Detection of DNA Damage and Identification of UV-Induced Photoproducts Using the CometAssay™ Kit

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ABSTRACT

We introduce the first commercially available comet assay for the detection and quantification of DNA damage in individual eukaryotic cells. The major difficulty of the comet assay is the preparation of the slides needed to immobilize the samples throughout the lysis and electrophoretic procedure. The CometAssay™ kit uses a proprietary technology to precoat glass microscope slides to allow direct application of the agarose embedded sample without any additional slide treatment. In this report, we discuss the detection of DNA damage in individual cells exposed to ultraviolet irradiation using the new CometSlides™ and their cost compared to traditional slides.

INTRODUCTION

The comet assay (6,16,17,20), also called single-cell gel electrophoresis (SCGE), is a simple yet powerful tool for demonstrating the damaging effects of different compounds or physical treatments on DNA at the individual cell level. In the last 20 years, advances in DNA damage detection have allowed scientists to determine the impact of certain environmental factors or medical treatments on DNA integrity. Although other methodologies exist for detecting damaged DNA, such as alkaline elution (8), supercoiled DNA unwinding (2,21) and DNA repair enzyme analysis (5), the comet assay is rapidly gaining in popularity.

Analysis of DNA damage by SCGE was first published in 1984 by Östling and Johanson (14). They demonstrated that DNA in cells embedded in low-melting point agarose migrated out of the cell in an electrophoretic field in a pattern deter-

mined by the extent of the damage. The migrated DNA often resembles a comet tail, hence the term comet assay. The comet assay has evolved from the use of neutral electrophoresis buffers that allow only the detection of double-stranded breaks to an alkali electrophoresis system (13,16) in which the DNA unwinds and denatures and permits the detection of single-stranded (ss)DNA breaks and alkali-labile sites, as well as double-stranded (ds)DNA breaks. Different modifications added to the comet assay were reviewed by Fairbairn et al. in 1995 (6).

The comet assay's major difficulty lies in the preparation of the glass microscope slide surface to keep the samples in place. Application of multiple layers of agarose on the slide, use of fully or partially frosted slides and even roughly damaging the slide surface by sand blasting have been reported in the literature as methods used for enhancing gel adhesion (6,9,18). These methods are time-consuming and may interfere with the fluorescent viewing and analysis. The CometAssay™ Kit (Trevigen, Gaithersburg, MD, USA) introduces a cost-effective, ready-to-use specially designed microscope slide (CometSlide™; Trevigen, patent pending) that allows direct application of cell samples. The slides are clear and have a hydrophobic boundary around the sample area to allow the application of multiple samples on one slide and the use of small volumes of enzymes or immunoreagents.

In addition to allowing qualitative and/or quantitative measurement of DNA damage in single cells, specific DNA adducts can be detected by using the comet assay in conjunction with immunological reagents (15). We demonstrate here how the CometAssay kit can be used in combination with an anti-UV ssDNA antibody to detect (6-4)-dipyrimidine photoproducts.

Table 1. Cost Comparison of Three Traditional Slide Preparation Methods to CometSlides

Method	Slide	Cost Per 5 Slides (\$)			Total	Cost per Sample ¹
		Agarose	Coverslips	Labor and Overhead at \$25/h		
Single layer²	Fully frosted ⁹ slides	Agarose ⁶ 0.7%	Coverslips ⁷ (3/slide) (to flatten base and 2 samples)			
	1.41⁵	0.05	2.04⁵	12.50	16.00	1.60
Sandwich³	Fully frosted slides ⁷	Agarose ⁸ base NMP 0.6% top LMP 0.5%	Coverslip ⁷ (1/slide) (to flatten base)			
	1.41⁵	0.10⁵	0.68⁵	12.50	14.69	2.94
Dried agarose⁴	Clear slides w/ frosted end ⁹	Agarose ⁸ coating 50 mL of 1% NMP	Coverslips ⁷ (1/slide)			
	1.10⁵	2.17⁵	0.68⁵	12.50	16.45	3.29
CometSlide	CometSlides (at 75/25 slides)					
	15	N/A	N/A	N/A	15.00	1.50
CometSlide	CometSlides (at 185/100 slides)					
	9.25	N/A	N/A	N/A	9.25	0.93

N/A: not applicable for this item.
¹Assuming a 100% success rate and not including cost of kit and other reagents.
²Components and vendors as described in Morris et al. (12).
³Components described in Kreja et al. (11).
⁴As described by Collins (3).
⁵Fisher Scientific, Pittsburgh, PA, USA.
⁶SeaKem® Gold; FMC, Rockland, ME, USA.
⁷Unspecified source, prices from Fisher Scientific 1998-1999 Catalog.
⁸Life Technologies.
⁹Richardson Supply, London, England, UK.
NMP: Normal melting point.
LMP: Low melting point.

