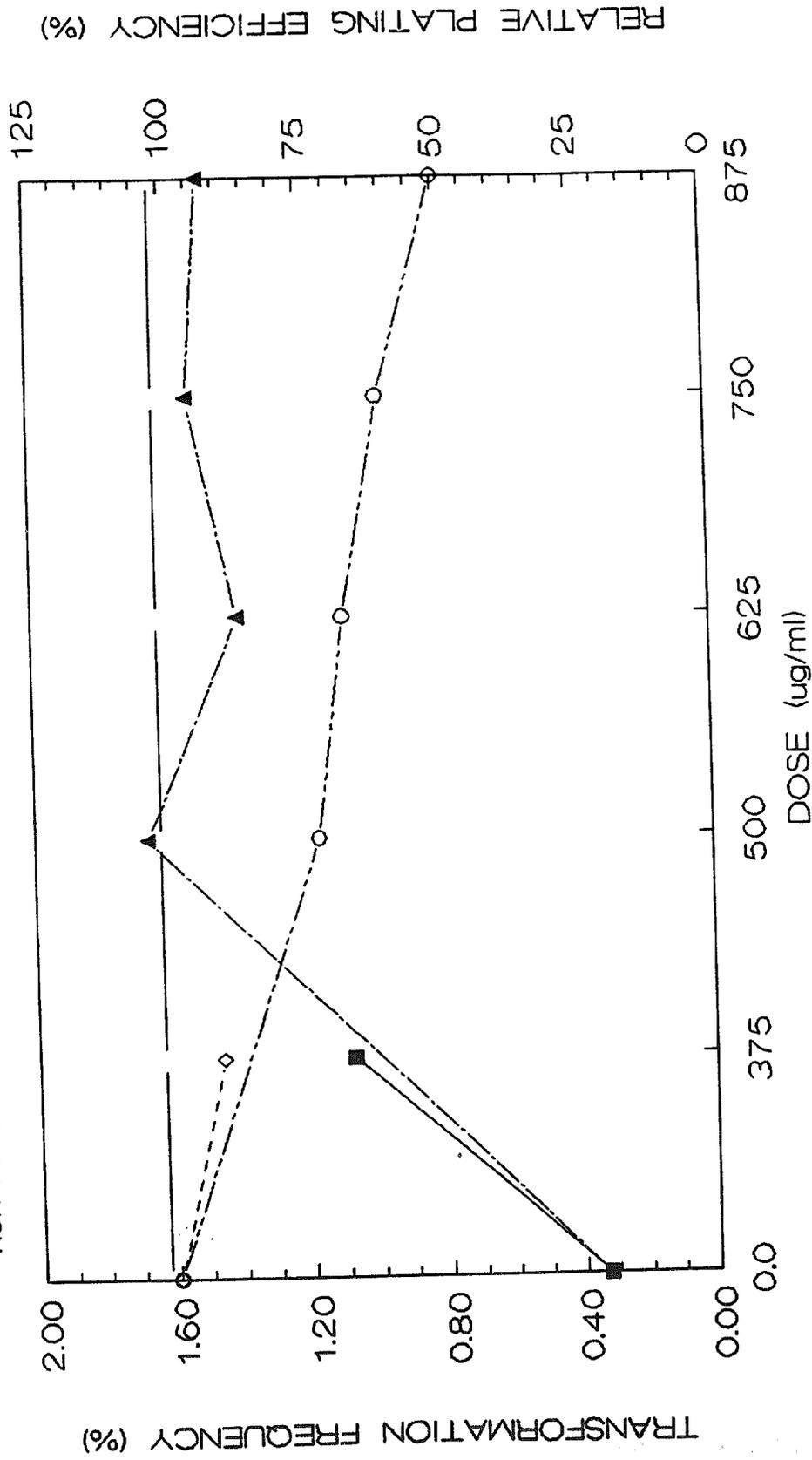


TABLE 1
 24 HR / REFEEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO447.01

