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Standard Guide for Determining DNA Single-Strand Damage in Eukaryotic Cells Using the Comet Assay[1]

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1. Scope

1.1 This guide covers the recommended criteria for performing a single-cell gel electrophoresis assay (SCG) or Comet assay for the measurement of DNA single-strand breaks in eukaryotic cells. The Comet assay is a very sensitive method for detecting strand breaks in the DNA of individual cells. The majority of studies utilizing the Comet assay have focused on medical applications and have therefore examined DNA damage in mammalian cells in vitro and in vivo (**1-4**).[2] There is increasing interest in applying this assay to DNA damage in freshwater and marine organisms to explore the environmental implications of DNA damage.

1.1.1 The Comet assay has been used to screen the genotoxicity of a variety of compounds on cells in vitro and in vivo (**5-7**), as well as to evaluate the dose-dependent anti-oxidant (protective) properties of various compounds (**3, 8-11**). Using this method, significantly elevated levels of DNA damage have been reported in cells collected from organisms at polluted sites compared to reference sites (**12-15**). Studies have also found that increases in cellular DNA damage correspond with higher order effects such as decreased growth, survival, and development, and correlate with significant increases in contaminant body burdens (**13, 16**).

1.2 This guide presents protocols that facilitate the expression of DNA alkaline labile single-strand breaks and the determination of their abundance relative to control or reference cells. The guide is a general one meant to familiarize lab personnel with the basic requirements and considerations necessary to perform the Comet assay. It does not contain procedures for available variants of this assay, which allow the determination of non-alkaline labile single-strand breaks or double-stranded DNA strand breaks (**8**), distinction between different cell types (**13**), identification of cells undergoing apoptosis (programmed cell death, (**1, 17**)), measurement of cellular DNA repair rates (**10**), detection of the presence of photoactive DNA damaging compounds (**14**), or detection of specific DNA lesions (**3, 18**).

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory requirements prior to use.*

1.4 This guide is arranged as follows:

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to express the strongest possible recommendation, just short of an absolute requirement. "Must" is only used in connection with factors that relate directly to the acceptability of the test. "Should" is used to state that the specific condition is recommended and ought to be met if possible. Although violation of on "should" is rarely a serious matter, the violation of several will often render the results questionable. Terms such as "is

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions:*

3.2.1 *CCD camera, n*—charge coupled device (CCD) camera is a light sensitive silicon solid state device composed of many small pixels. The light falling on a pixel is converted into a charge pulse which is then measured by the CCD electronics and represented by a number. A digital image is the collection of such light intensity numbers for all of the pixels from the CCD. A computer can reconstruct the image by varying the light intensity for each spot on the computer monitor in the proper order. Such digital images can be stored on disk, transmitted over a computer network, and analyzed using image processing techniques.

3.2.2 *cell lysis, n*—the process of breaking open a cell by disruption of the plasma membrane.

3.2.3 *DNA, n*—acronym for deoxyribonucleic acid, the substance that is the carrier of genetic information found in the chromosomes of the nucleus of a cell.

3.2.4 *DNA denaturation, n*—refers to breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce two single-stranded polynucleotide polymers.

3.2.5 *DNA lesion, n*—a portion of a DNA molecule which has been structurally changed.

3.2.6 *DNA supercoiling, n*—the condition of DNA coiling up on itself because its helix has been bent, overwound, or underwound.

3.2.7 *DNA supercoil relaxation, n*—upon denaturation, DNA strand breaks allow the supercoiled DNA to unwind or relax.

3.2.8 *double-stranded DNA, n*—a structural form of DNA where two polynucleotide molecular chains are wound around each other, with the joining between the two strands via hydrogen bonds between complementary bases.

3.2.9 *electrophoresis, n*—a method of separating large molecules (such as DNA fragments or proteins) from a mixture of similar molecules. An electric current is passed through a medium containing the mixture, and each kind of molecule travels through the medium at a different rate, depending on its electrical charge and size. Separation is based on these differences. Agarose and acrylamide gels are the media commonly used for electrophoresis of proteins and nucleic acids.

