MMS/JaCVAM Joint Seminar

—The Pros & Cons of Comet Assay—

Date: August 13, 2006 From 2:00 pm to 5:00 pm
Venue: Hokkaido-koseinenkin-kaikan 3F Horai-room (Sapporo)

Program

Chairpersons: Dr. M. Hayashi (NIHS)
Dr. L. Schechtman (ICCVAM)

Welcome address
Mr. Tsuguo Ikka (Safety Res. Inst. Chem. Compounds)

Opening remarks
Dr. Norihide Asano (President of MMS)

1. **The Pros & Cons of Comet Assay**
   Dr. Ray R. Tice (NICEATM)

2. **Utility of Alkaline DNA Strand Breakage Assays in Genotoxicity Testing**
   —Comparison of the Alkaline Elution and Comet Assays
   Dr. Rick Storer (Merck Research Laboratories)

3. **Use of the Comet Assay for Human Risk Assessment—A case study in the gastro-intestinal tract.**
   Dr. Phil Clay (Syngenta)

4. **Case study 2 – Sensitivity of the alkaline Comet Assay to buffer temperature during unwinding and electrophoresis**
   Dr. Patricia Escobar (BioReliance, invitrogen)

Coffee break

Chairpersons: Dr. H. Kojima (JaCVAM)
Dr. R. Tice (NICEATM)

5. **Validation of *in vivo* Comet Assay with Rat Hepatocytes (Preliminary test) by JEMS/MMS**
   Dr. Madoka Nakajima (Anpyo-Center)

6. ***In vitro* Comet assay—A possible candidate as a member of the standard test battery**
   Dr. Masamitsu Honma (NIHS) & Dr. K. Yamakage (FDSC)

7. **Limitation of the comet assay**
   Dr. Brian Burlinson (Huntingdon Life Sciences)

Closing remarks
Dr. Yasuo Ohno (JaCVAM/NIHS)

MMS/JaCVAM Joint Seminar
—The Pros & Cons of Comet Assay—

The host organization: *JEMS.MMS and JaCVAM*
The supporter: *Safety Research Institute for Chemical Compounds*
(Shinei, Kiyota-ku, Sapporo, Phone:011-885-5031)
Japan Food Research Laboratories, Chitose
(3 Bunkyo 2-chome, Chitose-shi, Hokkaido)
Supported by *the Health and Labour Sciences Research Grants*
(H18-Chemistry-General-003)

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The Pros & Cons of the Comet Assay

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Historically, regulatory agencies have used genotoxicity information as part of a weight-of-evidence (WOE) approach in the approval and registration of products (i.e., pesticides, pharmaceuticals, medical devices). The current default regulatory assumption is that "genotoxic/mutagenic" substances are capable of causing somatic and/or germ cell mutations in humans and, thus, adverse health outcomes (predominantly cancer) via a genotoxic/mutagenic mode of action (MOA). A MOA strategy proposed by Dearfield and Moore (EMM 46:236, 2005) first includes integration of all available information into a WOE analysis as to whether a substance is a mutagen and then, whether an adverse health outcome is mediated via a mutagenic MOA. The alkaline (pH>13) version of the Comet assay does not detect mutations per se but rather detects multiple kinds of DNA damage (i.e., strand breaks, crosslinking, alkali-labile sites) and incomplete excision repair events. Some but not necessarily all of these lesions or the incomplete repair events are likely to be pre-mutagenic. The MOA approach takes into account not only the intrinsic genotoxicity of a test substance, but also its ability to induce DNA damage or mutations in an appropriate "target tissue" *in vivo*, generally using a rodent-based test method. In this regard, the Comet assay can provide useful and meaningful information at two levels: (1) whether the test substance is genotoxic (i.e., does it induce DNA damage?), and (2) whether, under appropriate exposure conditions, DNA damage is induced *in vivo*. Consistent with all genetic toxicity test methods, the extent to which the Comet assay accurately predicts an adverse health outcome in humans depends on the mechanistic relevance of the endpoint measured (direct DNA damage vs DNA/chromosomal mutations) as well as the relevance of the test system (*in vitro* vs nonhuman *in vivo*) to human exposure conditions. While the Comet assay offers a number of advantages over many other genotoxicity test methods, especially in regard to evaluating the *in vivo* genotoxicity of a test substance, there are limitations that need to be considered. These include, among others, the potential for false positives associated with cytotoxicity, the potential for false negatives depending on chemical class and mechanism of action, and the reproducibility of the test method within and across laboratories using different protocols and different methods for collecting data.