MATERIALS AND METHODS

Cell Culture

WEHI 7.1 BALB/c mouse lymphomas were obtained from the ATCC (Rockville, MD, USA). Cells were grown in suspension in Dulbecco's modified Eagle media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Mediatech, Herndon, VA, USA), and 10 µg/mL gentamicin (Life Technologies, Gaithersburg, MD, USA). Cells were incubated at 37°C in a 5% CO₂ humidified atmosphere and maintained below 1 × 10⁶ cells/mL.

UV Irradiation

WEHI 7.1 cells were prechilled on ice in a microtube. In a prechilled, plastic petri dish, 3 × 10⁵ cells were irradiated with

0, 100 and 1000 J/m² of UV at 254 nm using a Model 1800 Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA, USA). The UV fluence was confirmed with a Blak Ray® UV Short-Wave Measuring Meter (UVP, Upland, CA, USA). After treatment, cells were maintained on ice for 10 min and shielded from ambient light until the CometAssay was performed.

Hydrogen Peroxide Treatment

WEHI 7.1 cells were diluted to 1 × 10⁵ cells/mL in cold phosphate-buffered saline (PBS). Hydrogen peroxide (H₂O₂) (Sigma, St. Louis, MO, USA) was added to the cell sample at a final concentration of 100 µM and incubated for 5 min on ice. Cells were centrifuged at 250× g, washed with cold PBS and maintained on ice for 5 min before being embedded in low-melting agarose to perform the comet assay.

CometAssay Protocol

The comet assay was performed using the CometAssay kit following the manufacturer's instructions. Briefly, an aliquot of 50 μL of cells (1×10^5 cells/mL) was added to 500 μL of molten LMAgarose (0.5% low-melting agarose) kept at 42°C. After mixing the sample, a 50- μL aliquot was pipetted onto an area of the CometSlide. The slide was incubated at 4°C for 10 min to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% (wt/vol) sodium lauryl sarcosinate, 0.01% (vol/vol) Triton® X-100] for 30 min at 4°C. A denaturation step was performed in alkali solution (0.3 M NaOH, 1 mM EDTA) at room temperature (RT) for 30 min, shielded from light. The slide was then transferred to 1× TBE (90 mM Tris-borate, 2 mM EDTA) for 10 min to neutralize before electrophoresis in a horizontal chamber in fresh 1× TBE at 1 V/cm for 10 min. The slide was fixed in ice-cold, 100% methanol for 5 min, then immersed in 100% ethanol at RT for 5 min and air dried. For observation, samples were stained with SYBR® Green (Molecular Probes, Eugene, OR, USA), diluted 1:10 000 in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and observed by epifluorescence microscopy with a 490-nm filter.

6-4 dipyrimidine Photoproduct Detection

After performing the comet assay, fixing and dehydrating the samples, the presence of (6-4)-dipyrimidine photoproducts in the DNA of the UV-treated cells was determined. Each sample area was covered with 50 μL of 12 $\mu\text{g/mL}$ anti-UV ssDNA antibody (Trevigen) in PBST (PBS, 0.05% Tween® 20), 1% bovine serum albumin (BSA), and incubated at 4°C for 16 h. The slides were washed 3 times in PBST. The samples were covered with 50 μL of 2.5 $\mu\text{g/mL}$ goat anti-mouse IgG (H+L) biotin-conjugated antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in PBST, 1% BSA, for 90 min at RT. After 3 washes in PBST, the samples were incubated with 3.3 $\mu\text{g/mL}$ of streptavidin-fluorescein (Trevigen) in PBS for 20 min at RT. After washing in PBS, the samples were covered with 50 μL of propidium iodide/ribonuclease solution (Trevigen) and immediately observed by epifluorescence microscopy with a 490-nm filter. Qualitative data analysis was performed by scoring the comets either positive or negative for the presence of tails for DNA damage, and positive or negative for antibody binding.