3.2.10 *eukaryotic cell, n*—cell with a membrane-bound, structurally discrete nucleus and other well-developed subcellular compartments. Eukaryotes include all organisms except viruses, bacteria, and cyanobacteria (blue-green algae).

3.2.11 *ocular micrometer, n*—a graduated grid placed between the viewer's eye and an object being observed under a microscope, to measure the object's size.

3.2.12 *single-stranded DNA, n*—linear polymers of DNA resulting from the breaking of hydrogen bonds between complementary base pairs in double-stranded DNA.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *comet, n*—name based on the appearance of individual stained nuclear DNA and associated relaxed or fragmented DNA migrating out from the nuclear DNA observed under the microscope following these assay procedures.

3.3.2 *DNA migration distance, tail length, comet tail length, n*—distance in microns between the leading edge of electrophoretically migrating DNA and the closest edge of the associated nuclear DNA (head).

3.3.3 *head, comet head, n*—portion of a comet comprised of the intact/immobile nuclear DNA.

3.3.4 *tail, comet tail, n*—portion of a comet comprised of the DNA migrating away from the intact/immobile nuclear DNA.

3.3.5 *tail moment, n*—a calculated value used to express the distribution of DNA migrating from the comet head. Image analysis software applies an algorithm to the digitized image of stained DNA and associated migrating DNA tail, which in essence defines the limits of the comet, subtracts background, and determines the boundaries and staining intensity of the nucleus and comet tail. The calculated product of the percent of DNA in the tail and the tail length is defined as the tail moment.

4. Summary of Guide

4.1 Cells collected from organisms under different levels or types of stress are dispersed and immobilized in agarose gel on microscope slides. The slides are placed in a solution to lyse and disperse cell components, leaving the cellular DNA immobilized in the agarose. The DNA is denatured for a specified period of minutes by immersing the slides in an alkaline solution. Strand breaks in the denatured cellular DNA results in higher degree of supercoil relaxation: the more breaks, the greater the degree of relaxation. Given a sufficient degree of relaxation, the application of an electric field across the slides creates a motive force by which the charged DNA may migrate through the surrounding agarose, away from the immobilized main bulk of cellular DNA. Following electrophoresis, the alkaline conditions are neutralized by rinsing the slides in a neutral pH buffer and fixation of slide and its contents in ethanol. The DNA in the fixed slides is stained with fluorescent DNA stain and visualized using a fluorescent microscope. Migration distance of DNA away from the nucleus, comet tail length, can be measured by eye using an ocular micrometer. Comet tail length, percent DNA in tail, tail moment, and other DNA migration values can be calculated with the use of image analysis software.

5. Significance and Use

5.1 A common result of cellular stress is an increase in DNA damage. DNA damage may be manifest in the form of base alterations, adduct formation, strand breaks, and cross linkages (**19**). Strand breaks may be introduced in many ways, directly by genotoxic compounds, through the induction of apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of

excision repair enzymes (20-22). In addition to a linkage with cancer, studies have demonstrated that increases in cellular DNA damage precede or correspond with reduced growth, abnormal development, and reduced survival of adults, embryos, and larvae (16, 23, 24).

5.1.1 The Comet assay can be easily utilized for collecting data on DNA strand breakage (9, 25, 26). It is a simple, rapid, and sensitive method that allows the comparison of DNA strand damage in different cell populations. As presented in this guide, the assay facilitates the detection of DNA single strand breaks and alkaline labile sites in individual cells, and can determine their abundance relative to control or reference cells (9, 16, 26). The assay offers a number of advantages; damage to the DNA in individual cells is measured, only extremely small numbers of cells need to be sampled to perform the assay (<10 000), the assay can be performed on practically any eukaryotic cell type, and it has been shown in comparative studies to be a very sensitive method for detecting DNA damage (2, 27).

