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

Test Type: Clonal Transformation

Conducting Laboratory and Location: P&G Miami Valley Laboratories, Biological Testing Facility, Cincinnati, OH

Test Substance(s): G0539.04 – Octopirox in DMSO

Species: Syrian Golden Hamster Embryo (SHE) Cells

Test Conditions: Primary assay modification was the length of time of SHE cell exposure to the test material. The exposure time was reduced to 24 hours from the usual exposure of 7 days then fixed/stained. 4 doses of OP tested ranging from 0.2 to 0.35 ug/ml.

Results: Octopirox caused a significant increase in morphological transformation frequency at four dose levels, compared to solvent controls and thus the test material is considered positive. Additionally, it can be concluded that this transformation represents a stable change in these cells, as evidenced by the persistence of this phenotype 7 days after the removal of the test material.

Study #: B90-3001
Report Date: 9/12/91
QA Statement/GLP compliance: Yes

Accession #: 36561

SHET

Standard Protocol # C45

BIOLOGICAL SAFETY TEST
SUMMARY REVIEW

Test Substance: G0539.05 Octopirox

Type of Study: Clonal Transfromation Assay Using Syrian Golden Hamster Embryo (SHE) Cells

Division: HAESD

DRD #: BY0856S

Report Date: September 12, 1991

Study #: B90-3001

Functional Use:

Notebook #: YH-1419

This is a ( ) non-regulated or (X) regulated study.

It is the responsibility of the divisional toxicologist to ensure that the summary reflects all significant findings of the report. This report has been reviewed for scientific quality and is summarized as follows:

Dose Range: 0.1-0.35 ug/ml

Results: Positive Negative/Positive/Inconclusive/Not Applicable

Remarks: see study summary

This report and summary are approved for entry into the WTDS. (Circulate in order.)

Divisional Toxicologist S. T. Springer Date 10/21/91

Study Director R. A. LeBoeuf Date 10/29/91

Return to QAU, MVL L. K. Klahn Date 10/25/91

Entered into WTDS M R Brown Date 10-29-91

Microfilming Completed _____ Date _____

THE PROCTER & GAMBLE COMPANY

MIAMI VALLEY LABORATORIES

P.O. BOX 39707, CINCINNATI, OHIO 45239-0707

QUALITY ASSURANCE STATEMENT

TEST FACILITY:	The Procter & Gamble Company Miami Valley Laboratories Cincinnati, Ohio 45247
STUDY NUMBER:	B90-3001
NOTEBOOK NUMBER:	YE 1419
DIVISIONAL REQUEST DOCUMENT:	BY0856S
TSIN:	G0539.05
TYPE OF STUDY:	Clonal Transformation Assay Using Syrian Golden Hamster Embryo (SHE)

PORTION(S) OF STUDY AUDITED:	AUDITOR:	DATE AUDITED:	DATE REPORTED TO STUDY DIRECTOR:	DATE REPORTED TO MANAGEMENT:
Dosing	L. K. Klahm	7/19/90	7/19/90	7/25/90
Study Data	L. K. Klahm	7/19/90	7/19/90	7/25/90

The protocol was audited for compliance to the GLP regulations.

The final study report was audited. The results presented in this report accurately reflect the raw data of the study.

Lynn K. Klahm 9/13/91

Quality Assurance Unit Date

CELL TRANSFORMATION REPORT
Human & Environmental Safety Division

R.A. LeBoeuf
G.A. Kerckaert

Study #:B90-3001
Notebook#:YE-1419

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

Testing Facility: Biological Testing Facility
Miami Valley Laboratories
The Procter & Gamble Company
P. O. Box 39175
Cincinnati, Ohio 45247

Date Study Initiated: 7/13/90

Date Study Completed: 1/15/91

Test Substance Identification Number: G0539.05

Division Request Document Number: BY0856S

Test Substance Description: Off-white Powder

Storage Conditions: Room Temperature

Positive Control Chemical: Benzo[a]pyrene Lot# 06601EX Aldrich

Sponsor's Divisional Toxicologist: S.T. Springer
Procter & Gamble

Study Director: R.A. LeBoeuf
Procter & Gamble

Study Technician: G.A.Kerckaert
Procter & Gamble

Archived at: Biological Testing Facility
Miami Valley Laboratories
The Procter & Gamble Company
P. O. Box 39175
Cincinnati, Ohio 45247

I. SUMMARY

G0539.05 was tested in a modified *in vitro* Syrian Hamster (SHE) cell transformation assay to determine its potential to induce morphological transformation. The primary assay modification was the length of time of SHE cell exposure to G0539.05. This exposure time was reduced to 24 hours (from the usual exposure of 7 days) to determine the stability of the morphologically transformed colony phenotype in the absence of test material. Treatment with 4 test material concentrations ranging from 0.2 ug/ml to 0.35 ug.ml resulted in a statistically significant increase in morphological transformation frequency, compared to solvent controls. All doses yielding a significant increase in morphological transformation frequency were associated with a decrease in plating efficiency. A concentration of 0.1 ug/ml did not cause a significant increase in morphological transformation frequency.

II. PURPOSE

The purpose of this study was to determine the potential for G0539.05 to cause a significant increase in morphological transformation frequency, compared to solvent controls, in the SHE transformation assay. The stability of the morphologically transformed phenotype was examined by removing test material from the cells 24 hours after initial exposure, and permitting colony growth for 7 days without test material.

III. METHODS

The procedures described in Protocol C45 (Clonal Transformation Assay Using SHE Cells) were used, with the following protocol modifications:

1. In the standard toxicity screen and cell transformation protocols, SHE cells are exposed to test material for 7 days. In this modified protocol, SHE cells are exposed to test material for 24 hours, at which time culture medium containing the test material was removed from the cultures and replaced with control culture medium for the remainder of the colony expression period (7 days). The reason for this change was to determine the stability of the morphologically transformed colony phenotype in the absence of test material.
2. In the standard cell transformation protocol a constant number of SHE target cells are seeded, prior to test material exposure. In this modified protocol, a preliminary cytotoxicity assay was done to determine the number of target cells/plate needed to yield a constant colony number/plate across all treatment groups, after chemical treatment and cell killing. Thus for the transformation study, an adjustment of the number of target cells/plate was made, based on the toxicity screen data, to achieve as close as possible, a constant number of colonies/plate across all treatment groups. The reason for this change is that colony number/plate has been observed to affect transformation frequency.

3. In the standard cell transformation protocol SHE cell colonies are permitted to grow for 7 days after initial test material exposure. In this modified protocol the colonies were grown for 8 days after initial test material exposure. The reason for this change is that refeeding the colonies 24 hours after dosing retards their growth rate, resulting in a reduced colony size after a 7 day growth period. Reduced colony size and number, have been associated with increased morphological transformation frequency.
4. In the standard cell transformation protocol the criteria used to determine whether a test material induced a positive response is either a statistically significant increase ($p \leq 0.05$) in morphological transformation at any <u>one</u> dose, compared to concurrent controls as indicated by a one-tailed Fisher's Exact Test, or a statistically significant positive Trend Test. In this modified protocol the test material was considered positive if it caused a significant increase in morphological transformation frequency ($p \leq 0.05$) in at least <u>two</u> dose groups compared to concurrent controls, or in <u>one</u> dose group, with an indication of a dose response. A positive Trend Test in the absence of a statistically significant increase in morphological transformation frequency <u>was not</u> considered to be a positive result, as is currently stated in protocol C45. The reason for this change is that to date, all of the rodent or human carcinogens tested in the assay have resulted in a statistically significant increase in morphological transformation frequency in at least one dose of test material with a positive trend, and all but one carcinogen at multiple doses of test material, as indicated by the Fisher's Exact Test. We have <u>not</u> encountered a carcinogen which gave only a Trend Test positive result.