Photography

Photographs were taken on 35-mm HG 1600 color film (Fuji) with a photomicroscopy system (Olympus America, Melville, NY, USA).

RESULTS AND DISCUSSION

Advantages of the CometSlides

The CometSlides provided in the CometAssay kit constitute a simple, efficient, ready-to-use and reliable alternative to the traditional slide. The preparation of slides by any of the traditional methods is relatively labor-intensive. Often the

slides have a high failure rate because of their need for repeated layering of agarose onto the slides and a need to use coverslips between layers and after application of the sample. The CometSlide has been extensively tested and, in more than 200 assays, there were few instances in which the applied sample did not remain adhered to the CometSlide throughout the assay, unless excessive force was used when tapping the slide in a vertical position. Additionally, there are reports of autofluorescence associated with the use of fully frosted slides. The clear viewing area of the CometSlide and the proprietary coating produces less background fluorescence than agarose and fully frosted slides. The reliability of the CometSlide provides an immediate advantage to the researcher by eliminating coating time and loss of precious experimental samples.

The cost of preparing slides using three different methods as seen in recent publications was compared with the cost of CometSlides (Table 1). Briefly, in a one-layer technique (12), agarose is applied to a glass microscope slide and flattened with a coverslip. This base layer is overlaid with 2 separate cell samples in agarose and covered with coverslips. Alternatively, a sandwich method (11) is also used in which the sample is positioned between a base layer and a top layer of agarose, which requires the application of a coverslip on both layers. The cost of a third method was evaluated where glass slides were immersed in molten agarose and allowed to solidify in a thin layer on the slide (3). Table 1 shows that assum-

Figure 1. Detection of DNA damage in WEHI 7.1 cells with the CometAssay kit. (A) Control cell sample and (B) 100 μM H_2O_2 -treated cells were processed with the CometAssay kit and submitted to electrophoresis for 10 min in TBE buffer at 1 V/cm. Samples were fixed, dried and stained with SYBR Green before observation.

ing a 100% success rate and familiarity with these methods, the CometSlide is of similar cost per slide when compared to other methods that typically allow treatment of one sample per slide. Furthermore, each CometSlide offers 2 sample areas per slide, which reduces the actual cost per sample by 50%. Finally, when the CometSlide is used in conjunction with immunoreagents and other enzyme preparations, the defined sample application area with the hydrophobic barrier reduces the quantities of expensive reagents required. Thus, the CometSlide provides several significant advantages over traditional slides for use in the comet assay: cost-effectiveness, reliability, simplicity and a standardized assay platform.

CometAssay Kit

The comet assay can detect double-stranded breaks, apyrimidinic/apurinic (AP) sites and single-stranded breaks when using an alkali treatment. The electrophoresis causes migration of the broken DNA, which migrates faster than intact DNA. When using neutral conditions, some renaturation of DNA results in an elongated comet shape when compared to the droplet shape of alkaline electrophoresis (9). Visualization of the comets can be achieved using DNA binding dyes such as SYBR Green, as offered with the CometAssay kit, ethidium bromide, propidium iodide (PI) or acridine orange. Data can be obtained by visual scoring (4) or by digital scanning and analysis using statistical software. The parameters

for analysis consist of measuring the length of the comet tail and estimating the distribution of the fluorescence between the head of the comet and the tail. The product of the comet length and the fraction of the fluorescence in the tail is called the tail moment and is typically used for comet assay analysis (7). The use of “neutral” electrophoresis buffer (i.e., TBE) allows the comet tail length alone to be used (10). The convenient CometAssay kit provides the basic reagents required to perform the assay and lets the researchers decide on the electrophoresis conditions according to the type of damage of interest. Figure 1, A and B, shows typical results obtained with the CometAssay kit. Untreated cells showed no comet tail, and the DNA remained confined within the nuclear membrane (Figure 1A). Extensive oxidative damage induced by treatment with H_2O_2 revealed long comet tails indicative of ssDNA and dsDNA breaks (Figure 1B). H_2O_2 treatment causes indirect oxidative damage in DNA through the formation of OH radicals by Fenton-like reactions that create extensive DNA damage at both the single-strand and the double-strand levels.