5.1.2 These are general guidelines. There are numerous procedural variants of this assay. The variation used is dependent upon the type of cells being examined, the types of DNA damage of interest, and the imaging and analysis capabilities of the lab conducting the assay. To visualize the DNA, it is stained with a fluorescent dye, or for light microscope analysis the DNA can be silver stained (28). Only fluorescent staining methods will be described in this guide. The microscopic determination of DNA migration can be made either by eye using an ocular micrometer or with the use of image analysis software. Scoring by eye can be performed using a calibrated ocular micrometer or by categorizing cells into four to five classes based on the extent of migration (29, 30). Image analysis systems are comprised of a CCD camera attached to a fluorescent microscope and software and hardware designed specifically to capture and analyze images of fluorescently stained nuclei. Using such a system, it is possible to measure the fluorescence intensity and distribution of DNA in and away from the nucleus (8). Using different procedural variants, the assay can be utilized to measure specific types of DNA alterations and DNA repair activity (1, 3, 8, 10, 13, 14, 17, 18). Alkaline lysis and electrophoresis conditions are used for the detection of single-stranded DNA damage, whereas neutral pH conditions facilitate the detection of double-strand breaks (31). Various sample treatments can be used to express specific types of DNA damage, or as in one method, to preserve strand damage at sites of DNA repair (10). Nuclease digestion steps can be used to introduce strand breaks at specific lesion sites. Using this approach, oxidative base damage can be detected by the use of endonuclease III (18), as well as DNA modifications resulting from exposure to ultraviolet light (UV) through the use of T4 endonuclease V (3). Modifications of this type vastly expand the utility of this assay and are good examples of its versatility.

5.2 A sufficient knowledge of the biology of cells examined using this assay should be attained to understand factors affecting DNA strand breakage and the distribution of this damage within sampled cell populations. This includes, but is not limited to, influences such as cell type heterogeneity, cell cycle, cell turnover frequency, culture or growth conditions, and other factors that may influence levels of DNA strand damage. Different cell types may have vastly different background levels of DNA single-strand breaks due to variations in excision repair activity, metabolic activity, anti-oxidant concentrations, or other factors. It is recommended that cells representing those to be studied using the SCG/Comet assay be examined under the light or fluorescent microscope using stains capable of differentially staining different cell types. Morphological differences, staining characteristics, and frequencies of the different cell types should be noted and compared to SCG/Comet damage profiles to identify any possible cell type specific differences. In most cases, the use of homogenous cell populations reduces inter-cell variability of SCG/Comet values. The procedures for this assay, using cells from many different species and cell types, have been published previously (1, 2, 3, 5, 8, 10, 13, 14, 17, 18, 32-38). These references and others should be consulted to obtain details on the collection, handling, storage, and preparation of specific cell types.

5.3 The experimental design should incorporate appropriate controls, reference samples, and replicates to delineate the influence of the major sources of experimental variability.

6. Equipment and Reagents

6.1 *Equipment:*

6.1.1 *Water Bath,* set at a temperature of 35 to 40°C to keep slide coating agarose liquefied during the preparation of slides.

6.1.2 *Centrifuge,* capable of exerting a 600X *g* force and of handling 1.5 mL microcentrifuge tubes. Lower *g* force will require longer centrifugation times and refrigeration to minimize stress to cells.

6.1.3 *Electrophoresis Chamber and Power Supply,* a submarine electrophoresis chamber, and a power supply able to deliver a constant current up to 300 mA and a voltage gradient of 0.4 to 1.3 V/cm.

6.1.4 *Fluorescent Microscope,* DNA is visualized by staining with one of a number of fluorescent DNA stains such as ethidium bromide (EtBr), propidium iodine, or YOYO®.[4] In this guide, EtBr is used with a fluorescent microscope with a 510 to 560 nm excitation filter and 590 nm barrier emission filter to view the EtBr-stained DNA.

6.1.5 *Image Analysis System,* an image analysis system comprised of a CCD camera attached to the microscope which is connected to a computer loaded with the systems software.[5]

6.2 *Reagents:*

6.2.1 *Slide Coating Agarose,* low-endo-osmotic agarose, melting point of ~ 30°C for slide base coat dissolved in TAE buffer, to a final concentration of 0.5 to 1.0 %.