A. Cytotoxicity by Relative Plating Efficiency
 (70 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	42	40	36	36	40	32	39	42	31	40	38	54 ± 1.8	100
10 µg/ml	39	42	37	37	41	40	38	31	55	C	40	57 ± 3.1	106
25 µg/ml	40	40	36	27	48	40	46	46	45	44	41	59 ± 2.8	109
50 µg/ml	34	42	41	35	37	36	47	44	38	40	39	56 ± 1.9	104
100 µg/ml	33	36	43	44	51	49	47	47	37	41	43	61 ± 2.7	113
175 µg/ml	49	44	40	38	46	35	35	53	28	48	42	60 ± 3.5	111
250 µg/ml	39	38	38	47	34	32	40	40	33	44	39	55 ± 2.1	102
500 µg/ml	28	26	29	18	20	16	24	19	24	26	23	33 ± 2.0	61
1000 µg/ml	17	15	15	12	12	13	12	14	15	18	14	20 ± 1.0	37
1750 µg/ml	3	0	4	3	2	6	8	9	4	8	5	7 ± 1.3	13
2500 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0 ± 0.0	0
5000 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0 ± 0.0	0

TABLE I
 24 HOUR / REFEEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO447.01

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	8.13x10 ⁶ / 5 ml	5	1.63x10 ⁶	4.28x10 ⁴	100
10 µg/ml	7.80x10 ⁶ / 5 ml	5	1.56x10 ⁶	3.90x10 ⁴	91
25 µg/ml	7.07x10 ⁶ / 5 ml	5	1.41x10 ⁶	3.45x10 ⁴	81
50 µg/ml	8.67x10 ⁶ / 5 ml	5	1.73x10 ⁶	4.45x10 ⁴	104
100 µg/ml	7.54x10 ⁶ / 5 ml	5	1.51x10 ⁶	3.51x10 ⁴	82
175 µg/ml	7.83x10 ⁶ / 5 ml	5	1.57x10 ⁶	3.73x10 ⁴	87
250 µg/ml	6.81x10 ⁶ / 5 ml	5	1.36x10 ⁶	3.49x10 ⁴	82
500 µg/ml	3.21x10 ⁶ / 5 ml	5	6.42x10 ⁵	2.79x10 ⁴	65
1000 µg/ml	1.25x10 ⁶ / 5 ml	5	2.50x10 ⁵	1.79x10 ⁴	42
1750 µg/ml	4.50x10 ⁵ / 5 ml	5	9.00x10 ⁴	1.80x10 ⁴	42
2500 µg/ml	2.80x10 ⁵ / 5 ml	5	5.60x10 ⁴	0.00	0
5000 µg/ml	5.00x10 ⁴ / 5 ml	5	1.25x10 ⁴	0.00	0

TABLE 1A
 24 HR / REFEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO447.01

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

Dose	PLATE COUNTS										Average P.E. ¹ ± S.E. ²	Relative Plating Efficiency %	
	1	2	3	4	5	6	7	8	9	10			Average number Colonies/Dish
MEDIUM	43	27	31	27	32	30	27	38	33	40	33	47 ± 2.5	100
250 µg/ml	35	25	38	37	35	36	40	30	26	31	33	48 ± 2.3	102
375 µg/ml	27	19	33	29	25	25	25	23	28	33	27	38 ± 1.9	81
500 µg/ml	27	25	21	23	17	29	20	18	24	20	22	32 ± 1.7	68
625 µg/ml	29	20	24	27	22	25	23	24	26	17	24	34 ± 1.6	72
750 µg/ml	23	19	29	23	26	22	21	15	24	21	22	32 ± 1.7	68

TABLE I
 24 HOUR / REFEED
 SUMMARY OF PRELIMINARY CYTOTOXICITY USING
 SYRIAN HAMSTER EMBRYO CELLS
 RO447.01

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	1.14×10^7 / 5 ml	5	2.28×10^6	6.91×10^4	100
250 $\mu\text{g/ml}$	9.85×10^6 / 5 ml	5	1.97×10^6	5.97×10^4	86
375 $\mu\text{g/ml}$	7.43×10^6 / 5 ml	5	1.49×10^6	5.52×10^4	80
500 $\mu\text{g/ml}$	6.31×10^6 / 5 ml	5	1.26×10^6	5.74×10^4	83
625 $\mu\text{g/ml}$	5.74×10^6 / 5 ml	5	1.15×10^6	4.78×10^4	69
750 $\mu\text{g/ml}$	4.45×10^6 / 5 ml	5	8.90×10^5	4.05×10^4	59

