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

Objective: To determine the potential transforming activity of a test chemical in cryopreserved Syrian hamster embryo (SHE) cells.

Testing Facility or Sponsor:

Test Substance:

Vehicle Control:

Positive Control:

Solvent for Test Substance: 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. Check which solvent(s) the test substance is soluble in if known.

[ ] Culture medium
[ ] DMSO
[ ] Acetone
[ ] Ethanol
[ ] Other

Rationale for Test Substance Selection:
(e.g. human carcinogen, human non-carcinogen, rodent non-genotoxic carcinogen)

Route of Administration of Test Substance: *In Vitro*. Route specified by test procedure.

Personal Safety Data:
Test Substance Handling:
Vehicle Control Handling:
Positive Control Handling:

Test System: Syrian hamster embryo (SHE) cells

Test System Source: Charles River, Wilmington colony.
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

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%. A positive control of 1.25 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 1 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.

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 stereomicroscope 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 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 corraborated by data generated in this toxicity screen. Due to normal variability in toxicity encountered from one assay to another, an extra treatment group of one concentration higher than the concentration anticipated to give approximately 50% relative toxicity should be considered to ensure that appropriate levels of toxicity are achieved. 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. 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 call 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 X 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 ±1 C and 10 ± 0.5% $C0_2$ for 24 hours.

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 X 10^4 feeder cells. Dishes are incubated at 37 ± 1 C and 10 ± 0.5% $C0_2$ 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 O.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 ± 1 C and 10 ± 0.5% $C0_2$ 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.

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) ± SEM.
   $$PE = \frac{\text{number of colonies/dish}}{\text{number of cells seeded}} \times 100$$
4. Relative plating efficiency (RPE).
   $$RPE = \frac{\text{real group PE}}{\text{solvent control PE}} \times 100$$
5. Number of morphologically transformed (MT) colonies.
6. $$\text{MT frequency} = \frac{\text{Number of MT colonies}}{\text{Total number of colonies}} \times 100$$
7. $$\text{Ave. No. of cells/dish} = \frac{\text{Total No. of cells}}{\text{Total No. of dishes counted}} \times 100$$
8. $$\text{Colony Density} = \frac{\text{Ave. No. cells/dish}}{\text{Ave. No. colonies/dish}}$$
9. $$\text{Relative Colony Density} = \frac{\text{Colony Density}}{\text{Colony Density of solvent control}} \times 100$$

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 Test[1]. 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 test[2] (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 all groups must be greater than 1000.
2. With pooled data from all trials, the plating efficiencies of all groups must be greater than or equal to 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 less than 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%.
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.

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 less than 0.05) in morphological transformation 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 less than 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, additional studies may be warranted.

Study Director Date

[1]Armitage, P. (1971) Statistical Methods in Medical Research, Blackwell Scientific Publications, Oxford pp 135-138.
[2]Statxact, Cytel Software Corp., Cambridge, MA 02139.