

## Endo III FLARE™ Assay Kit

Reagent Kit for Analysis of DNA Damage in  
Single Cells Using the Trevigen CometAssay®  
and *E. coli* Endonuclease III

Cat#: 4045-01K-FK  
4045-01K-FM

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## I. Background

Trevigen's **Endo III FLARE™** (Fragment Length Analysis using Repair Enzymes) **Assay Kit** provides the ability to detect DNA damage in single cells using *E. coli* Endonuclease III (Thymine Glycol-DNA Glycosylase) in conjunction with Trevigen's **CometAssay®** single cell gel electrophoresis kits. To assess the type of DNA damage induced by a putative mutagen, drug, or treatment regimen, cells are harvested after treatment and immobilized in low melting point agarose (LMAgarose) on FLARE™ Slides (Cat# 3950-075-02). The cells are gently lysed and then incubated with Endonuclease III to assess the type of damage present. Endonuclease III (Endo III) catalyzes the excision of the following forms of DNA damage:

1. Cis- and trans-thymine glycol
2. 5,6-dihydrothymine
3. 5,6-dihydroxydihydrothymidine
4. urea
5. 5-hydroxy-5-methylhydantoin
6. methyltartronylurea
7. 6-hydroxy-5,6-dihydropyrimidines
8. 5-hydroxycytosine & 5-hydroxyuracil
9. 5-hydroxy-6-hydrothymine
10. 5,6-dihydrouracil
11. alloxan
12. uracil glycol
13. 5-hydroxy-6-hydrouracil
14. AP sites

The slides are then immersed in an alkali solution, to unwind and denature the DNA strands prior to alkali gel electrophoresis. Denatured, cleaved DNA fragments migrate out of the cell under the influence of an electric field, whereas undamaged supercoiled DNA remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern after staining with a fluorescent DNA intercalating dye, or silver staining, allows for assessment of the extent of DNA damage. The type of DNA damage is inferred from the substrate specificity of Endonuclease III listed above.

Qualitative data may be generated if the comets are scored according to categories of small to large comet tail DNA content. Quantitative and statistical data can readily be generated by analysis of the results using one of several commercially available image analysis software packages which calculate tail length, percent DNA in the tail, and tail moment (see Section X, Data Analysis). Trevigen's exclusive FLARE™ Slide, available as a separate order or as part of the FLARE™ Assay Kits (<http://www.trevigen.com/flare.php>), promotes adherence of LMAgarose, eliminating the time consuming and unreliable traditional method of preparing base layers of agarose. These slides shorten assay time and permit the rapid and reliable analysis of large numbers of samples in a standard format.

## II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the products contained within Trevigen's **FLARE™ Assay Kits** may not have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.

3. Lysis Solution contains 1% sodium lauryl sarcosinate which is an irritant and precipitates with long term storage at 4°C. In case of eye or skin contact, wash thoroughly under running water. In case of ingestion, rinse mouth with water and seek medical advice.
4. SYBR® Gold contains DMSO. Please refer to manufacturer.

## III. Materials Supplied

### Module 3950-075-SP

<u>Component</u>	<u>Amount Provided</u>	<u>Storage</u>	<u>Catalog #</u>
Lysis Solution	2 x 500 ml	Room temp.	4250-050-01
Comet LMAgarose	15 ml	4 °C	4250-050-02
FLARE™ Slides	25 each	Room temp.	3950-075-02

### Module 4045-01K-FM

<u>Component</u>	<u>Amount Provided</u>	<u>Storage</u>	<u>Catalog #</u>
<i>E. coli</i> Endonuclease III	1000 units	-20 °C *	4045-01K-01
25X FLARE™ Buffer 1	40 ml	Room temp.	3950-040-01
REC™ Dilution Buffer	10 ml	-20 °C	3950-010-03
100X BSA Additive	100 µl	-20 °C	3950-100-04

\*For optimal stability, freeze at -80 °C in working aliquots

## IV. Additional Materials/Equipment Required but not Supplied

### Equipment:

1. 1-20 µl, 20-200 µl, 200-1000 µl pipettors, and tips
2. Serological pipettor and pipets
3. Boiling water bath and 37 °C water bath
4. CometAssay® Electrophoresis System (cat# 4250-050-ES)\*
5. Epifluorescence microscope equipped with Fluorescein filter or light transmission microscope when using silver staining kit.
6. 1 L graduated cylinder
7. 4 °C refrigerator/cold room
8. Humidity chamber
9. Hydrophobic Coverslips (cat# 4867-100)\*