ABSTRACT

Japanese Environmental Mutagen Society
Mammalian Mutagenicity Study Group Meeting
August 13, 2006
Sapporo, Japan

Utility of Alkaline DNA Strand Breakage Assays in Genotoxicity Testing: Comparison of the Alkaline Elution and Comet Assays

Richard D. Storer, Ph.D., Genetic and Cellular Toxicology, Dept. of Laboratory Sciences and Investigative Toxicology, Safety Assessment, Merck Research Laboratories West Point, PA USA

Several alkaline DNA strand breakage assays have been developed since the 1970s and used to assess the in vitro and in vivo effects of DNA damaging agents. The alkaline elution and alkaline comet assays have been the most widely utilized in genotoxicity testing and offer the ability to assess genotoxicity in many different target tissues in vivo. The Comet assay has become the more widely adopted method in recent years since it is easier to perform, has minimal equipment requirements, and a greater sensitivity for certain classes of agents. This is particularly true for agents such as alkylating agents that produce alkali-labile sites (ex. N7-gunanine adducts) that are more rapidly converted to single-strand breaks in the pH 13 alkaline denaturation and electrophoresis buffer used in the Comet assay as compared to the pH 12.1-12.5 buffers used in alkaline elution assays. The use of more alkaline denaturation conditions is likely enabled in the Comet assay by the lysis of cells in agar, rather than in solution, which minimizes the physical effects of sheer stress on DNA strand break induction. The Comet assay also offers the advantage of being able to visualize damage in individual cells, allowing assessment of the degree of heterogeneity in the response and of the presence of dead cells with extensive DNA degradation. The alkaline elution assay has sufficient sensitivity to detect the genotoxicity of a variety of classes of DNA damaging agents. The assay can be fully automated in a 96-well microtiter plate format offering advantages in high throughput screening applications. The alkaline elution/rat hepatocyte assay validated in our laboratory incorporates a gravity-flow neutral elution step (the lysis/rinse fraction). Quantitation of the DNA in this fraction, together with the first alkaline fraction, allows for a sensitive measure (the Y-intercept parameter) of the percent of dead/and dying cells with highly fragmented DNA in the treated cell population. This parameter together with parallel cytotoxicity assay such as trypan blue dye exclusion and ATP levels allows for an integrated assessment of cytotoxicity and genotoxicity. This is especially important for in vitro DNA strand break assays where nuclease-mediated DNA strand breakage in dead and/or dying cells can confound the assessment of genotoxicity and lead to false positives. Validation of in vitro DNA strand break assays need therefore to include a carefully selected panel of non-genotoxic non-carcinogens which are cytotoxic to the target cells in order to properly assess specificity and set criteria for accurate scoring of the assay. Interpretation of effects in in vivo assays are less likely to be confounded by DNA degradation in necrotic and/or apoptotic cells but increased elution rates or migration rates in Comet assays may still be seen with treatment causing extensive tissue damage. Careful assessment of cytotoxicity is therefore a requisite component of any in vitro or in vivo genotoxicity assay using DNA strand breaks as an endpoint.

Validation of *in vivo* Comet Assay with Rat Hepatocytes (Preliminary test) by JEMS/MMS

- **THE ORGANIZERS**

Madoka Nakajima (Chief)
Yoshifumi Uno
Makoto Hayashi
Norihide Asano
Kohji Yamakage
Wakako Ooyama
Takashi Omori

VALIDATION STUDY

- **PARTICIPANTS** (TESTING FACILITIES)
  - Maya Ueda (Biosafety Research Center, Foods, Drugs and Pesticides [**An-Pyo**])
  - Yuzuki Nakagawa (Hatano Research Institute, Food and Drug Safety Center [**FDSC**])
  - Munehiro Nakagawa (Mitsubishi Chemical Safety Institute Ltd. [**MCSI**])

PROTOCOL (1)

- **PURPOSE**

Comparison between mesh method (cell) and homogenizer method (nuclei) in preparation of tissue samples.

PROTOCOL (2)

- **ANIMALS**

Species: Rat

Strain/Age: Crj:CD(SD)IGS [SPF], 8 weeks

- **CHEMICAL**

Ethyl methanesulfonate (EMS) ,

Dose: 250 mg/kg×1 (po)

- **ORGAN**

Liver

PROTOCOL (3)

EXPERIMENTAL DESIGN

Compound	Dose (mg/kg)	Number of treatments	Number of animals*		
			1st	2nd	3rd
Negative control	0	1	1	1	1
EMS	250	1	1	1	1

*: Single dose of negative control and test substance were administered to 3 animals each on the different days in the same week to observe the inter-day variation of animals after administration.

PROTOCOL (4)

PREPARATION OF CELL NUCLEI OR SINGLE CELLS

After liver tissues were minced with scissors (cut into about 5 millimeters cubic pieces at random), cell nuclei or single cells were prepared by using either of the following two methods.

PROTOCOL (5)

- **HOMOGENIZE METHOD (Dauns type)**

Three milliliters of the homogenize buffer was added into the tube containing minced liver, then it was gently homogenized once using a homogenizer.

- **MESH METHOD**

The minced liver was placed on the nylon mesh that fixed to a 50-mL tube. The liver cell mass was pushed out through the tube with a plunger of a disposable syringe, and about 3 mL of homogenize buffer was flashed into the tube.

PROTOCOL (6)

- **ELECTROPHORESIS**

Platform: Submarine type

Alkali unwinding: 10 min.

Electrophoresis: 1 V/cm for 15 min.