[4] The sole source of supply of the apparatus known to the committee at this time is Molecular Probes Inc., 4849 Pitchford Ave. Eugene, OR 97402-9165. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

[5] Information on many of the available systems can be found at www.geocities.com/cometassay/index.html.

6.2.1.1 *Stock TAE Buffer (50X),* Tris base 242 g, glacial acetic acid 57.1 mL, 0.5 M EDTA pH 8.0 100 mL, mix in distilled water to final volume of 1 L.

6.2.1.2 *TAE Working Buffer (1X),* dilute 50X TAE (6.2.1.1) 1:49, 10 mL 50X TAE in 490 mL distilled water.

6.2.2 *Cell Suspension Agarose (CSA),* low melting point agarose (LMA) for cell suspension and slide application, melting point of 30°C. LMA dissolved to 0.65 to 1.0 % in buffer solution suitable for the cells being studied. Kenny's salt solution (6.2.2.1) has been successfully used with marine invertebrate cells **(13, 14, 17)** and phosphate buffered saline for most vertebrate cells **(8, 31)**.

6.2.2.1 *Kenny's Salt Solution,* to 900 mL of distilled water add 23.5 g NaCl (0.4 M), 0.7 g KCl (9 mM), 0.1 g K_2HPO_4 (0.7 mM), and 0.2 g $NaHCO_3$ (2 mM). Adjust pH to 7.5 with NaOH, bring to a final volume of 1 L and filter through 0.45 µm filter to sterilize. Store at 4°C.

6.2.2.2 *Phosphate Buffered Saline Stock,* 10X PBS, 1.361 g KH_2PO_4 (0.01 M), 14.2 g Na_2HPO_4 (0.1 M), 80.1 g NACl, (1.37 M), 20.1 g KCl (0.027M), adjust to pH 7.0, bring to a final volume of 1 L and filter through 0.45 µm filter to sterilize. Store at 4°C.

6.2.2.3 *PBS Working Solution,* dilute 10X PBS (6.2.2.2) 1:9, 10 mL 10X PBS in 90 mL distilled water. After dilution pH should be 7.4.

6.2.3 *Stock Lysis Solution,* 146.4 g NaCl (2.5 M), 37.2 g EDTA (0.1 M), 1.2 g TrisHCl (0.01 M), combine chemicals in 500 mL of distilled water; while stirring slowly, add 8 to12 g NaOH to facilitate the dissolution of EDTA and bring the pH to 10. Adjust volume to 1 L, filter through 0.45 µm filter to sterilize and store at 4°C.

6.2.3.1 *Working Lysis Solution,* 89 mL Lysis stock solution (6.2.3), 10 mL DMSO, 1.0 mL Triton X-100, final volume 100 mL. Make fresh on the day of use. Refrigerate to 4°C prior to use.

6.2.4 *Electrophoresis/Unwinding Solution,* to 968 mL distilled water add 30 mL (10 N) NaOH and 2 mL (0.5 M) EDTA, pH 8.0, final volume 1 L. Make fresh on the day of use.

6.2.5 *Stock 1 M Tris Buffer, pH 7.5,* dissolve 121.1 g Tris Base, in 800 mL distilled water, add 63 mL concentrated HCl, adjust pH to 7.5 and bring to the final volume of 1L.

6.2.5.1 *Working 0.4 M Tris Neutralization Buffer, pH 7.5,* mix 200 mL (1 M) Tris pH 7.5 (6.2.5) with 300 mL distilled water for 500 mL final volume. Store at 4°C.

6.2.6 *Ethidium Bromide Stock Solution,* (**Warning—**Use extreme caution when handling. Ethidium bromide is a mutagen. Wear appropriate safety protection. Dispose of contaminated items and waste properly.), dissolve 10 mg ethidium bromide (EtBr) in 1 mL distilled water, store in light-protected container at 4°C.

6.2.6.1 *Ethidium Bromide Working Solution,* 10 µL EtBr stock solution (6.2.6) in 5 mL distilled water. Store in a light-protected container at room temperature.