### Reagents:

1. Deionized water
2. 10X PBS, Ca<sup>2+</sup> and Mg<sup>2+</sup> free (cat# 4870-500)\*
3. NaOH Pellets
4. 0.5M EDTA (pH 8.0)
5. 95% Ethanol (reagent grade)
6. TE buffer (10 mM Tris (pH 7.5), 1 mM EDTA)
7. 10,000X SYBR® Gold in DMSO (see Appendix B: *DNA Stains*)

### Optional Reagents:

1. Silver staining kit (cat# 4254-050-K)\*
2. Dimethylsulfoxide

\*Available from Trevigen

## V. Reagent Preparation

### 1. 1X PBS Ca<sup>2+</sup> and Mg<sup>2+</sup> free

Dilute 10X PBS with deionized water to prepare 1X PBS and store at room temperature. (10X PBS is available from Trevigen, Cat# 4870-500.)

### 2. Lysis Solution

For up to 10 slides (3 samples per slide) prepare:

Lysis Solution (Cat#4250-050-01)	40 ml
DMSO	4 ml (optional)

Cool to 4 °C for at least 20 minutes before use. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. The buffer formulation is proprietary.

### 3. Comet LMAgarose

The Comet LMAgarose is ready to use once molten. Loosen the cap to allow for expansion then heat the bottle in a 90-100 °C water bath for 5 minutes, or until the agarose is molten (Caution: Microwaving is not recommended). Place the bottle in a 37 °C water bath for 20 minutes to cool. The LMAgarose will remain molten at 37 °C for sample preparation indefinitely. The LMAgarose formulation is proprietary.

### 4. Alkali Unwinding and Electrophoresis Solution, pH > 13 (200 mM NaOH; 1 mM EDTA)

Prepare a stock solution of 500 mM EDTA, pH8.  
For 1 liter solution:

NaOH pellets	8 g
500 mM EDTA, pH 8	2 ml
dH <sub>2</sub> O (after NaOH is dissolved) add to:	1 liter

Use of freshly prepared solution is recommended.  
Store 100 ml at room temperature for alkaline unwinding and cool remaining 900 ml to 4 °C for alkaline electrophoresis.

### 5. Endo III Enzyme Dilutions

Upon receipt, prepare aliquots of Endo III (cat# 4045-01K-01) and store at -80°C. The final concentration of Endo III to be used in FLARE must be optimized for your particular cell line and type of exposure to maximize the difference in comet size between drug or mitogen-exposed cells treated with Endo III and those exposed to reaction buffer only. **We recommend starting with undiluted enzyme (please see section VIII C). Typically, 1 µl of enzyme added to 75 µl FLARE™ Reaction Buffer is used per sample area (see #8 below).**

Suggested dilutions for Endo III in REC™ Dilution Buffer (cat#3950-010-03) may include 1:2, 1:5, or 1:10. The stability of diluted forms of Endo III has not been established, therefore, diluted enzyme is

recommended for use immediately or within 1 week. Store the diluted enzyme at -80°C.

### 6. 1X FLARE™ Buffer 1

Prepare the 1X FLARE Buffer 1 (sufficient for 10 slides):

25X FLARE Buffer 1, Cat# 3950-040-01	6 ml
Deionized water	144 ml

### 7. Endo III FLARE™ Reaction Buffer

Prepare the following Endo III FLARE Reaction Buffer (sufficient for 10 samples):

25X FLARE Buffer 1, Cat# 3950-040-01	40 µl
100X BSA Additive, Cat# 3950-100-04	10 µl
Deionized water	to 1 ml

Store at 4 °C for up to 1 week, or freeze at -20 °C for long-term storage.

### 8. Working Endo III Enzyme Solutions

Each sample area on the FLARE™ Slide requires 75 µl of working Endo III Enzyme Solution prepared as follows (for 1 sample area):

Endo III FLARE™ Reaction Buffer	75 µl
Endo III Enzyme (e.g. straight or 1:2, step 5)	1 µl

Place on ice and use immediately.

### 9. SYBR® Gold Staining Solution (see Section IV: *Materials Not Supplied*)

The diluted stock is stable for several weeks when stored at 4 °C in the dark.

10,000X SYBR® Gold in DMSO	1 µl
TE Buffer, pH 7.5	30 ml
(TE: 10 mM Tris-Cl (pH 7.5), 1 mM EDTA)	

Note: Alternative dyes are described in Appendix B: *DNA Stains*.