Electric current: 300 mA

Temperature: Below 4°C

Neutralization: 10-20 min

PROTOCOL (7)

- **OBSERVATION OF SAMPLES**

All remaining samples were centrifuged, and supernatant was removed. Cells were fixed by adding 5 mL of 10% neutral buffer formalin solution. The Acridine orange (A.O.) solution and cell suspension were mixed at a rate of 1:1. Then, cells were observed using a fluorescence microscope.

PROTOCOL (8)

- **SLIDE ANALYSIS**

The slides to be made at each facility were analyzed at An-Pyo Center. The comets were measured via a CCD camera linked to a PC with a Rainbow Star System analysis board.

Fifty nuclei or cells in migrating image per slide were analyzed.

PROTOCOL (9)

- **COMET PARAMETERS**
  - Olive tail moment
  - =(Tail mean-Head mean) ×Tail%DNA/100
  - Tail%DNA
  - =100-Head%DNA
  - Head%DNA
  - =(Head optical intensity/(Head optical intensity+ Tail optical intensity)) ×100

Comparison between Mesh and Homogenize

Tail Moment	Saline	EMS
MCSI -M	0.73 ± 0.15	1.07 ± 0.23
MCSI -H	0.77 ± 0.27	0.85 ± 0.18
FDSC-M	0.72 ± 0.18	1.69 ± 0.35
FDSC-H	0.60 ± 0.13	1.99 ± 0.43
An-Pyo Ⅰ -M	0.58 ± 0.14	1.61 ± 0.86
An-Pyo Ⅰ -H	0.46 ± 0.10	0.99 ± 0.26
An-Pyo Ⅱ -M	0.53 ± 0.05	1.24 ± 0.34
An-Pyo Ⅱ -H	0.43 ± 0.02	0.91 ± 0.24

Tail % DNA	Saline	EMS
MCSI -M	4.92 ± 0.30	8.93 ± 0.59
MCSI -H	5.03 ± 1.45	7.57 ± 1.34
FDSC-M	4.84 ± 0.79	10.43 ± 1.91
FDSC-H	4.42 ± 0.44	11.73 ± 1.36
An-Pyo Ⅰ -M	5.72 ± 0.94	11.17 ± 4.02
An-Pyo Ⅰ -H	4.90 ± 1.09	8.00 ± 1.64
An-Pyo Ⅱ -M	4.26 ± 0.46	9.23 ± 1.36
An-Pyo Ⅱ -H	3.59 ± 0.48	7.54 ± 0.97

M : Mesh method
H : Homogenize method

Summary data on validation of *in vivo* Comet Assay (Preliminary test)

Saline

Mesh	Tail % DNA	Tail Moment
MCSI	4.9	0.7
FDSC	4.8	0.7
An-Pyo I	5.7	0.6
An-Pyo II	4.3	0.5
Homogenize	**Tail % DNA**	**Tail Moment**
MCSI	5.0	0.8
FDSC	4.4	0.6
An-Pyo I	4.9	0.5
An-Pyo II	3.6	0.4

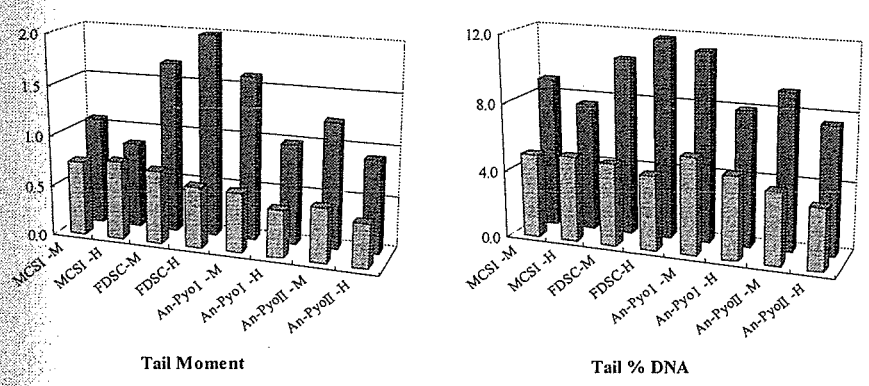
EMS

Mesh	Tail % DNA	Tail Moment
MCSI	8.9	1.1
FDSC	10.4	1.7
An-Pyo I	8.0	1.0
An-Pyo II	9.2	1.2
Homogenize	**Tail % DNA**	**Tail Moment**
MCSI	7.6	0.8
FDSC	11.7	2.0
An-Pyo I	11.2	1.6
An-Pyo II	7.5	0.9

M : Mesh method

H : Homogenize method

Comparison between Mesh and Homogenize



Percentage of whole cells from three preparation methods

- Mesh and Homogenize Method

	Mesh	Homogenize
MCSI	17.2%	17.3%
FDSC	9.4%	10.7%
An-Pyo I	10.9%	18.3%
An-Pyo II	11.3%	13.7%

- Mince (FDSC)

20 times	15.2%
100 times	6.3%

Mincing with a fine scissors

TO SUM UP

- Basically, there is no difference between mesh method (cell) and homogenizer method (nuclei).

From now on, need no discussion on the matter of preparation method for assay (?).