6.2.7 *95 % Ethanol,* reagent grade 95 % ethanol, stored in a freezer at -20°C.

7. Assay Procedures

7.1 *Slide Preparation:*

7.1.1 Slides on which agarose cell suspensions may be applied are prepared as follows.

NOTE 1—Gloves should be worn whenever handling slides and slidecovers, skin oils reduce the adherence of agarose to the microscope slides.

Prepare 100 mL of melted slide coating agarose (6.2.1). Solid agarose preparations can be liquefied in a microwave oven with alternating short pulses of microwaves followed by gentle swirling. Using 3 in. by 1 in. by 1 mm clear glass microscope slides with a frosted label area, grasp the slide at the label area, dip into the melted agarose, and leave immersed for at least 10 s. Remove from the agarose and wipe excess agarose off the back of the slide, and then place on a level surface and allow to dry for 2 h at room temperature or for 30 min in a drying oven at 37°C. Store dry slides in a moisture-free slide storage box at room temperature. Slides prepared in this manner may be stored almost indefinitely and require no further preparation before sample application.

7.2 *Sample Preparation:*

7.2.1 This assay requires very small quantities of cells. Researchers should be familiar with maintenance media most likely to reduce stress on the cell types used. Cells suspended in 50 to 1000 µL maintenance media are placed in a 1.5 mL centrifuge tube and the cells are pelleted (for example, at 600 × g for 2 min). Supernatant is carefully drawn off and discarded. In most cases, a cell pellet ~1 to 2 mm in diameter is more than sufficient to yield cell densities of between 5 to 20 cells per microscopic field of view at 200×, when resuspended in 50 to 400 µL of cell suspension agarose (CSA, 6.2.2). Experience estimating and adjusting cell densities will reduce the number of overlapping and superimposed nuclei encountered during scoring. CSA is melted and placed in a 35 to 40°C water bath and the temperature is allowed to stabilize. The pellet is gently resuspended in the appropriate volume of CSA at 35 to 40°C, as determined by the researcher. Before the agarose solidifies, an aliquot of the cell suspension (for example, 50 to 75 µL) is transferred to an agarose-coated slide (7.1). The applied sample is then uniformly spread over the slide by placing a clean glass coverslip on top of the still liquefied sample. The slide is then placed on a level ice-cold metal or glass surface until the agarose has solidified. Once solidified, a top-coat of 50 to 75 µL CSA is added by slipping the coverslip off the slide, applying agarose and replacing the coverslip on top. The agarose is allowed to solidify as before. After the agarose has solidified, the coverslip is slipped off and the slide placed in a glass slide jar filled with ice-cold working Lysis solution (6.2.3.1), so that the solution completely immerses the area of the slide on which the sample was applied. The minimum lysis period is usually one hour, with no apparent maximum. However, gels can become more fragile with extended lysis times.

7.3 *Unwinding and Electrophoresis Conditions:*

7.3.1 Slides processed through the lysis step, 7.2, should be rinsed in distilled water or neutralizing buffer to remove the lysis salts and detergents. This can be accomplished by immersing the slides for 2 min and replacing with fresh distilled water/neutralizing buffer three times. The slides are

placed in the electrophoresis chamber and the chamber filled with electrophoresis/unwinding solution (6.2.4) to a depth of 3 to 4 mm above the slides. The slides should be set close together; multiple rows may be formed on the chamber platform. Optimum unwinding and electrophoresis times can be determined by comparing the extent of migration in untreated control target cells and target cells exposed to a DNA damaging agent (for example, gamma-radiation, hydrogen peroxide, methlymethane sulphonate). In order for historical data to be useful, the negative control cells should exhibit some level of DNA migration. The slides should be left undisturbed in the electrophoresis/unwinding solution for from 15 to 60 min to unwind or denature the nuclear DNA. The power supply is set to run for from 5 to 60 min at 300 mA constant current, applying a voltage gradient that may range from 0.4 to 1.3 V/cm. After electrophoresis, the power is turned off, the slides are removed from the chamber, and the alkali neutralized by three rinses of 2 min duration in 0.4 M Tris, pH 7.5 (6.2.5.1). The DNA in the agarose gel is then fixed by soaking for 5 min in ice cold 95 % ethanol (6.2.7). Fixed slides can then be dried at room temperature or in a 37°C oven. The dried slides can be stored in slide boxes until stained and scored.