Five test material dose groups (0.1, 0.2, 0.25, 0.3 and 0.35 ug/ml), a solvent control group (DMSO), a positive control group (10 ug/ml Benzo(a)pyrene), and a nontreated control group were tested in the modified cell transformation assay (24 hour exposure). These five test material dose groups were identical in concentration to those dose groups tested previously in a Cytotoxicity and Transformation assay (Study # B89-0174). Three additional control groups (DMSO solvent control, 10 ug/ml Benzo[a]pyrene control and a nontreated control) were tested with an eight day exposure period, to establish overall study validity. Following test material and control substance exposure, the cultures were fixed and stained. Colony counts were done to determine plating efficiency, and morphologically transformed colonies were scored to assess transformation frequency. The transformation assay was run in duplicate, and the resultant data were pooled.

IV. RESULTS

The raw data and summary tables from the two transformation assays (#4 and #5) are appended to this report. Assays #1, #2, and #3 were not included in this report as they were considered to be unacceptable because their control groups were out of historical range. Table 1 summarizes the pooled plating efficiency and number of colonies/plate data. While a concentration of 0.1 ug/ml G0539.05 had little effect on 3BE cell plating efficiency (98.1% of control), 0.35 ug/ml G0539.05 reduced the plating efficiency to 54.5% that of the DMSO solvent control. Overall, the G0539.05 effect on plating efficiency was dose dependant. A constant number of colonies/plate across dose groups was achieved by varying the number of target cells seeded/dose group. This is indicated by the fact that the mean number of colonies/plate of <u>all</u> eight refed test groups combined (5 test material plus 3 control groups) = 31.15 colonies/plate ± 2.39 SD. Also, the plating efficiency and number of colonies/plate data from the nonrefed control groups are in accord with the criteria for a valid study (plating efficiency ≥ 20% with 20-45 colonies/plate).

Table 2 and Figure 1 summarize the pooled morphological transformation data. G0539.05 at doses of 0.2 ug/ml, 0.25 ug/ml, 0.3 ug/ml, and 0.35 ug/ml caused a statistically significant increase ($p \leq 0.05$) in morphological transformation frequency, compared to the solvent control. A reduction in plating efficiency compared to controls was observed at all doses of test chemical. A concentration of 0.1 ug/ml did not significantly affect transformation frequency. The transformation frequency of both 24 hour and 8 day exposed positive controls (10 ug/ml Benzo[a]pyrene) was significantly increased ($p \leq 0.05$) over that of the solvent controls. The transformation frequencies of all control groups (nontreated control, solvent control, and positive control) for both 24 hour and 8 day exposure were not statistically different ($p \leq 0.05$) than the historical transformation frequency values for this cell lot. This study, then, meets the validity criteria, as defined by the protocol.

V. CONCLUSION

G0539.05 caused a significant increase in morphological transformation frequency at four dose levels, compared to solvent controls and thus the test material is considered positive in this study (see point 4 in METHODS). Additionally, it can be concluded that this transformation represents a stable change in these cells, as evidenced by the persistence of this phenotype 7 days after removal of the test chemical.

R.A. LeBoeuf 4/30/91
R.A. LeBoeuf
Study Director

G.A. Kerckaert 4/8/91
G.A. Kerckaert
Study Technician

Table 1

G0539.05 Plating Efficiency Summary

Treatment (ug/ml)	Plating Efficiency[a]	Plating Efficiency (% of Control)[b]	Number Target Cells Seeded/Plate[c]	Number Of Colonies/Plate[d]
Solvent	41.2±1.46	100	80	33.0
Nontreated	38.0±1.45	92.2	80	30.4
10 BaP	45.2±1.26	110	80	36.1
0.1 G0539.05	40.4±1.74	98.1	74	29.9
0.2 "	27.6±0.92	67	112	30.9
0.25 "	24.1±0.94	58.5	118	28.4
0.3 "	24.5±0.94	59.5	126	30.9
0.35 "	22.4±1.03	54.4	132	29.6
Solvent[e]	37.5±1.08	100	80	30.0
Nontreated[e]	34.1±1.31	90.9	80	27.3
10 BaP[e]	43.8±0.87	117	80	35.0

[a]Colony Number/Plate X 100. Values are mean ± S.E.M. with 40 replicates.
Number Target Cells Seeded
[b]Plating efficiency expressed as % of solvent control.
[c]Target cell seeding density was determined by cell and colony counts in the Cytotoxicity Assay.
[d]Values shown are the mean of 40 replicates.
[e]No refeed, 8 day exposure.

Table 2

G0539.05 Transformation Frequency Summary[a]

Treatment (ug/ml)	Total Colonies Scored	Altered Colonies	Transformed Colonies	% Altered Colonies	% Transformed Colonies
Solvent	1320	7	5	0.53	0.38
Nontreated	1216	6	5	0.49	0.41
10 BaP	1444	11	23	0.76	1.59[b]
0.1 G0539.	1196	5	7	0.42	0.59
0.2 "	1236	8	21	0.65	1.70[b]
0.25 "	1136	2	15	0.18	1.32[b]
0.3 "	1236	5	24	0.41	1.94[b]
0.35 "	1184	8	23	0.68	1.94[b]
Solvent[c]	1200	7	3	0.58	0.25
Nontreated[c]	1092	4	2	0.37	0.18
10 BaP[c]	1400	7	15	0.50	1.07[b]

[a]Pooled data from two assays.
[b]Value is significantly greater ($p \leq 0.05$) than solvent control.
[c]No refeed, 8 day exposure.