Utility of Alkaline DNA Strand Breakage Assays in Genotoxicity Testing: Comparison of the Alkaline Elution and Comet Assays

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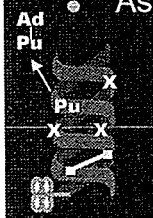
Dept. of Laboratory Sciences and Investigative Toxicology
Safety Assessment
Merck Research Laboratories
West Point, PA USA

Alkaline DNA Strand Breakage Assays

Background

- Assays for primary DNA damage
  - DNA single and double strand breaks (x)
  - alkali-labile lesions: abasic sites (Ad / Pu)
  - Some assays have protocols for DNA interstrand crosslinks (▬) and protein-DNA crosslinks(● –)
- Need to include assessment of cytotoxicity and necrosis
  - to identify cytotoxic dose levels
  - to exclude effects due to DNA degradation in dead or dying cells



Alkaline DNA Strand Breakage Assays

History

- Alkaline sucrose density gradient centrifugation
  - McGrath & Williams, 1966
- Alkaline DNA unwinding
  - Ahnstrom & Erixon, 1973
- Alkaline elution*
  - Kohn & Ewig, 1973
- Viscoelastometry
  - Parodi et al., 1981
- Alkaline comet assay*
  - Singh, 1988

* Most extensively used in genotoxicity testing

Merck Elution Assays

In vitro
- *rat hepatocytes*
- *TK6, L1210 cells*

In vivo
- *liver*
- *glandular stomach*
- *kidney*
- *lung*
- *bladder*
- *testes*
- *small intestine*
- *bone marrow*
- *WBC*

Comparison of Alkaline Elution and Comet Assays

Alkaline Elution	Alkaline Comet Assay
in vitro or in vivo assays	in vitro or in vivo assays
cells/nuclei filter loaded	cells/nuclei agarose gel embedded
pH10 lysis with Prot. K	pH 10 lysis
– DNA DS fragments in lysis quantitated	- DNA DS fragments in lysis lost/not quantitated
alkalinization (pH 12.1-12.5)	alkalinization (pH 13)
separation by elution through 2.0 μ filter pores (pH 12.1-12.5)	Separation by gel electrophoresis (pH 13)
quantitation by fractionation and fluorescence assay for DNA	quantitation of separation by fluorescence and image analysis
primary DNA damage evident as increased elution rates	primary DNA damage evident as increased DNA migration
well characterized to control confounding effects of cytotoxicity	Highly-damaged cells (ghosts) can be recognized

Comparison of Alkaline Elution and Comet Assays: Advantages of Comet Assays

- Sensitivity
  - ability to work at very alkaline pH (≥13) while maintaining acceptable migration of DNA from control cells
    - increased rat of conversion of ALS to SSBs
  - immobilization of cells in agar for lysis, denaturation, and unwinding likely minimizes physical effects (shear stress)
  - detection of as little as 50 mGy of radiation-induced DNA strand breaks (Singh et al., 1994)
    - with radical scavengers, YOYO-1 dye, uniform electric field, image analysis system
  - compares to ~ 300 mGy limit of detection for alkaline elution (Kohn et al., 1976)
  - differences in sensitivity likely to be agent-dependent
    - pH-dependent ALS with alkylating agents AC >AE
    - NER- or Topo II inhibitor-induced DSB AC~=AE?

Comparison of Alkaline Elution and Comet Assays

Other Advantages of Alkaline Comet Assays

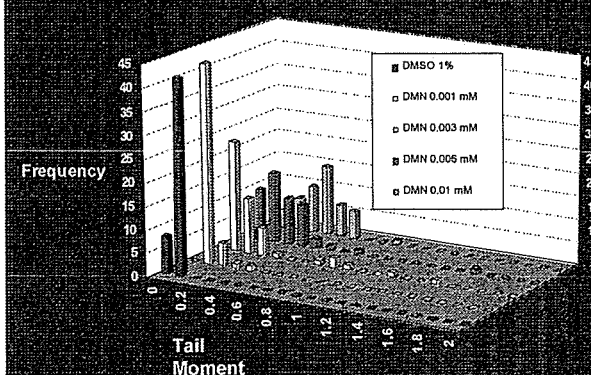
- Technically easier to perform
  - minimal equipment requirements
- In standard assay formats, more cells required for alkaline elution
  - can limit utility of in vivo elution for tissues for which only small numbers of cells/nuclei can be obtained
  - not a problem with microtiter plate alkaline elution assay
- Standardized, validated image analysis software commercially available
- Wider adoption: large and growing user community
- Assay and its interpretation will ultimately be more familiar to regulatory authorities

Comparison of Alkaline Elution and Comet Assays: Advantages of Comet Assays

- Visualization of individual cells/nuclei
  - ability to image differences in the extent of DNA damage between individual cells allows for
    - analysis of heterogeneity in the response of individual cells to DNA damaging agents
    - recognition and quantitation of necrotic and apoptotic cells with extensive DNA fragmentation
      - for this to be a true advantage, it needs to be developed and validated as a quantitative parameter of cytotoxicity
      - can necrotic cells in earlier stages of enzymatic DNA fragmentation be distinguished from live cells with treatment-induced DNA strand breaks?