7.4 *Staining:*

7.4.1 There are many commercially available fluorescent DNA stains that may be used to stain Comet slides. The user must determine which is the most suitable for use. Ethidium bromide will be the stain referred to in this guide, though stains such as propidium iodide, and YOYO®-1,[4] have been used successfully (**1**, **8**, **31**). To stain the DNA, 40 µL of EtBr working stain solution is placed on each slide, a coverslip is placed on top, and excess stain removed with an absorbent tissue. The slides are now ready for scoring under a fluorescent microscope. Following scoring, the coverslips of scored slides can be removed and the slides dried and stored in slide storage boxes as permanent records.

NOTE 2—Gloves should be worn throughout these procedures, and whenever the slides are handled in the future.

7.5 *Scoring:*

7.5.1 Stained slides are examined using a fluorescent microscope under 200× magnification. A magnification range of 100× to 1000× has been reported, but more generally from between 200× and 400×. Optimal magnification will depend on the size of the cells being assessed for DNA damage. Ethidium bromide bound to double-stranded DNA has fluorescence excitation and emission maxima of 518 nm and 605 nm respectively. Appropriate filters allowing excitation wavelength light on to the sample, with barrier filters allowing the passage of emission wavelength light to be viewed through the microscope should be used. Slides viewed in this way reveal stained comets as brightly fluorescent orange balls 10 to 40 µm in diameter. Scoring may be conducted in several ways: (*1*) by eye, using a calibrated ocular micrometer (7.5.2) or by categorizing comets into four or five classes based on the extent of migration (**29**, **30**), or (*2*) using an image analysis system (7.5.3), comprised of a CCD camera attached to an epifluorescent microscope and software and hardware designed specifically to capture and analyze images of fluorescently stained nuclei. With the use of an image analysis system, it is possible to measure the fluorescence intensity and distribution of DNA throughout the comet (**32**). In this way, the percentage of DNA in the comet tail, the length of the tail, tail moment (which is the product of the fraction of DNA in the tail and tail length), as well as numerous other measures can be determined.

7.5.1.1 Regardless of the method used for collecting data on DNA migration, the number of comets measured per slide must be determined by the researcher to yield adequate statistical power. Generally, the recommended number of cells to score per sample is from 50 to 100 (**39**, **40**). Fewer cells would eliminate the ability to identify the presence of subpopulations of cells with altered migration among a larger population of cells with migration patterns not different from the control sample. Because of inherent variability within and across electrophoresis runs, it is recommended that two slides be scored per sample, with 50 % of the data obtained from each replicate slide. Comets are scored in different sectors of the slide. It is important to avoid scoring comets near the edge of the slide and not to score slides with high background levels of staining. Once the field of view is randomly moved to a sector, a systematic method of scoring is used, such as scoring comets from left to right starting in the upper left-hand corner until a predetermined number of comets have been scored. Overlapping and superimposed comets are not counted. For the sake of objectivity, slide scoring should be "blind" (that is, that the sample identity of the slide not be known during scoring).

7.5.2 The first method involves measuring with an ocular micrometer the length of the "comet" tail trailing the nucleus of 25 to 100 nuclei on each slide. The number of comets measured per slide must be determined by the researcher to yield adequate statistical power. The diameter of the head and the distance the stained DNA has migrated from the head are recorded.

7.5.3 The second method incorporates the use of an image analysis system (6.1.4). These systems are comprised of a CCD camera attached to the microscope and software designed specifically to capture and analyze images of fluorescently stained DNA. Using such a system, it is possible to measure the fluorescence intensity and distribution of DNA in and away from the head. In this way, the percent of DNA in a comet tail, length of the tail, tail moment (which is the product of the % DNA in the tail and tail length), as well as numerous other measures can be determined. The method of picking comets to be scored is the same as in 7.5.1.