Application of the CometAssay Kit: Immunodetection

To demonstrate the utility of the CometAssay kit in immunodetection of specific DNA adducts, comparison was made between control, H_2O_2 -treated and UV-irradiated cells. UV light is known to generate bipyrimidine DNA photoproducts.

ucts that are potentially cytotoxic and mutagenic. There are two major classes of dimeric photoproducts: cyclobutane pyrimidine dimers and (6-4)-photoproducts. The anti-UV ssDNA antibody recognizes (6-4)-dipyrimidine photoproducts in ssDNA, but (6-4)-dicytidines and cyclobutadipyrimidines are not recognized.

Immunodetection was performed subsequent to the CometAssay protocol. Staining of total cellular DNA in the immunodetection procedure was achieved using PI (red). Colocalization of PI with fluorescein generates a yellow-orange fluorescence. In control cells, no evidence of antibody binding was detected (data not shown). H₂O₂ treatment, followed by both CometAssay and immunodetection of (6-4)-dipyrimidine photoproducts, showed no evidence of antibody-binding to DNA retained in the nucleus or present into the comet tail (Figure 2A). PI staining resembled cells subjected to the CometAssay alone, indicating that processing cells for immunodetection does not significantly interfere with dye uptake. Detection of (6-4)-dipyrimidines with antibody on UV-treated cells showed significant antibody binding to DNA in both nucleus and tail (Figure 2B). The distribution of (6-4)-pyrimidine dimers appears to be concentrated mostly in the head of the comet.

These data indicate that the incorporation of photoproducts in the DNA strand does not nick the DNA by itself, but the alkali treatment of the (6-4)-dipyrimidine photoproducts

can generate some ssDNA breaks as seen by the presence of a comet tail. Also, DNA damage repair involving excision of photoproducts may have begun in the cell samples before cell lysis, because repair of single-strand breaks induced by ionizing radiation is believed to be complete in 1–2 h in mammalian cells (21). Repair has been shown to occur even at cold temperatures in a human B-lymphoblast cell line (1).

The addition of immunodetection to the comet assay allows for type-specific recognition of DNA damage. Cells containing oxidative damage in DNA after H₂O₂ treatment exhibited comet tails after electrophoresis without antibody recognition. The result rules out nonspecific antibody binding to damaged DNA or to DNA that has been processed through the CometAssay. Steady state levels of (6-4)-dipyrimidine photoproducts are below the level of detection by the immunoassay. The presence of the (6-4)-dipyrimidine photoproduct adducts in WEHI 7.1 cells DNA following UV was confirmed by using the anti-UV ssDNA antibody (19). Presence of green fluorescence in the tail of the comet of UV-treated cells suggests that on ice, DNA repair has been initiated through the introduction of nicks adjacent to the adducts. The more intense fluorescence in the head indicates that the nicking involved in the mechanisms of repair of the DNA in the nucleus had recently been initiated, i.e., as repair continues, the amount of DNA and antibody binding in the comet tail is expected to increase. Recently, Sauvaigo et al. (15) followed repair of cyclobutane pyrimidine dimers in UVB-irradiated human fibroblast using the SCGE with alkali electrophoresis conditions and an antibody labeled with Cy3TM fluorescent dye (Amersham Pharmacia Biotech, Piscataway, NJ, USA). They were able to demonstrate the disappearance of 80% of the antibody sites after 48 h, showing the progression of the DNA repair.

CONCLUSION

The CometAssay kit provides a convenient and cost-effective way of performing the comet assay while reducing the risks of losing samples. The detection of DNA damage is the principal use of the kit, but when combined with immunodetection, it becomes a powerful tool for identifying different types of DNA damage and DNA repair occurring in a cell without the use of HPLC or GC/MS. As new antibodies become available against different DNA adducts and their intermediates, the utility of this versatile assay will be expanded.

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Figure 2. Detection of (6-4)-dipyrimidine photoproducts in WEHI 7.1 cells after comet assay. Cells were treated with (A) 100 μ M H₂O₂ or with (B) 1000 J/m² of UV irradiation at 254 nm, before being processed with the CometAssay kit and anti-UV ssDNA antibody. Immunodetection of the (6-4)-dipyrimidine photoproducts was performed using a biotinylated goat, anti-mouse antibody and streptavidin-fluorescein isothiocyanate (FITC). Counterstaining with DNA-binding PI emits the red fluorescence.

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