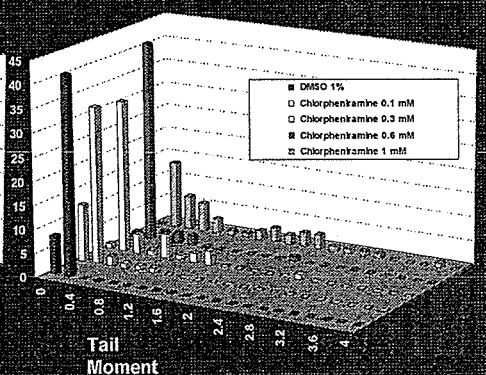
Comparison of a Genotoxic Carcinogen and Non-Genotoxic Non-Carcinogen in the Alkaline Comet Assay in Rat Hepatocytes

N-nitrosodimethylamine



Dose (mM)	0	0.001	0.003	0.006	0.01
Viability	100	90	90	90	90

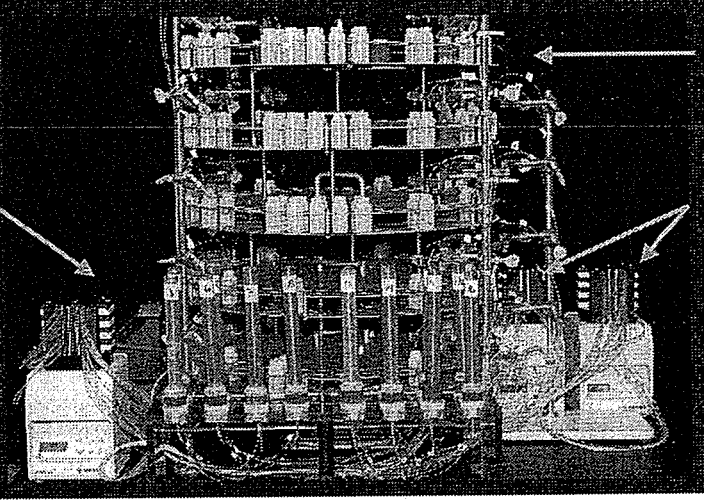
Chlorpheniramine maleate



Dose (mM)	0	0.1	0.3	0.6	1.0
Viability	100	90	80	54	14

Alkaline Elution Assay

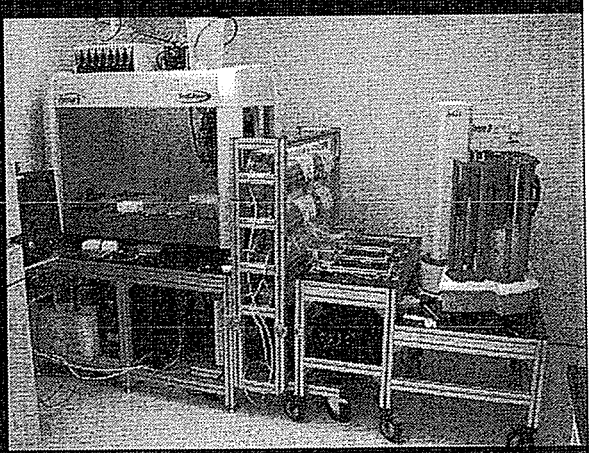
- Technically challenging/extensive equipment requirements



Comparison of Alkaline Elution and Comet Assays

Advantages of Alkaline Elution Assays

- Alkaline elution assays (in vitro and in vivo) can be automated in a 96-well plate high-throughput format
  - minimal cell/nuclei requirements
  - compound requirements for in vitro screening: <50 mg
- Systems built around a 4 x 96-well format liquid handler can assay 384 samples in 2 days
- Assays developed for both in vitro and in vivo samples