Figure 1

GO539.05 TRANSFORMATION

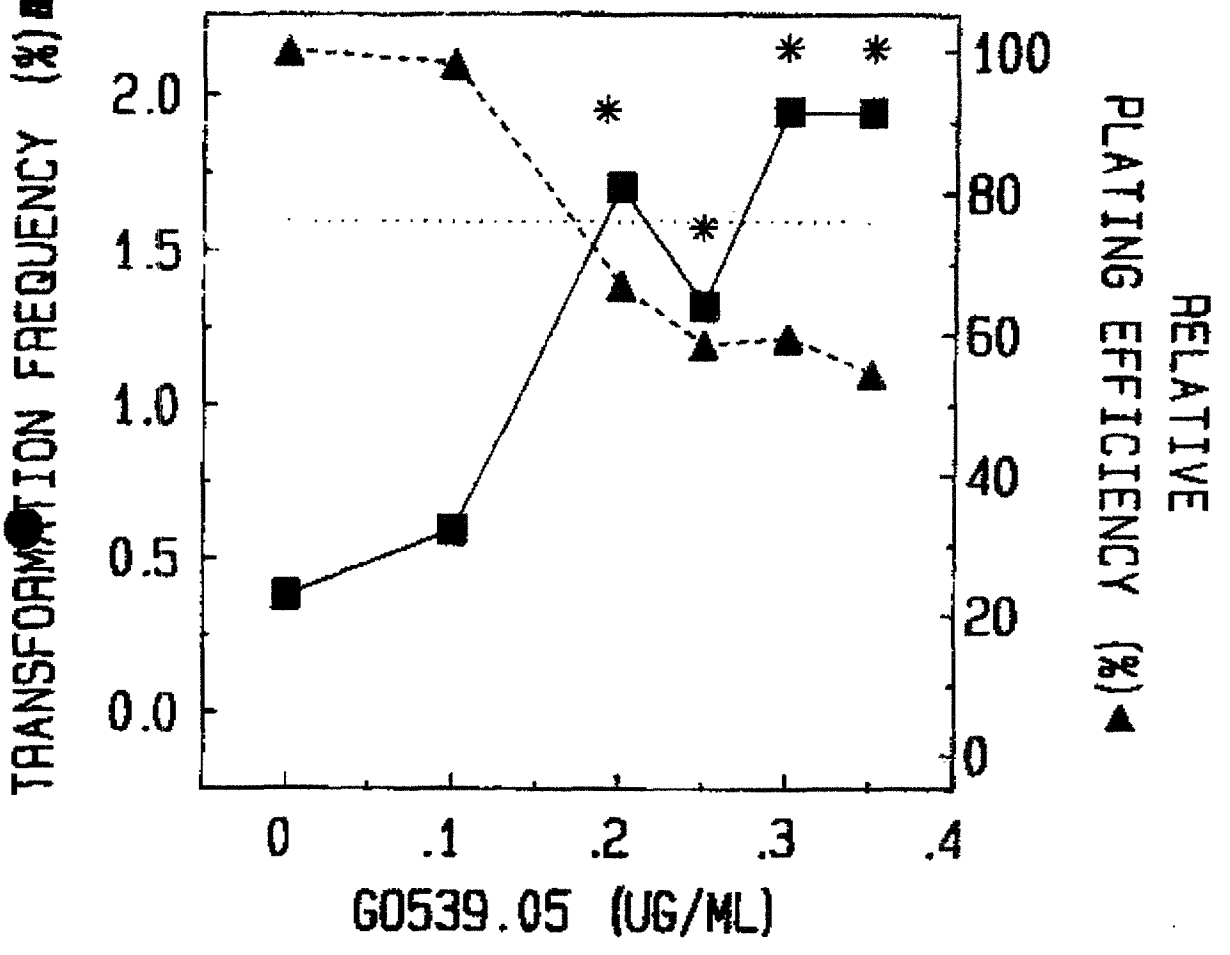

Figure 1. GO539.05 transformation of SHE cells. ▲, plating efficiency expressed as % of solvent control. ■, transformation frequency expressed as number of transformed colonies/total colonies scored X 100. * denotes that the indicated data points are statistically different (P≤0.05) from the solvent control point (0 ug/ml GO539.05) on the curve. Horizontal dotted line indicates the transformation frequency of the 10 ug/ml benzo[a]pyrene control.

ASSAY # 4 SUMMARY TABLE

TREATMENT (ug/ml)	PLATING EFFICIENCY[a]	ALTERED COLONIES[b]	TRANSFORMED COLONIES[c]	% ALTERED COLONIES	%TRANSFORMED COLONIES
Solvent	48.3±1.27	1/774	2/774	0.13	0.26
Nontreated	43.1±2.67	1/690	2/690	0.43	0.29
10 BaP	50.1±1.33	8/802	11/802	1.00	1.37
0.1 GO539	49.0±1.60	1/726	2/726	0.14	0.28
0.2 "	31.1±1.23	3/698	8/698	0.43	1.15
0.25 "	28.3±0.94	1/668	9/668	0.15	1.35
0.3 "	28.2±1.01	3/710	11/710	0.42	1.55
0.35 "	28.1±0.72	4/742	10/742	0.54	1.35
Solvent[d]	42.3±1.22	3/676	0/676	0.44	0.00
Nontreat[d]	40.6±1.20	3/650	2/650	0.46	0.31
10 BaP[d]	44.8±1.23	4/716	9/716	0.56	1.26

[a]Colony Number/Plate / No.Target Cells Seeded X 100 Values are mean ± S.E.M. of 20 plates.
[b]Number of altered colonies/total colonies.
[c]Number of transformed colonies/total colonies.
[d]No refeed, 8 day exposure.

ASSAY # 5 SUMMARY TABLE

TREATMENT (ug/ml)	PLATING EFFICIENCY[a]	ALTERED COLONIES[b]	TRANSFORMED COLONIES[c]	% ALTERED COLONIES	%TRANSFORMED COLONIES
Solvent	34.1±1.36	6/546	3/546	1.10	0.55
Nontreated	31.3±0.93	3/502	3/502	0.60	0.60
10 BaP	40.5±1.50	3/648	12/648	0.46	1.85
0.1 GO539	31.8±1.47	4/472	5/472	0.85	1.06
0.2 "	24.1±0.79	5/540	13/540	0.93	2.41
0.25 "	19.9±0.92	1/470	6/470	0.21	1.28
0.3 "	20.8±1.09	2/524	13/524	0.38	2.48
0.35 "	16.8±0.72	4/444	13/444	0.90	2.93
Solvent[d]	32.8±0.95	4/524	3/524	0.76	0.57
Nontreat[d]	27.5±1.02	1/440	0/440	0.23	0.00
10 BaP[d]	42.8±1.24	3/686	6/686	0.44	0.88

[a]Colony Number/Plate / No.Target Cells Seeded X 100 Values are mean ± S.E.M. of 20 plates.
[b]Number of altered colonies/total colonies.
[c]Number of transformed colonies/total colonies.
[d]No refeed, 8 day exposure.