TABLES 1 and 1A
24 HR / REFEEED
SUMMARY OF PRELIMINARY CYTOTOXICITY USING
SYRIAN HAMSTER EMBRYO CELLS
OF RO447.01

¹Average PE = Average PE of combined dishes

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

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

³Total No. of Cells (Volume) = Cell Suspension Concentration x Suspension Volume

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

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

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
 RO447.01

Dose	Total Colonies Counted	MT ¹ / MT Freq ² .	Average P.E. ± S.E. ^{3,4}	Relative P.E. (%) ⁵	MT Value 1 Tail Fisher's ⁶
MEDIUM	1240	4 / 0.328	44 ± 1.1	100	-----
DMSO (0.2%)	1362	3 / 0.220	49 ± 1.5	111	-----
B(a)P 2.5 µg/ml	1229	20 / 1.627	44 ± 1.2	90	0.0001 ⁷
Dose: 375 µg/ml	1115	12 / 1.076	40 ± 1.0	91	0.0252 ⁷
500CA µg/ml	1320	22 / 1.667	32 ± 0.7	73	0.0005 ⁷
625CA µg/ml	1148	16 / 1.394	30 ± 0.8	68	0.0039 ⁷
750CA µg/ml	1112	17 / 1.529	27 ± 0.8	61	0.0018 ⁷
875CA µg/ml	1144	17 / 1.486	22 ± 0.4	50	0.0022 ⁷

TABLE 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL
TRANSFORMATION ASSAYS OF
RO447.01

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.

⁸CA = Cell Adjustment of dose equalized to controls:

$$\text{No. Target cells needed} = \frac{(\text{No. Target Cells seeded for Controls}) \times (100)}{\text{RPE of dose level (from cytotoxicity)}}$$

For the 500 µg/ml dose:

$$103 = \frac{(70 \text{ Cells seeded for Controls}) \times (100)}{68 \text{ (RPE of 500 } \mu\text{g/ml dose group)}}$$

For the 625 µg/ml dose:

$$97 = \frac{(70 \text{ Cells seeded for Controls}) \times (100)}{72 \text{ (RPE of 625 } \mu\text{g/ml dose group)}}$$

For the 750 µg/ml dose:

$$103 = \frac{(70 \text{ Cells seeded for Controls}) \times (100)}{68 \text{ (RPE of 750 } \mu\text{g/ml dose group)}}$$

For the 875 µg/ml dose:

$$132 = \frac{(70 \text{ Cells seeded for Controls}) \times (100)}{53 \text{ (RPE of 875 } \mu\text{g/ml dose group)}}$$

TABLE 3
 TRIAL 1
 24 HR / REFEEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO447.01

Dose	Total Colonies Scored	MT ¹ / MT Freq. ²	Average P.E. ³ ± S.E. ⁴	Relative P.E. (%) ⁵
MEDIUM	599	1 / 0.167	43 ± 1.9	100
DMSO (0.2%)	688	2 / 0.291	49 ± 2.6	114
B(a)P 2.5 µg/ml	562	10 / 1.779	40 ± 1.6	82
Dose: 375 µg/ml	519	6 / 1.156	37 ± 1.2	86
500CA µg/ml	631	13 / 2.060	31 ± 0.7	72
625CA µg/ml	535	6 / 1.121	28 ± 1.0	65
750CA µg/ml	536	9 / 1.679	26 ± 0.9	60
875CA µg/ml	559	6 / 1.073	21 ± 0.5	49