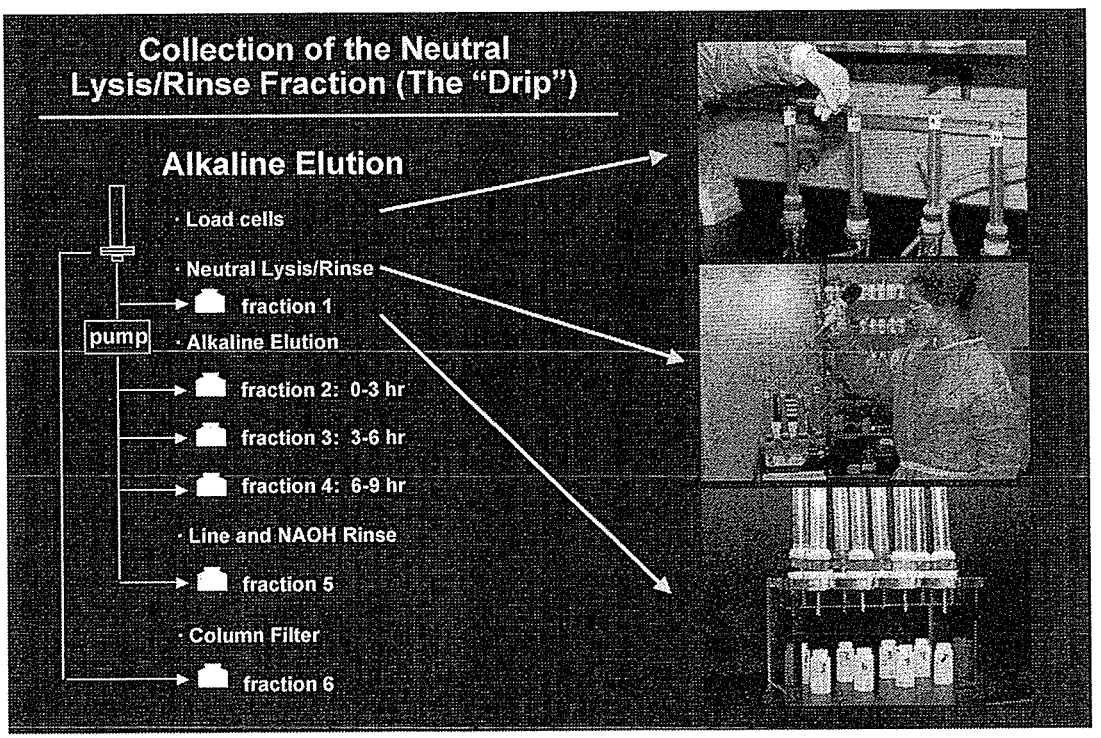
Merck Custom Zymark Staccato System for Alkaline Elution Assays

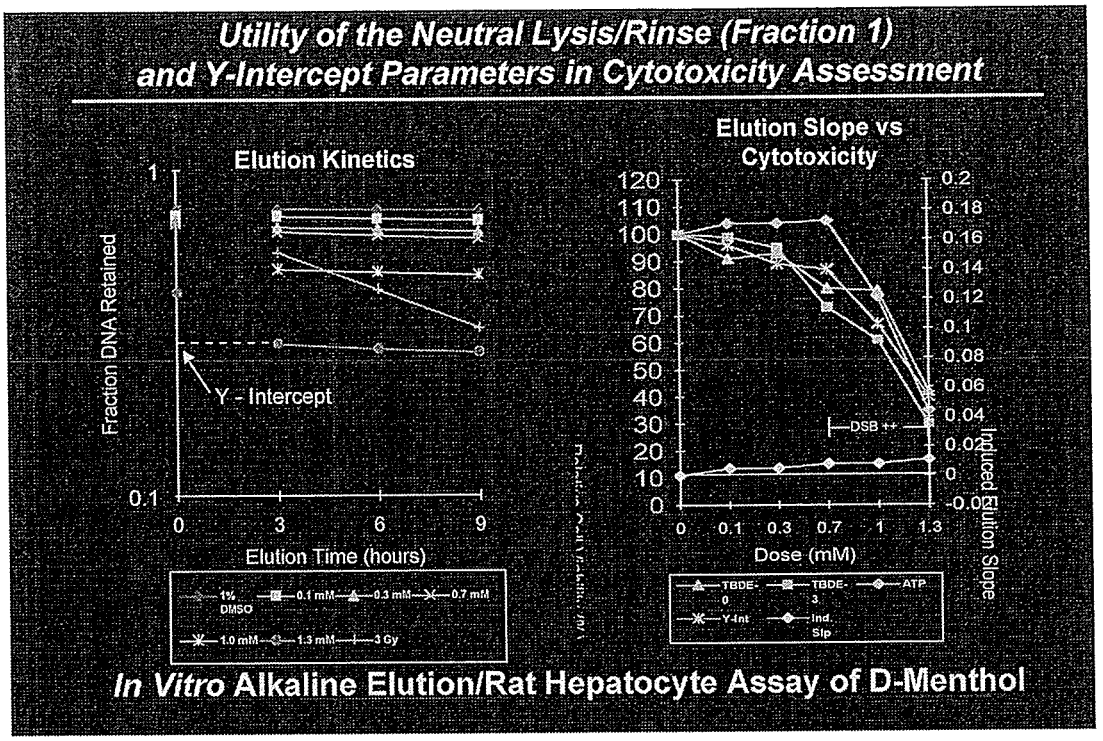
Comparison of Alkaline Elution and Comet Assays