WORKSHEET FOR TRANSFORMATION ASSAY #4

FIXING-STAINING OF PLATES AND COLONY COUNTS

DATE 11/22/90 PERFORMED BY G. Herchant

EIGHT (SEE PROTOCOL MODIFICATION #1) [illegible]

On ~~seven~~ days post dosing, plates were NaOH fixed and Giemsa stained, using the procedures described in Protocol 8C45, section III B6, pp 12-13. Colonies in each plate were counted and scored manually, using a stereo-microscope. ( ) = ALTERED COLONY; (*) = TRANSFORMED COLONY

	REFEED							
	DMSO Solvent Control	10 ug/ml B a P	Non-Treated Control	0.10 ug/ml	0.20 ug/ml OCTOPIROX	0.25 ug/ml	0.30 ug/ml	0.35 ug/ml
	44(1)	34(2*)	36(1)	37(1)	26(1*)	27(1*)	36(1*)	36(1*)
	42	29(1*)	31(1)	36	29(1*)	21(1*)	31(1*)	34(1*)
	35	46(1)	34	42	39	38	46	29(1)
	35	39	34	22	38	37	28	36
	45	36	41	43	32	41	46	42
	35	38(3*)	31(1*)	36	44(1*)(1)	37(1*)	28(1*)(1)	43(2*)
	36	39(1*)	27	35	32(1*)	34(1*)	30(1*)	34(1*)(1)
	40	39(4)	30	37	30	40(1)	37	34(1*)(1)
	35	48	35	36	24	31	38	42
	33	45	40	28	39	32	35	42
	45(1*)	44(1*)	47(1)	39(1*)	47(1*)	33(2*)	44(3*)(1)	35(1*)
	46	39(1*)	35	41	36(1*)	36(1*)	36(2*)	40(1*)
	35	41(1)	40	37	39(1)	33	38	36
	44	40	22	29	27	30	38	41
	36	43	41	33	42	31	41	33
	43(1*)	40(2*)	40(1*)	40(1*)	38(1*)	42(1*)	32(1*)	35(1*)
	40	46(1)	46	43	38(1*)	31(1*)	27(1*)(1)	33(1*)
	33	37(1)	39	38	31(1)	30	35	44(1)
	35	39	30	33	35	33	32	39
	36	C	37	40	31	31	32	33
PLATING EFF. $\bar{X}$	48.3	50.1	43.1	49.0	31.1	28.3	28.2	28.1
SEM	1.27	1.32	2.67	1.60	1.23	0.94	1.01	0.72
# COLONIES/PLATE	38.7	40.1	34.5	36.3	34.9	33.4	35.5	37.1
# TOT. COLONIES	774	802	690	726	698	668	710	742
# ALT. COLONIES	1	8	3	1	3	1	3	4
# TRANS. COLONIES	2	11	2	2	8	9	11	10
% AC	0.13	1.00	0.43	0.14	0.43	0.15	0.42	0.54
% TF	0.26	1.37	0.29	0.28	1.15	1.35	1.55	1.35

G. Herchant

FIXING-STAINING OF PLATES AND COLONY COUNTS

DATE 11/22/90 PERFORMED BY [illegible]

EIGHT (SEE PROTOCOL MODIFICATION #1) DAYS

On seven days post dosing, plates were both fixed and tissue stained, using the procedures described in Protocol BCAS, section III B6, pp 12-13. Colonies in each plate were counted and scored manually, using a stereo-microscope. ( ) = ALTERED COLONY; (*) = TRANSFORMED COLONY

	8 DAY		
	DMSO Solvent Control	Non-Treated Control	10 ug/ml B a P
	34(1)	31(1)	38(1)*
	37	39	39(1)*
	36	24	35
	35	29	39
	33	25	40
	35	32(1)	33(2)*
	34	34	36(1)
	32	30	40(1)
	41	28	43
	39	33	38
	37(1)	33(1)*	34(3)*
	24	37(1)	35(1)
	29	37	33(1)
	37	34	36
	27	34	27
	34(1)	36(1)*	36(1)*
	30	28	28(1)*
	28	40	40
	39	33	29
	35	33	35
PLATING EFF. $\bar{X}$	42.3	40.6	44.8
SEM	1.22	1.20	1.22
# COLONIES/PLATE	33.8	32.5	35.8
TOT. COLONIES	676	650	716
# ALT. COLONIES	3	3	4
# TRANS. COLONIES	0	2	9
% AC	0.44	0.46	0.56
% TF	0	0.31	1.26

[illegible] 11/15/91

[illegible] #5

FIXING-STAINING OF PLATES AND COLONY COUNTS

DATE 12/6/80 PERFORMED BY H. Herbert

EIGHT (SEE PROTOCOL MODIFICATION #1) HH

On seven days post dosing, plates were both fixed and Giemsa stained, using the procedures described in Protocol ECAS, section III 36, pp 12-13.

Colonies in each plate were counted and scored manually, using a stereo-microscope. ( ) = ALTERED COLONY ; (*) = TRANSFORMED COLONY.

	REFEED							
	DMSO Solvent Control	10 ug/ml 3 & 7	Non-Treated Control	0.10 ug/ml	0.20 ug/ml	0.25 ug/ml	0.30 ug/ml	0.35 ug/ml
				←	α toxiphox			→
	31(1)	32(3)*	19(1)	19(2)*	29(3)*	24(1)	24(3)*	23(1)*
	23	42(2)*	25	23	27(1)	28	27(1)	26(3)*
	38	34	27	25	30	22	32(1)	18(1)
	18	26	28	23	25	19	27	30
	28	35	23	22	33	32	22	25
	27(1)	41(1)*	27(1)	15(1)*	20(1)	27(3)*	34(2)*	27(1)
	28	29(1)*	24	32(1)	30(1)(1)	16	31(3)*	26(1)*
	27	23	27	21	24	28	24(1)	19(1)
	26	29	20	30	24	16	26	28(1)
	24	34	29	27	23	15	31	15
	27(2)(1)	33(3)*	29(1)*	26(1)*	31(1)(1)*	21(1)	25(1)*	24(1)*
	28(1)	34(1)(1)	17(1)	17(1)	25(3)*	32(1)	24(3)*	23(1)*
	32	26(1)	26	22	28	29	21	21(1)(1)*
	29	37	27	19	24	21	33	17
	26	33	28	19	24	23	18	21
	17(1)(1)	31(1)*	24(1)*	32(1)(1)*	32(3)(2)*	23(1)*	28(1)*	19(2)*
	30(1)	29(1)(1)	27(1)	20(1)	22(2)*	27	37(1)*	15(2)*
	24	27(1)*	26	25	27(1)	23	12	20(1)*
	34	41	33	30	27	22	22	24
	29	29	25	24	35	18	38	22
PLATING EFFICIENCY	34.1	40.5	31.3	31.8	24.1	19.9	20.8	16.8
SEM	1.36	1.50	0.92	1.47	0.79	0.92	1.09	0.72
# COLONIES/PLATE	27.3	32.4	25.1	23.6	27.0	23.5	26.2	22.2
TOT. COLONIES	546	648	502	472	540	470	524	444
# ALT. COLONIES	6	3	3	4	5	1	2	4
# TRANS. COLONIES	3	12	3	5	13	6	13	13
2 AC	1.10	0.46	0.60	0.85	0.93	0.21	0.38	0.90
2 TC	0.55	1.85	0.60	1.06	2.41	1.28	2.48	2.93

H. Herbert 1/15/81

[illegible] #5

FIXING-STAINING OF PLATES AND COLONY COUNTS

DATE 12/6/90 PERFORMED BY [illegible]

EIGHT (SEE PROTOCOL MODIFICATION #1) [illegible]

On ~~seven~~ days post dosing, plates were NaOH fixed and Giemsa stained, using the procedures described in Protocol SC45, section III B6, pp 12-13. Colonies in each plate were counted and scored manually, using a stereo-microscope. ( ) = ALTERED COLONY; (*) = TRANSFORMED COLONY.

