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STUDY OUTLINE

Single Cell Gel Electrophoresis in vitro Comet Assay on L5178Y/TK+/- Mouse Lymphoma Cell

Objective	The Comet assay is a simple method for measuring DNA strand breaks in eukaryotic cells. Cells embedded in agarose on microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks and the size of the resulting fragments.
Cell line	L5178Y TK+/- 3.7.2 C mouse lymphoma cells
Medium	RPMI 1640 buffered with 2g/L $NaHCO_3$ and supplemented with 0.3g/L L-Glutamine, 0.01mL/mL Antibiotic-antimycotic solution (10'000U/mL penicillin, 10mg/mL streptomycin and 25µg/mL amphotericin-B); 0.5mg/mL Pluronic-F68, 0.2mg/mL Pyruvic acid and 10% (v/v) heat inactivated horse serum
Metabolic activation	The experiments are performed in the presence and absence of a post mitochondrial supernatant (S9) prepared from livers of phenobarbital/β-naphthoflavone-induced rats. The concentration of S9 Mix components during the treatment: S9: 5.90mM (=2%) glucose-6-phosphate; 0.32mM NADP; 1.50mM KCl.
Dose levels	Maximum dose level: 5mg/mL or 5µL/mL. One test is performed with at least four dose levels approximately half log or smaller intervals.
Controls	Negative (vehicle) controls; positive controls: methylmethane sulfonate (MMS, in absence of metabolic activation) and cyclophosphamide (CP, in presence of metabolic activation).
Number of parallels	Two
Cell number	1 x 10^6
Treatment volume	20mL
Incubation	37°C; 5% CO_2, treatment duration: 4 hours
Post-treatment observations	Presence of precipitate, medium changes
Cytotoxicity	Trypan blue dye exclusion method just after the treatment, measurement of relative cell growth for 24 hours after the treatment, counting of "hedgehogs" non detectable cell nuclei.
Processing of slides	Pre-treatment of microscope slides, embedding of cells at 10^4 order (using low and normal melting point agarose).
The Comet assay	Lysis (Lysing solution: 2.5M NaCl, 100mM EDTA, 10mM Tris, 12g/L NaOH; 10g/L N-Laurylsarcosyl; 10% DMSO, 1% Triton X-100; 1 hours ≤, at ~4°C); Unwinding (Electrophoresis solution: 300mM NaOH, 1mM EDTA; pH>13; 20-45 min at pH>13; at ~4°C) Electrophoresis (Electrophoresis solution; 20-40 min, at 25V/300 mA) Neutralization (0.4M Tris, pH=7.5; 3 x 5 min.; dehydration: abs. ethanol) Staining (2µg/mL Ethidium bromide)

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Number of slides prepared	At least 3 slides per parallel per dose level (per control)
Number of slides evaluated	50 cells per slide will be randomly scored i.e. 150 cells per treatment x 2 parallels (at least 300 analyzed cells per test item treatments and controls).
Evaluation	The slides are examined at an appropriate magnification using fluorescent microscope equipped with an appropriate excitation filter. For Image Analysis (analysis of tail length, tail moment, tail intensity) the Komet 6.0 F (Andor Technology) is used.
Draft report	Approximately 4 months from the arrival of the test item.
Archiving	Study Plan, Amendment(s), original Final report and all raw data, and one sample of the test item for 5 years
References	Burlinson B.: The in vitro and in vivo Comet Assays, Methods Mol Biol. 817: 143-63 (2012) Tice R.R., Agurell E., Anderson D., Burlinson B., Hartmann A., Kobayashi., Miyamae Y., Rojas E., Ryu J,C., Sasaki Y.F.: Single Cell/Comet Assay: Guidelines for In vitro and in vivo Genetic Toxicology Testing, Environ. Mol. Mutagen. 35:206-221 (2000) International Pre-Validation Study of the in vitro Alkaline Comet Assay (Version 5.1, July 26, 2008)

Option 1	**Extension of archiving:** Study Plan, Amendment(s), original Final report and all raw data for further 10 years; biological samples for further 7 years.