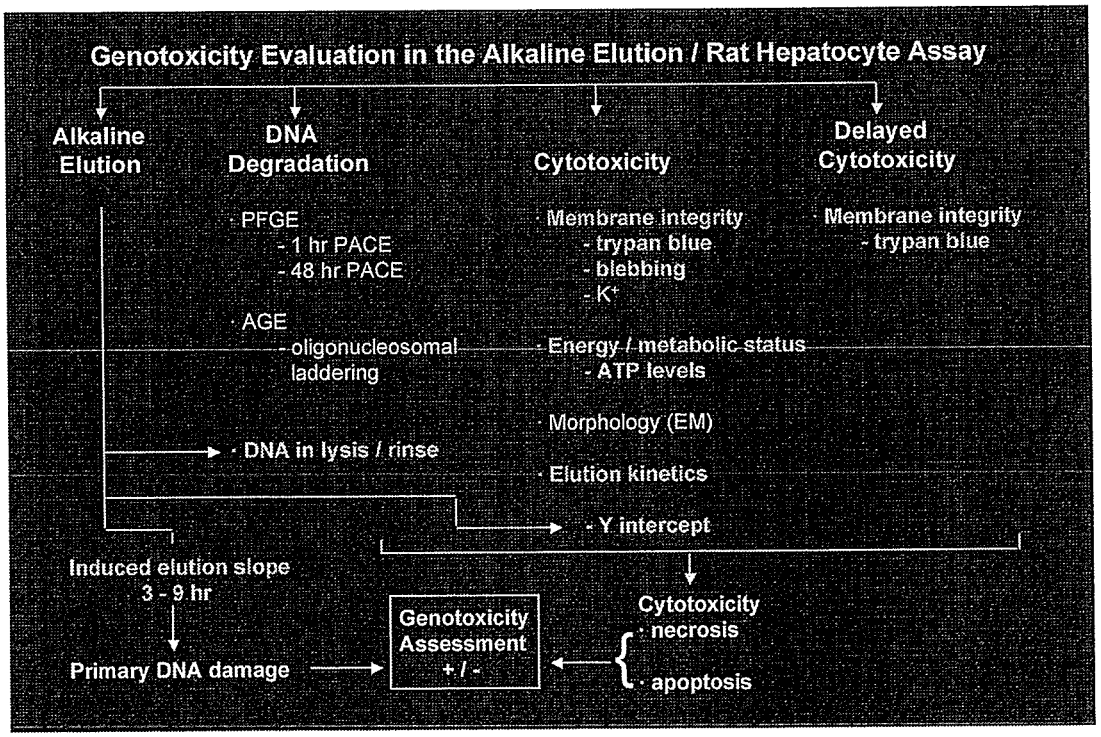
Advantages of Alkaline Elution Assays

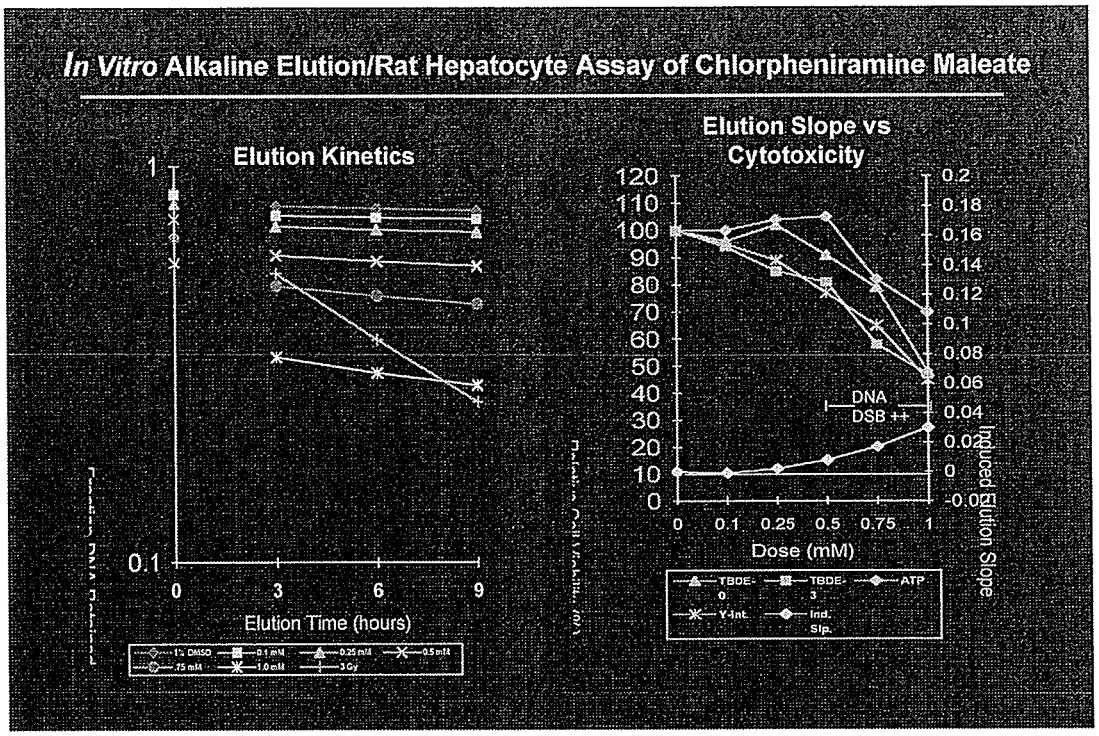
- Alkaline elution assay incorporates a quantitative gravity-flow neutral elution step for DNA double-strand breaks
  - fraction 1 is the gravity flow eluate from the combined lysis and rinse steps of the cells lysed on the column filter
  - quantitation of the DNA content of this fraction provides a sensitive measure of the percentage of cells in the population of treated cells that were dead or dying at the time of lysis
  - thus an integrated analysis of data from the neutral elution and alkaline elution steps provides an assessment of both
    - primary DNA damage (genotoxicity) and
    - damage (fragmentation) secondary to apoptotic of necrotic cell death (cytotoxicity)