	8 DAY		
	DMSO Solvent Control	Non-Treated Control	10 ug/ml B a P
	28(*1)	19(1)	27(*1)
	25(1)	26	37(1)
	23	21	40
	31	27	28
	23	19	39
	30(*1)	25	37(*1)
	24	20	31(*1)
	26	24	35
	28	25	38
	26	17	31
	23(1)	24	32(*1)
	21(1)	18	36(1)
	23	21	24
	27	26	36
	34	30	34
	26(*1)	17	39(*2)
	30(1)	21	33(1)
	25	19	37
	29	20	39
	22	21	32
PLATING EFFICIENCY	32.8	27.5	42.8
SEM	0.95	1.02	1.24
# COLONIES/PLATE	26.2	22.0	34.3
TOT. COLONIES	524	440	686
# ALT. COLONIES	4	1	3
# TRANS. COLONIES	3	0	6
% AC	0.76	0.23	0.44
% TF	0.57	0	[illegible]

[illegible] 1/15/91

PROTOCOL NO. C45

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

Issue Date: February 23, 1989

Supersedes Issue Dated: November 14, 1988

Test Substance Identification Number (TSIN) # 6053905

Divisional Request Document Number (DRD) # B40856S

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

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

Study# B90-3001
(To be filled in by Testing Facility)

Purpose: To determine the potential transforming activity of a test chemical in cryopreserved Syrian Golden 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 reconstruct the study and demonstrate adherence to the protocol.

Test Substance(s):

TSIN #	DRD Number	Description: Color	Description: Physical Form	Expiration Date
6053905	B40856S	off-white	powder	6/20/91

Storage Conditions:
(Check one)

[✓] Room Temperature [ ] Freezer
[ ] Refrigerator [ ] Other

Hazards:
(Check one)

[ ] None known. Take ordinary precautions in handling.
[✓] As follows: corrosive

PROTOCOL NO. C45
Clonal Transformation Assay Using Syrian Golden Hamster Embryo (SHE) Cells
Issue Date: February 23, 1989
Superseded Issue Dated: November 14, 1988

Special Instructions:
(Check one)

[ ] None
[X] As follows: PLEASE SEE ATTACHED PROTOCOL MODIFICATIONS.

Solvents:
(In order of choice)

[ ] Medium
[1] DMSO
[ ] Acetone
[ ] Ethanol
[ ] Other

Test System Identification: Individual cultures are to be identified according to the label on the freezer canes or vials.

Test System: Syrian Golden Hamster Embryo (SHE) cells obtained according to the procedures described in Section II-1-3 of this protocol. *SEE PROTOCOL MODIFICATIONS, POINT 2. GAK 6/6/90

Test System Storage: Frozen stocks of SHE cells are prepared and maintained in a liquid nitrogen freezer according to the procedure described in Section II-4 of this protocol.

Dose Preparation: Doses will be determined by results of the Preliminary Cytotoxicity Test - described in Section III of this protocol. Pre-weighed samples of chemical are taken to a Type II, Class II hood and solvent is added to the chemical.

Serial dilutions of the chemical are made in medium or solvent. The final concentration of chemical in medium is 2X that concentration to be used in the assay or if dissolved in solvent is 500X concentration to be used in the assay.

Five dilutions of the test chemical-medium/solvent stock are made in medium immediately prior to use.

Two positive controls ranging from 1.25 µg to 10.0 µg Benzo(a)pyrene per ml complete medium are employed.

A 0.20% solvent control (v/v) is made in complete medium.

Unless the solubility properties of the test substance are provided by the Sponsor or the solubility properties are available from another source, a suitable solvent must be found for the test substance prior to testing using the Standard Operating Procedures of the Test Facility.

PROTOCOL NO. C45
Clonal Transformation Assay Using Syrian Golden Hamster Embryo (SHE) Cells

Issue Date: February 23, 1989
Supersedes Issue Dated: November 14, 1988

SEE PROTOCOL MODIFICATIONS POINTS 1-5. GAK 6/6/90

* I. Transformation Assay

A. Preparation of feeder cells

1. Reconstitute cryopreserved cells in 30 ml of complete medium (see Section II-3) in a T150 flask. Refeed in 4 hours if DMSO is not removed by centrifugation. Alternatively, reconstitute frozen cells in complete medium in a 15 ml centrifuge tube. Centrifuge refrigerated until pellet forms. Resuspend the cells and seed into a T150 flask.

2. Grow cells for 1-6 days subculturing up to 2 times, feeding 2-3 times/week with complete medium if necessary.

3. On the day of irradiation, rinse cells twice with CMF-HBSS (see Section II-3) and collect by trypsinization with detachment solution.

4. Resuspend the cells in complete medium.

5. Expose the cells to x-irradiation on ice so that they are no longer capable of replication. Confirmation of no feeder cell replication is made by culturing feeder cells in the transformation assay (see B-7 of the Section).

6. Transfer cells to centrifuge tubes, centrifuge in refrigerated unit to form pellet, remove medium then resuspend pellet in 5 ml complete medium. Enumerate the viable cells using the trypan blue exclusion test and a hemocytometer.

7. Adjust the cells to 2×10^4 cells per ml "complete" (see Section II-3) medium and add 2 ml aliquots per 60 mm petri dish.

8. Incubate at 37±1°C in 10~~±1.0%~~ 0.5% GAK 6/6/90 CO_2 in humidified air for 24 hrs before adding target cells.

B. Preparation of target cells.

1. Thaw a tube of cells from a cryopreserved cell pool (the same pool used for feeder cells) according to the procedure outlined for preparation of feeder cells.

2. Carefully transfer cells to a 15 ml centrifuge tube using a 1 ml pipette. Centrifuge to form pellet to remove DMSO (alternatively add cells directly to dish containing 4 ml complete medium; refeed in 4 hours).

Seed 2.5×10^6 cells in T25 containing 4 ml complete medium.

0.5% GAK 6/6/90

3. Incubate at 37±1.0°C and 10~~±1.0%~~ CO_2 in humidified air for 24 hrs.

4. Rinse cells twice with CMF-HBSS and collect by trypsinization with detachment solution.

5. Resuspend cells in 1-5 ml complete medium and enumerate viable cells by trypan blue exclusion using a hemocytometer.

6. Adjust cell concentration to 50 cells/ml complete medium and add 2 ml to each 60x15 mm petri dish containing 4×10^4 lethally irradiated feeder cells put down 24 hrs previously. The number of target cells plated should be adjusted if average colonies/plate in control cultures exceed 40.

7. Five feeder cell only plates are included in each experiment to verify no replication of feeder cells.

8. Incubate target cells for 24 hrs at 37±1°C and 10~~±1.0%~~ CO_2 in humidified air.

0.5% 6/6/90 GAK

C. Transformation of SHE cells.

1. Following this 24 hr incubation, 4 ml aliquots of 2% test chemical:solvent (0.4%) in complete medium are added to each petri dish.

2. Positive controls are given 4 ml of a 2% concentration of benzo(a)pyrene:0.4% solvent in medium. Two final concentrations ranging from 1.25 µg/ml to 10 µg/ml B(a)P in the petri dish are used.

