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

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 This guide is arranged as follows:

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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 (Withdrawn 2022)[4]

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to

[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.

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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.

[4] The last approved version of this historical standard is referenced on www.astm.org.

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 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.