TABLE 4
 TRIAL I
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO447.01

Cytotoxicity by Relative Colony Density

Dose	Total No. of Cells/Volume ⁶	Average No. Cells/Dish ⁷	Average No. Colonies/Dose	Colony Density ⁸	Relative Colony Density ⁹
Medium	8.69x10 ⁶ / 5 ml	1.74x10 ⁶	30	5.80x10 ⁴	100
DMSO (0.2%)	8.26x10 ⁶ / 5 ml	1.65x10 ⁶	34	4.85x10 ⁴	84
B(a)P 2.5 µg/ml	6.25x10 ⁶ / 5 ml	1.25x10 ⁶	28	4.46x10 ⁴	92
Dose: 375 µg/ml	5.30x10 ⁶ / 5 ml	1.06x10 ⁶	26	4.08x10 ⁴	70
500CA µg/ml	5.11x10 ⁶ / 5 ml	1.02x10 ⁶	32	3.19x10 ⁴	55
625CA µg/ml	4.69x10 ⁶ / 5 ml	9.38x10 ⁵	27	3.47x10 ⁴	60
750CA µg/ml	4.05x10 ⁶ / 5 ml	8.10x10 ⁵	27	3.00x10 ⁴	52
875CA µg/ml	3.09x10 ⁶ / 5 ml	6.18x10 ⁵	28	2.21x10 ⁴	38

TABLE 5
 TRIAL 2
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO447.01

Dose	Total Colonies Scored	MT/ MT Freq.	Average P.E. ± S.E.	Relative P.E. (%)
MEDIUM	621	3 / 0.483	44 ± 1.2	100
DMSO (0.2%)	674	1 / 0.148	48 ± 1.7	109
B(a)P 2.5 µg/ml	667	10 / 1.499	48 ± 1.5	100
Dose: 375 µg/ml	596	6 / 1.007	43 ± 1.4	98
500CA µg/ml	689	9 / 1.306	33 ± 1.0	75
625CA µg/ml	613	10 / 1.631	32 ± 1.0	73
750CA µg/ml	576	8 / 1.389	29 ± 1.3	66
875CA µg/ml	585	11 / 1.880	22 ± 0.7	50

TABLE 6
 TRIAL 2
 24 HR / REFEED
 SUMMARY OF SYRIAN HAMSTER EMBRYO CELL
 TRANSFORMATION ASSAY OF
 RO447.01

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	1.10×10^7 / 5 ml	2.20×10^6	31	7.10×10^4	100
DMSO (0.2%)	1.07×10^7 / 5 ml	2.14×10^6	34	6.29×10^4	89
B(a)P 2.5 μ g/ml	8.96×10^6 / 5 ml	1.79×10^6	33	5.42×10^4	86
Dose: 375 μ g/ml	5.58×10^6 / 5 ml	1.12×10^6	30	3.73×10^4	53
500CA μ g/ml	6.12×10^6 / 5 ml	1.22×10^6	34	3.59×10^4	51
625CA μ g/ml	4.35×10^6 / 5 ml	8.70×10^5	31	2.81×10^4	40
750CA μ g/ml	4.43×10^6 / 5 ml	8.86×10^5	29	3.06×10^4	43
875CA μ g/ml	4.04×10^6 / 5 ml	8.08×10^5	29	2.79×10^4	39

TABLES 3 through 7

24 HR / REFEED
INDIVIDUAL TRIAL SUMMARY
OF
RO447.01

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

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

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

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

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

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

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

VIII. APPENDIX A

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose: MEDIUM				DMSO (0.2%)			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	40	57	1	1	32	46	1
2	43	61		2	38	54	1
3	30	43		3	35	50	
4	25	36		4	31	44	
5	34	49		5	25	36	
6	23	33		6	43	61	
7	34	49		7	26	37	
8	25	36		8	27	39	
9	28	40		9	21	30	
10	33	47		10	43	61	
11	26	37		11	47	67	
12	41	59		12	38	54	
13	29	41		13	22	31	
14	28	40		14	38	54	
15	27	39		15	41	59	
16	28	40		16	24	34	
17	30	43		17	38	54	
18	22	31		18	39	56	
19	29	41		19	33	47	
20	24	34		20	47	67	
TOTAL COLONIES		599		688			
AVERAGE COLONIES		30		34			
PE ±S.E.:		43 ± 1.9		49 ± 2.6			
MT/FREQ.		1 / 0.167		2 / 0.291			