3. Solvent control (final concentration of 0.2%) is determined for the test chemical.

4. Medium control is always included.

5. All doses are tested > 15 plates per condition.

6. All are incubated at 37±1°C and 10~~±1.0%~~ CO_2 in humidified air for 7 days. 0.5% GAK 6/6/90

7. Following seven days of chemical treatment, remove treatment media, then rinse ~~twice~~ once with PBS, and add absolute methanol per plate to fix the cells. (Plates may be screened before addition of methanol and colonies with desired morphology cloned for subsequent testing.) GAK 6/6/90

8. Stain the cells with 10% buffered aqueous Giemsa.

9. Plates are washed in tap water to remove stain and inverted until dry.

10. Discard tops of prelabeled dishes.

D. Collection and presentation of data.

1. Dishes are scored visually for percent cloning efficiency (number of colonies per number of viable cells seeded).

2. The percent survival is calculated relative to the solvent controls.

3. Using a stereo microscope, dishes are screened at 12-20X magnification and individual colonies evaluated at 32-50X magnification. Criteria for morphological transformation are colonies which possess: piled up cells normally at the edge of the colony, extensive random-oriented three dimensional growth, crisscrossing cells with increased cytoplasmic basophilia. Morphologically altered colonies have similar characteristics to the morphologically transformed but to a lesser degree.

4. Number of normal, morphologically altered and morphologically transformed colonies are recorded.

5. See Section IV. for report protocol.

II. Preparation of cryopreserved stocks of Syrian Golden Hamster Embryo (SHE) cells.

A. Materials

1. Hamsters

a) Time-pregnant Syrian golden hamsters are obtained from an approved source.

Example:

Strains	Source
LVG	Charles River Breeding Laboratories, Inc. 251 Ballardvale Street Wilmington, MA 01887

2. Supplies.

a) Underpads, gloves, shoe covers, hair covers, paper towels, bell jar, dry ice, 70% ethanol.

b) Sterile beakers.

c) Sterile instruments - scissors, forceps, scalpels, etc.

d) Sterile glass trypsinization flask (Melnick) with teflon-coated magnetic stirring bar.

e) Sterile, capped conical centrifuge tubes (50 and 250 ml).

f) Sterile glass funnel covered with 1 layer fine-mesh gauze.

g) Sterile glass Pasteur pipets.

h) Hemocytometer.

i) 100 mm sterile, prescreened, plastic tissue culture petri dishes.

j) Sterile serological pipets, assorted sizes.

3. Biological Reagents. (prepared according to SOP 4 of the laboratory.
S AZ 12/20/90

a) "HBSS" - Hanks' Balanced Salt Solution, 1X.

b) "CMF-HBSS" Calcium, Magnesium-free Hanks' Balanced Salt Solution

c) "Wash Solution" - Calcium and magnesium free Hanks' Balanced Salt Solution. "CMF-HBSS" containing 200 U/ml penicillin and 200 µg/ml streptomycin.

d) "Complete Media" - Quality Biological, Inc. or other suitable supplier (LeBoeuf's DMEM modification) containing 0.75 gm/l $NaHCO_3$ plus 20% fetal bovine serum and 4 mM L-glutamine. GAK 6/6/90

e) Fetal bovine serum, "FBS," prescreened.

f) Dissociation solution - CMF-HBSS containing 1.25% (v/v) trypsin (40X Enzar-T) and 2.5% (v/v) pancreatin (4X National Formulary Units) and antibiotics.

g) Detachment solution - 0.05% trypsin containing 0.02% Na_2EDTA in CMF-HBSS. (1/50 dilution of GIBCO-10X trypsin/EDTA solution).

h) "Cryoprotective media" - complete media supplemented with 7.5% (v/v) dimethyl sulfoxide (DMSO).

B. Methods

1. Obtaining the Fetuses/Sacrifice of Hamsters.

a) Sacrifice pregnant hamsters at 13.0-13.5 days gestation by CO_2 asphyxiation.

b) Swab ventral surface of hamster with 70% ethanol.

c) Incise and retract abdominal skin.

d) Open peritoneal cavity.

e) Remove both uterine horns and place in pre-numbered sterile beaker containing approximately 50 ml HBSS containing antibiotics; place beaker on ice.

f) Rinse individual fetuses in wash solution to remove blood and placental tissues.

g) Fetuses are decapitated, eviscerated and delimbed.

h) Rinse carcasses with fresh wash solution.

i) Finely mince 12-15 carcasses with curved scissors.

3. Preparation of SHE Cells.

a) Transfer minced tissue to a trypsinization flask containing a magnetic stirring bar.

b) Rinse the minced tissue with wash solution to remove as many red blood cells as possible. Allow tissue to settle and pipette off wash solution.

c) Add a 100 ml aliquot of dissociation solution to the flask. Gently agitate the tissue on a magnetic stirrer for 5 minutes at room temperature (25°C).

d) Allow tissue to settle; pipette and discard the upper 50-75 ml of trypsin solution.

e) Add a 50-75 ml aliquot of dissociation solution and gently stir for 10 minutes at room temperature.

f) Meanwhile, add 2 ml PBS (4°C) to a series of 50 ml sterile plastic centrifuge tubes.

Keep tubes on ice.

g) After trypsinization allow tissue to settle; carefully pipette 50-75 ml of the dissociation solution into the prepared centrifuge tubes.

h) Add a fresh 50-75 ml aliquot of dissociation solution to the minced tissue and gently agitate it for 10 minutes. The upper 50-75 ml is collected as described in step g above.

i) Dissociation procedures are continued for 2-4 collections.

j) Centrifuge the cell suspension at 300 x g (1000 rpm for 10 min). Resuspend in 20-50 ml complete media containing antibiotics; pool pellets when resuspending.

k) Enumerate the viable cells by trypan blue dye exclusion and seed $2x10^7$ viable cells/T150 flask containing 30 ml complete media with antibiotics.

l) Refeed flasks with 30 ml of antibiotic containing complete media 4-6 hours after cell seeding.

m) After 24 hrs, aspirate the medium from the flasks. Refeed the cultures with fresh complete media containing antibiotics if they are not 60-80% confluent. Otherwise, cryopreserve.

n) Discard the cultures if they have not reached 60-80% confluence by 48 hrs of incubation.

4. Cryopreservation of SHE Cells.

a) When the cells are 60-80% confluent, aspirate the medium from the dishes and rinse the cell sheet with CMF-HBSS. Detach the cells with detachment solution. Immediately add cold complete medium to the flasks and pool the cells in sterile centrifuge tubes.

b) Collect cells by centrifugation. Resuspend the pellet in 20-50 ml complete media.

c) Manually enumerate the viable cells by trypan blue dye exclusion with a hemocytometer and microscope and dilute to $5x10^6$ cells/ml.

d) Add an equal volume of complete medium which contains 15% DMSO.

e) Freeze at 2.5×10^6 cells/vial (1.0 ml per vial).

f) Step freeze the cells by freezing chilled vials for 4 hrs in a -70°C freezer before transfer to the liquid nitrogen freezer.

g) Maintain the cells in a liquid nitrogen freezer until used.