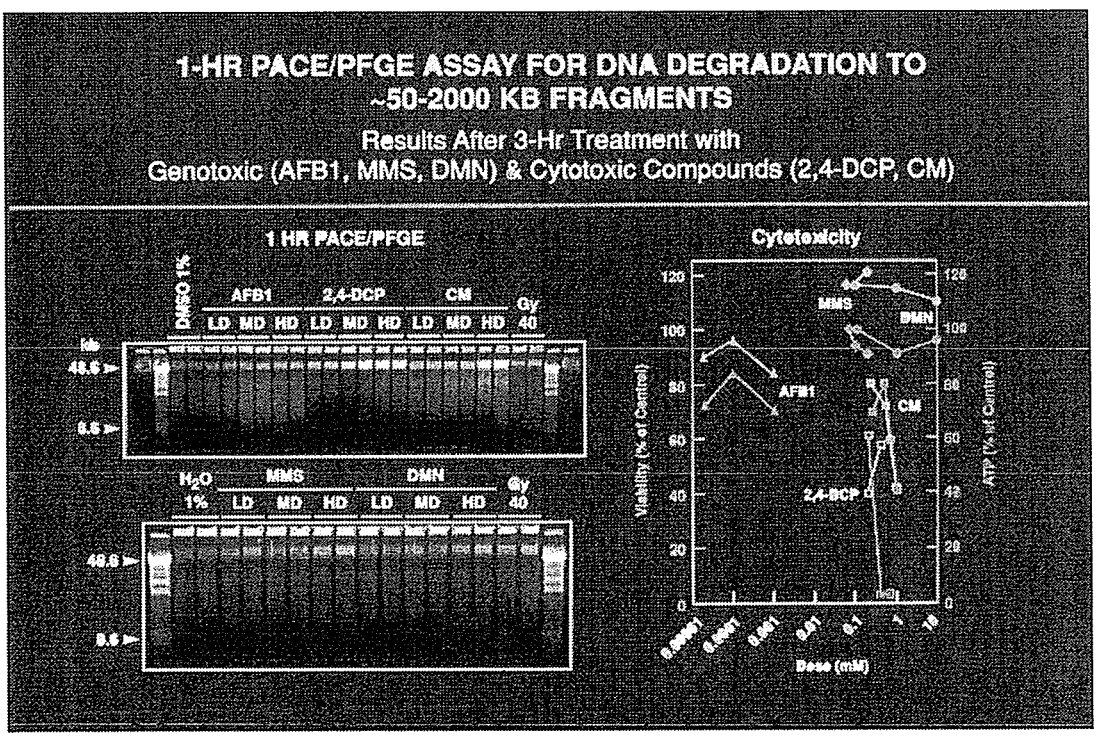
Collection of the Neutral Lysis/Rinse Fraction (The "Drip")




Utility of the Neutral Lysis/Rinse (Fraction 1)
and Y-Intercept Parameters in Cytotoxicity Assessment
Elution Kinetics
Fraction DNA Retained
Y - Intercept
Elution Time (hours)
1% DMSO
0.1 mM
0.3 mM
0.7 mM
1.0 mM
1.3 mM
3 Gy
Elution Slope vs Cytotoxicity
Dose (mM)
DSB ++
TBDE-0
TBDE-3
ATP
Y-Int
Ind. Slp
Induced Elution Slope
In Vitro Alkaline Elution/Rat Hepatocyte Assay of D-Menthol


Genotoxicity Evaluation in the Alkaline Elution / Rat Hepatocyte Assay
Alkaline Elution
DNA Degradation
Cytotoxicity
Delayed Cytotoxicity
PFGE
- 1 hr PACE
- 48 hr PACE
AGE
- oligonucleosomal laddering
Membrane integrity
- trypan blue
- blebbing
- K+
Energy / metabolic status
- ATP levels
Morphology (EM)
Membrane integrity
- trypan blue
- DNA in lysis / rinse
Elution kinetics
- Y intercept
Induced elution slope
3 - 9 hr
Primary DNA damage
Genotoxicity Assessment
+ / -
Cytotoxicity
- necrosis
- apoptosis


In Vitro Alkaline Elution/Rat Hepatocyte Assay of Chlorpheniramine Maleate
Elution Kinetics
Elution Time (hours)
Elution Slope vs Cytotoxicity
DNA DSB ++
Dose (mM)
Induced Elution Slope


1-HR PACE/PFGE ASSAY FOR DNA DEGRADATION TO ~50-2000 KB FRAGMENTS
Results After 3-Hr Treatment with
Genotoxic (AFB1, MMS, DMN) & Cytotoxic Compounds (2,4-DCP, CM)
1 HR PACE/PFGE
Cytotoxicity
AFB1
2,4-DCP
CM
MMS
DMN
Viability (% of Control)
ATP (% of Control)
Dose (mM)