8. Treatment of Data

8.1 *Interpretation of Data:*

8.1.1 Depending on the method of scoring, different types of data may be gathered, including but not limited to, ocular measurement of comet image or tail lengths, the fraction of cells with different patterns of migration, or image analysis based comet tail lengths, percentage of migrated DNA, tail moment, or other measurements. The distribution of migration patterns can be graphically expressed in histograms by plotting frequency of comets (*Y* axis) and the corresponding DNA damage measurement gathered for those comets (*X* axis). In addition, dose response plots can be constructed showing DNA damage (mean and standard deviation of data, *Y* axis) and test compound concentration (*X* axis).

8.2 *Acceptability of Data:*

8.2.1 DNA damage levels in controls and if the study design allows, the damage resulting from reference toxicants should be compared to previously gathered data to determine acceptability.

8.2.2 If studying cells from organisms or cell-lines where it is possible to perform laboratory exposure tests, then experiments can be designed to incorporate positive controls and the performing laboratory can construct control charts for the purpose of evaluating test performance (see Test Method E1706, Section 17.4.4). As stated in Test Method E1706, Section 17.4.4, a control chart can be prepared for each combination of reference toxicant and test organism. Each control chart should include the most current data. Endpoints from five tests are adequate for establishing the control charts. Control Charts are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits (±2 standard deviation, (SD)) are recalculated with each successive test result.

8.2.3 If the value from a given test with the reference toxicant falls more than two standard deviation outside the expected range, the sensitivity of the organisms and the overall credibility of the test system may be suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

8.2.4 It is recognized that, in many instances, samples will be collected from field populations, or from organisms on which controlled-exposure experiments cannot be performed, that is, humans or protected or endangered species. Under such circumstances, it is difficult or impossible to evaluate test performance using positive controls, and the researcher must rely on the collection of appropriate reference samples.

8.3 *Statistics:*

8.3.1 Appropriate statistical methods for analyzing the data should be used as an aid in evaluating the test results but should not be the only determining factor for identifying a positive response. The statistical methods to be used and the data requirements for those methods should be established during initial design of the experiment. The unit of exposure for in vitro studies is the culture, while it is the animal for in vivo studies. This means that multiple cultures or multiple animals are needed per dose group in order to conduct an appropriate statistical analysis. In most instances, the homogeneity of variance between treatments should be examined to determine whether parametric or non-parametric analysis is appropriate. Transformation of non-homogenous data can be explored as well. If homogeneity is not achieved using transformed data, non-parametric procedures should be used. Linear regression analysis can used to establish dose response relationships, while pairwise comparisons of each treatment group against the concurrent control can be conducted. (see Practice E1847)

8.4 *Evaluation and Interpretation of Results:*

8.4.1 In the event that a positive Comet assay response is obtained, it is critical that the investigator(s) assesses the possibility that the increase in migration is not associated with genotoxicity. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose response curve, the inter-cellular distribution of comet response at each dose, and the presence or absence of necrotic or apoptotic cells in the treated cell population may be useful in this regard. In the event that a negative Comet assay response is obtained, it is critical that the investigator(s) assesses the validity of the assay and the dose selection procedure. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Reproducibility in independent experiments is considered the strongest evidence for a positive or negative call.

9. Reporting Data

9.1 In addition to the provisions previously described in this guide, a concise, written report of the assay should be prepared and should include the following information:

9.1.1 All information pertaining to the organism(s) the cells were collected from, type of cells used and their treatment, collection, handling and storage conditions.

9.1.2 Specific to the assay, all information about agarose concentrations, DNA denaturation conditions and duration, electrophoresis times and conditions, stain used and method of analysis, and other material must be reported to ensure that individuals reading the report can accurately reproduce the assay conditions.

9.1.3 Any unique or extreme characteristics associated with the scored comets should be photographed or a digitized image made and incorporated into the report.

10. Keywords

10.1 biomarker; cellular; Comet assay; DNA damage; DNA strand breaks; stress effects; toxic