SEE ATTACHED PROTOCOL MODIFICATION POINT 3.
GAK 6/6/90

* III. Preliminary Cytotoxicity Assay Using Syrian Golden Hamster Embryo (SHE) Cells.

A. Materials.

1. Cells.

a) Target cells and feeder cells are from cryopreserved cell pools stored at -196°C.

2. Biological Reagents or Solutions.

a) Complete media - see Section II-3.

b) Detachment solution - see Section II- 3.

c) Methanol, absolute, reagent grade.

d) Giemsa, 10% in distilled water.

e) Intact hepatocytes freshly prepared (see Section IV) for studies requiring a source of metabolic activation.

3. Chemicals.

a) Solvent for test article (Medium, DMSO, etc.).

b) Test article, positive control.

4. Equipment and Supplies.

a) Sterile 15 ml plastic tubes with screw caps.

b) Disposable plastic-backed paper.

c) Sterile pipettes, assorted sizes.

d) Wet ice bath.

e) (15 x 60) mm plastic petri dishes.

f) 60 mm plastic petri dishes.

g) 25 and 150 cm^2 plastic flasks.

h) Sterile, capped, 50 ml conical plastic centrifuge tubes.

B. Methods

1. Preparation of Feeder Cells. Same procedure as described in part I-A for transformation assay.

2. Preparation of Target Cells. Same procedure as described in part I-A for transformation assay.

3. Preparation of Dosing Solutions/Suspensions of Test Article.

   a) Prepare test article stock by dissolving at concentrations not exceeding maximum solubility as determined by laboratory SOP or not exceeding 10 mg/ml in culture medium or 2.5 g/ml in DMSO (or other solvent).

   b) From test article stock, prepare 2X concentrations of test article by serially diluting chemical either in medium if medium is used as the primary solvent or if solvent is used make serial dilutions in solvent to 500X the desired final concentrations and then dilute 1:250 with culture medium (0.4% v/v solvent), such that upon addition to the target cells final concentrations attained are 1X (see below). If solvent is used, final solvent concentration in cultures is not to exceed (0.2%). At least five concentrations depending upon the solubility of the test article will be used.

4. Preparation of Solvent Control

   Prepare 2X concentration of solvent by diluting 1:250 with treatment medium giving 4 µl/ml (0.4%).

5. Treatment.

   a) Each treatment is performed with at least five plates per treatment group.

   b) At least five concentrations will be selected dependent upon the solubility of the test article, e.g. 1, 10, 30, 100, 300, and 1000 ug/ml.

   c) The dose (final concentration) of solvent to be used in the negative (solvent) control will be 0.2%.

   d) Add 2X concentrations of the compound (test chemical or solvent control) 1:1 (v/v) to cultures 20-24 hrs after seeding, (i.e. 4 ml of 2X). Incubate cells at 37±1.0°C in 10~~±1.0%~~ CO_2 in air for 7 days.
   0.5% GAK 6/6/90

6. Staining Plates.

   a) Seven days after addition of the test chemical to the plates, wash plates once with balanced salt solution.

   b) Add absolute methanol and let stand for 20 min. to fix cells. Remove methanol.

   c) Immediately stain for 15 min. with 10% aqueous Giemsa stain.

   d) Remove stain.

   e) Wash plates and invert to dry.

   f) Manually count the number of colonies and record.

7. Counting cell number/plate (Optional - to be requested by Sponsor).

   a) Seven days after addition of the test chemical to the plates, wash 5 plates with 2 ml of CMF-HBSS 2X, keeping the second wash. Add 3 ml of 0.05% trypsin, 0.02% Na_2EDTA in CMF-HBSS and detach the cells. This cell suspension is pooled with the second CMF-HBSS rinse to give 5 ml of cell suspension. Cells/ml is then determined and multiplied by 5 to give total cells/plate.

C. Evaluation of Test Results.

SEE ATTACHED DRAFT MODIFICATION POINT 3. GAK 6/6/90

1. The Study Director will determine the doses to be used in the SHE transformation assay.

   a) Doses tested should include one which causes: 1) either ~~50%~~ 10% GAK 6/6/90 or greater decrease in plating efficiency or macroscopic insolubility for the high dose, 2) the highest concentration which has no effect on plating efficiency and qualitatively does not affect colony size (alternatively the Sponsor can request cell counts and confirm that the low dose causes less that a 25% reduction in cell number) and 3 concentrations between these upper and lower limits which cause less than a ~~50%~~ 10% GAK 6/6/90 decrease in plating efficiency.

   b) If the compound is not toxic, five concentrations will be used to a maximum of 5000 µg/ml, solubility permitting.

IV. Report of Transformation Results

A. Criteria of Acceptable Assay

1. A table showing plating efficiency and cell number data from the toxicity screen must be included. Based on these data or chemical solubility considerations, a statement adjacent to this table must be made describing what doses of chemical were chosen for the transformation studies based on the criteria described for the preliminary cytotoxicity studies.

2. Plating efficiency of untreated and solvent controls will be 20% or greater giving an average of between 20-45 colonies/60 mm plate.

3. The total colony count of control groups must be ≥ 400 per assay.

4. The rate of spontaneous transformants in media or solvent controls for pooled duplicate assays will be as expected for the cell pool in use and not statistically different from historical controls as determined by Fisher's Exact Test for that cell pool.

5. The number of transformed colonies observed in the positive control (BaP) at must be statistically significantly greater ($p < 0.05$) than that number in the media or solvent control as indicated by Fisher's Exact Test on pooled data from duplicate assays. In addition, the positive control can not be statistically different from historical B(a)P treated group as indicated by Fisher's Exact test for that cell pool.

B. Report data consists of:

1. dose preparation information,

2. colony number per plate,

3. total colony number per treatment group,

4. number of morphologically transformed and altered colonies per treatment group, and qualitative data regarding the effect of a treatment on colony size relative to colonies in the solvent or untreated controls.

C. Calculations based on the raw data will include:

1. plating efficiency (colony number per plate/number of viable cells plated),

2. the treatment group mean plating efficiency and standard error of the mean,

3. the frequency of morphologically transformed colonies per treatment group (total number of morphologically transformed colonies per treatment group/total colony number per treatment group).

4. The frequency of morphologically altered colonies per treatment group (total number of morphologically altered colonies per treatment group/total colony number per treatment group).

5. Individual assays within the study will be run in duplicate. Therefore, an overall data summary will also be made and included on a separate sheet. The overall data summary will include for each treatment group:

   a. Total colonies scored (i.e. the sum of the two assays).

   b. Average plating efficiency (i.e. the mean of the two assays).

   c. Total transformed colonies.

   d. Overall transformed colony frequency (i.e. total transformed colonies/total colonies scored).

   e. Total altered colonies.

   f. Overall altered colony frequency.

6. If more than duplicate assays are conducted, data from all assays should be pooled for statistical analysis.

D. Statistical Analysis

1. Statistical tests for significant treatments effects on transformation frequency are to be conducted using Fisher's Exact Test (Armitage, 1971) and a Cochoran Armitage Trend Test with continuity correction (Thomas, 1977) on pooled data from two individual assays.

