



Designation: E2186 – 02a(Reapproved 2010)

Standard Guide for Determining DNA Single-Strand Damage in Eukaryotic Cells Using the Comet Assay¹

This standard is issued under the fixed designation E2186; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

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:

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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

¹ This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

Current edition approved March 1, 2010. Published May 2010. Originally approved in 2002. Last previous edition approved 2002 as E2186–02A. DOI: 10.1520/E2186-02AR10.

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 by the action 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.