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:	B(a)P	2.5 µg/ml	
Dish #	Total Colonies	PE	MT
1	25	36	2
2	26	37	1
3	29	41	1
4	28	40	1
5	34	49	1
6	23	33	2
7	18	26	2
8	30	43	
9	32	46	
10	21	30	
11	30	43	
12	32	46	
13	21	30	
14	30	43	
15	27	39	
16	35	50	
17	35	50	
18	34	49	
19	28	40	
20	24	34	

TOTAL COLONIES/DOSE:	562		
AVERAGE COLONIES/DOSE:	28		
PE ±S.E.:	40	±	1.6
MT/FREQ.	10	/	1.779

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose: 375 μ g/ml				500CA μ g/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	26	37	1	1	36	35	1
2	30	43	1	2	32	31	1
3	21	30	1	3	31	30	1
4	32	46	1	4	28	27	1
5	25	36	1	5	28	27	2
6	25	36	1	6	34	33	1
7	29	41		7	32	31	1
8	30	43		8	31	30	1
9	25	36		9	37	36	2
10	28	40		10	28	27	1
11	29	41		11	34	33	1
12	32	46		12	31	30	
13	25	36		13	38	37	
14	26	37		14	32	31	
15	22	31		15	32	31	
16	19	27		16	33	32	
17	21	30		17	27	26	
18	28	40		18	28	27	
19	20	29		19	30	29	
20	26	37		20	29	28	
TOTAL COLONIES/DOSE:						519	631
AVERAGE COLONIES/DOSE:						26	32
PE \pmS.E.:						37 \pm 1.2	31 \pm 0.7
MT/FREQ.						6 / 1.156	13 / 2.060

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:		625CA		µg/ml	750CA		µg/ml	
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT	
1	28	29	1	1	18	17	1	
2	24	25	1	2	33	32	1	
3	16	16	1	3	30	29	1	
4	28	29	1	4	25	24	1	
5	18	19	1	5	25	24	1	
6	30	31	1	6	32	31	2	
7	26	27		7	28	27	1	
8	34	35		8	35	34	1	
9	29	30		9	20	19		
10	28	29		10	28	27		
11	28	29		11	26	25		
12	23	24		12	25	24		
13	22	23		13	28	27		
14	31	32		14	30	29		
15	27	28		15	22	21		
16	28	29		16	25	24		
17	28	29		17	27	26		
18	29	30		18	26	25		
19	25	26		19	26	25		
20	33	34		20	27	26		
TOTAL COLONIES/DOSE:		535				536		
AVERAGE COLONIES/DOSE:		27				27		
PE ±S.E.:		28 ± 1.0				26 ± 0.9		
MT/FREQ.		6 / 1.121				9 / 1.679		

TRIAL 1
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:	875CA	$\mu\text{g/ml}$	
Dish #	Total Colonies	PE	MT
1	30	23	2
2	27	20	1
3	28	21	1
4	27	20	1
5	26	20	1
6	27	20	
7	33	25	
8	29	22	
9	30	23	
10	25	19	
11	28	21	
12	24	18	
13	28	21	
14	24	18	
15	33	25	
16	30	23	
17	28	21	
18	27	20	
19	23	17	
20	32	24	
TOTAL COLONIES/DOSE:		559	
AVERAGE COLONIES/DOSE:		28	
PE \pmS.E.:		21 \pm 0.5	
MT/FREQ.		6 / 1.073	