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

Thomas, D. G., N. Breslow and J.J. Gart (1977). Trend and homogeneity analysis of proportions and life table data. Comput. Biomed. Res. 10, p. 373-381.

SEE ATTACHED PROTOCOL MODIFICATIONS, PART 5 GPL 6/6/90

* 2. Criteria used to determine whether a chemical induced a positive response is either a statistically significant increase ($p<0.05$) in morphological transformation at any dose compared to concurrent controls as indicated by a one-tailed Fisher's Exact Test or a statistically significant positive trend test.

V. Protocol Changes

A. 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.

B. 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: S.T. [illegible]
Divisional Toxicologist

Date Approved by Sponsor's Divisional Toxicologist: 6/6/90

Proposed Starting Date: ~~6/18/90~~ 7/13/90) HK

Defined as DATE TRANSFORMATION STUDY INITIATED)

Proposed Completion Date: ~~9/14/90~~ 3/15/91) HK

Defined as DATE STUDY RESULTS ANALYSED) To be completed by the Test Facility

Study Director: R.A. [illegible])

Date: 6-8-90)

Study Cost: _____)

Modifications To Cell Transformation Protocol #45 To Be Implemented For Test Article G0539.05.

1. The primary modification of Protocol #45 for this study will be the length of time of chemical exposure to SHE cells. In the standard toxicity screen and cell transformation protocols, SHE cells are exposed for 7 days to test material. In the modified protocol, SHE cells will be exposed to test article for 24 hours for both the toxicity screen and transformation assay, at which time medium containing the test article will be removed from the cultures and replaced with control medium for the remainder of the colony expression period (7 days). Solvent controls and benzo[a]pyrene positive control groups will also follow the 24 hour refeed protocol. The reason for this change is to determine the stability of the morphologically transformed colony phenotype in the absence of the test article.

2. The use of crypopreserved cells prepared prior to receipt of this particular protocol is standard procedure and therefore acceptable for the purpose of this study.

3. A preliminary cytotoxicity assay will be done to determine the number of target cells/plate needed to yield a constant colony number/plate across all treatment groups. The reason for this change is that colony number/plate has been observed to affect transformation frequency. For the transformation study, an adjustment of target cell plating number will be made based on the toxicity screen data, to achieve as close as possible, a constant colony number across all treatment groups. Cell counts will also be done to determine the number of cells/plate across all treatment groups. A consideration of both cell number/plate and plating efficiency data will be made, for selecting the doses for the transformation study. The top dose tested in the transformation assay should result in at least a 10% reduction in plating efficiency compared to concurrent controls, unless reduction in colony size at this top dose precludes scoring the colonies for transformation.

4. Seven day treatment with 0.3 and 0.35 µg/ml test article will <u>not</u> be done. This was originally put into the protocol as a reference point to the initial 7 day exposure study. However, in the initial trial in this study, these dose levels resulted in 100% toxicity in the target cells currently in use and the target cells used in the initial seven day study have been depleted. A difference in toxicity with different cell isolates or serum lots is not unusual. However, seven day exposure solvent controls and benzo[a]pyrene positive controls will be included in this study.

5. The statistical method employed to test for a significant treatment related effect will be a one sided Fisher's Exact Test. Test material will be considered positive if it causes a significant increase in morphological transformation frequency (i.e. a p value equal to or less than 0.05) at at least two doses, compared to concurrent controls, or at one dose with an indication of a dose response. A positive trend analysis in the absence of a statistically significant increase in morphological transformation frequency will <u>not</u> be considered as a positive result as is currently stated in Protocol #45. The reason for this change is that to date, <u>all</u> of the rodent or human carcinogens tested in this assay have resulted in a statistically significant increase in transformation frequency at at least one dose of test chemical and all but one at multiple doses of test chemical, as indicated by Fisher's Exact Test. We have not encountered a carcinogen which only gave a trend test positive result. Thus, based on this experience, a revision of the criteria for calling a test chemical positive in this assay is justified.

STUDY DIRECTOR R. A. [illegible] DATE 10/5/90

DIVISIONAL TOXICOLOGIST [illegible] DATE 10/5/90

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

DRD: BY BY 0856 S

TSIN: ~~G30~~ G 0539.

1. Test Article
   Product (Ingredient): OCTOPIROX
2. Brand Notebook Ref:
   pH: 8-10 (1% SOLUTION IN H
   Expiration Date: 6/20/91
   Hazards: IRRITANT (SKIN
3. Physical Form: POWDER
   Color: OFF-WHITE
   Storage Conditions:

   [X] Room Temperature [ ] Other

4. Stability Testing is:

   [X] Completed [ ] In Progress

5. Shipping Information
   D.O.T. Shipping Name OCTOPIROX Flash Point N/A °F
   D.O.T. Hazard Classification: NON-HAZARDOUS Identification No N/A
   Hazard Waste No N/A
   Chemical/test articles checked against TSCA inventory list [X] YES [✓] NO
   Regulatory Liaison Initials

6. Formulated Composition

Component[a]	(C.A.S.)[b] (Number)	Nominal Component Level	Nominal Calculated Assay	Actual Calculated Assay	Stock Cod No. (Lot
OCTOPIROX	68890-66-4	100%	100%	100.07%	8X288

(a) Use chemical or CTFA names which uniquely identifies each component. Common, non-chemical names such as (Tergitol 15-S-0 or yellow dye #10) should be indicated in parentheses following the chemical name. Non-definitive identification (e.g., SC-Base, Arquad) is not acceptable.

(b) CAS numbers must be included whenever available. They are especially important fo main components of the formulation. For fragrances the "XP" number is required.

SCR/I/C:jvm
6/90

DRD: BY 08565

TSIN: G0539.05

7. ANALYSIS

Date Submitted	Submitter Code Or Lab Notebook	Analysis	(X) *	Measured Value	Acc. Limits	Data Source	Y/N**
6/21/89	8917 1036	MCT	X	PASS	PASS-FAIL	SWTC MICRO. GMP	
6/21/89	8917 1036	APPEARANCE		PASS	PASS-FAIL	SWTC ANALY.	
7/6/89	8917 1036	ID-IR		PASS	STD. SPECTRUM MATCH	SWTC ANALY.	
6/30/89	8917 1036	ASSAY		100.07	98.0-101.5	SWTC ANALY.	

* Analysis required by toxicologist or microbiologist
** Analytical method validated in this matrix (Must be initialed by Analytical Dept. liaison)

9.
Acceptable Microbial Susceptibility: (Signature) 7/5/90 Date

Made By: (Signature) N/A (Phone No.) (Date Made)

Approval for Release: The test substance as made and characterized is a representative example of the intended formulation. Mating records for plant-made product are being/have been obtained and evaluated by Products Research.

Process Development: (Signature) 7/5/90 Date

Products Research: (Signature) N/A Date

GMP Quality Assurance: (Signature) 7/5/90 Date

(Attach batch records for GMP review)

0 finished products will be retained by Quality Assurance
(# samples)

9.
This substance has been appropriately characterized and is acceptable for testing outlined in the attached DRD.

Division Toxicologist: (Signature) 7/5/90 (Date)

TSCR/2/C:jwm
8/90