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:		MEDIUM		DMSO (0.2%)			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	38	54	2	1	33	47	1
2	37	53		2	37	53	
3	37	53	1	3	30	43	
4	30	43		4	32	46	
5	31	44		5	30	43	
6	26	37		6	37	53	
7	31	44		7	39	56	
8	37	53		8	30	43	
9	28	40		9	27	39	
10	31	44		10	27	39	
11	28	40		11	29	41	
12	27	39		12	30	43	
13	28	40		13	39	56	
14	31	44		14	32	46	
15	31	44		15	31	44	
16	31	44		16	34	49	
17	32	46		17	37	53	
18	31	44		18	39	56	
19	26	37		19	33	47	
20	30	43		20	48	69	
TOTAL COLONIES		621		674			
AVERAGE COLONIES		31		34			
PE ±S.E.:		44 ± 1.2		48 ± 1.7			
MT/FREQ.		3 / 0.483		1 / 0.148			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:	B(a)P	2.5 μ g/ml	
Dish #	Total Colonies	PE	MT
1	34	49	1
2	36	51	2
3	28	40	2
4	45	64	1
5	27	39	1
6	31	44	1
7	30	43	2
8	30	43	
9	30	43	
10	30	43	
11	33	47	
12	28	40	
13	43	61	
14	37	53	
15	37	53	
16	30	43	
17	33	47	
18	33	47	
19	36	51	
20	36	51	
TOTAL COLONIES/DOSE:		667	
AVERAGE COLONIES/DOSE:		33	
PE \pmS.E.:		48	\pm 1.5
MT/FREQ.		10	/ 1.499

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose: 375 µg/ml				500CA µg/ml			
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT
1	32	46	1	1	33	32	1
2	28	40	1	2	42	41	1
3	30	43	1	3	36	35	2
4	21	30	1	4	27	26	1
5	29	41	1	5	34	33	1
6	32	46	1	6	39	38	1
7	24	34		7	43	42	1
8	31	44		8	31	30	1
9	27	39		9	33	32	
10	23	33		10	37	36	
11	30	43		11	30	29	
12	27	39		12	41	40	
13	27	39		13	31	30	
14	37	53		14	31	30	
15	37	53		15	31	30	
16	29	41		16	30	29	
17	38	54		17	31	30	
18	31	44		18	33	32	
19	32	46		19	39	38	
20	31	44		20	37	36	
TOTAL COLONIES/DOSE:		596		689			
AVERAGE COLONIES/DOSE:		30		34			
PE ±S.E.:		43 ± 1.4		33 ± 1.0			
MT/FREQ.		6 / 1.007		9 / 1.306			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:		625CA		µg/ml		750CA		µg/ml	
Dish #	Total Colonies	PE	MT	Dish #	Total Colonies	PE	MT		
1	35	36	1	1	28	27	1		
2	28	29	1	2	24	23	1		
3	26	27	1	3	31	30	1		
4	28	29	2	4	33	32	1		
5	34	35	2	5	34	33	1		
6	30	31	1	6	36	35	3		
7	30	31	1	7	27	26			
8	25	26	1	8	26	25			
9	34	35		9	25	24			
10	20	21		10	30	29			
11	31	32		11	31	30			
12	39	40		12	27	26			
13	31	32		13	24	23			
14	28	29		14	34	33			
15	36	37		15	34	33			
16	35	36		16	33	32			
17	35	36		17	22	21			
18	28	29		18	35	34			
19	27	28		19	15	15			
20	33	34		20	27	39			
TOTAL COLONIES/DOSE:		613				576			
AVERAGE COLONIES/DOSE:		31				29			
PE ±S.E.:		32 ± 1.0				28 ± 1.3			
MT/FREQ.		10 / 1.631				8 / 1.389			

TRIAL 2
24 HR / REFEED
SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION ASSAY
OF RO447.01

Dose:		875CA $\mu\text{g/ml}$	
Dish #	Total Colonies	PE	MT
1	25	19	2
2	28	21	1
3	25	19	2
4	30	23	1
5	25	19	1
6	27	20	1
7	29	22	1
8	34	26	2
9	27	20	
10	40	30	
11	27	20	
12	26	20	
13	28	21	
14	34	26	
15	27	20	
16	30	23	
17	30	23	
18	27	20	
19	33	25	
20	33	25	

TOTAL COLONIES/DOSE: 585
AVERAGE COLONIES/DOSE: 29
PE \pm S.E.: 22 \pm 0.7
MT/FREQ. 11 / 1.880

Appendix A

24 HR / REFEED
INDIVIDUAL TRIAL SUMMARY
OF RO447.01

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

²MT = Combined total no. of morphologically transformed colonies

³Average PE = Average PE of combined trials

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

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

⁶C= contaminated plate

VIII. APPENDIX B

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