### 10. Anti-fade Solution (optional)

Prepare if fading of samples occurs. In a 50 ml tube, mix until dissolved:

p-Phenylenediamine dihydrochloride	500 mg
1X PBS	4.5 ml

Add approximately 400 µl of 10 N NaOH dropwise with stirring until pH of solution reaches 7.5-8.0. Add 1X PBS to increase the volume to 5 ml, and 45 ml of glycerol for a final volume of 50 ml. Vortex mixture thoroughly and apply 10 µl per sample, covering samples with coverslip. Nail polish may be used to seal coverslip. Restaining of slides is not recommended. Anti-fade solution is stored at -20°C for one month. Darkening of solution may occur. Alternatively a fluorescent aqueous based mounting medium can be used (for example cat#4866-20).

## VI. Sample Preparation and Cell Storage

Cell samples should be prepared immediately before starting the assay, although success has been obtained using cryopreserved cells (see below). Cell samples should be handled under dimmed or yellow light to prevent DNA damage from ultraviolet light. Buffers should be cooled to 4 °C to inhibit endogenous damage occurring during sample preparation and to inhibit repair in cells. PBS must be calcium and magnesium free to inhibit endonuclease activities. The appropriate controls should also be included (see below). Optimal results in the FLARE™ Assay are usually obtained with 400-800 cells per FLARE™ Slide sample area. Using 40 µl of a cell suspension at  $1 \times 10^5$  cells per ml combined with 400 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 40 µl per sample.

### Suspension Cells

Cell suspensions are harvested by centrifugation. Suspend cells at  $1 \times 10^5$  cells/ml in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free). Media used for cell culture can reduce the adhesion of LMAgarose to the FLARE™ Slide.

### Adherent Cells

Gently detach cells from flask surface. Transfer cells and medium to a centrifuge tube, perform cell count, and then pellet cells. Wash cells once in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free). Suspend cells at  $1 \times 10^5$  cells/ml in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free). If high level of damage is seen in healthy population, reduce cell exposure to Trypsin or try alternative detachment methods such as scraping using a rubber policeman.

Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA) Protocol: Wash the monolayer of cells with sterile PBS, warmed to 37 °C. Add enough Trypsin-EDTA to coat entire monolayer. Incubate flask at 37 °C for 2 minutes or when cells easily detach upon tapping of flask. Add 10 ml of complete media (containing fetal bovine serum) to inactivate trypsin. Transfer cells and medium to centrifuge tube, perform cell count, and pellet cells. Wash once in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free). Suspend at  $1 \times 10^5$  cells/ml in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free).

### Tissue Preparation

Place a small piece of tissue into 1-2 ml of ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free), 20 mM EDTA. Using small dissecting scissors mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet by centrifugation, and suspend at  $1 \times 10^5$  cells/ml in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free).

For blood rich organs (e.g., liver, spleen), chop tissue into large pieces ( $1-2 \text{ mm}^3$ ), let settle for 5 minutes then aspirate and discard medium. Add 1-2 ml of ice cold 20 mM EDTA in 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free), mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet, and suspend at  $1 \times 10^5$  cells/ml in ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free).

## Method for Cryopreservation of Cells Prior to FLARE™ Assay

Certain cells, e.g. lymphocytes, may be successfully cryopreserved prior to performing CometAssay® (Visvardis *et al.*). A pilot study should be performed to determine if cryopreservation is appropriate for the cells in use.

1. Centrifuge cells at 200 x g for 5 minutes.
2. Suspend cell pellet at  $3 \times 10^5$  cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer 50 µl aliquots into freezing vials.
4. Freeze at -70 °C with -1 °C per minute freezing rate overnight.
5. Transfer to liquid nitrogen for long term storage.
6. Recover cells by submerging in 37 °C water bath until the last trace of ice has melted.
7. Add 500 µl ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) to tube.
8. Centrifuge at 200 x g for 10 minutes at 4 °C.
9. Suspend in 100 µl ice cold 1X PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) at  $\sim 1 \times 10^5$  cells/ml and proceed with FLARE™ Assay.

## VII. FLARE™ Assay Protocol

Modifications may be necessary for use of the Endo III FLARE™ Assay Kit with other types of slides. The protocol utilizes alkali electrophoresis conditions, which detects single-stranded DNA breaks, double-stranded DNA breaks, and apurinic and apyrimidinic sites. Prior to performing the FLARE™ Assay, a viability assay should be performed to determine the dose of the test substance or treatment that gives at least 90% viability. False positives may occur when high doses of cytotoxic agents are used.

The FLARE™ Assay requires approximately 3-4 hours to complete. Once the cells or tissues have been prepared, the procedure is not labor intensive. The Lysis Solution may be cooled and the LMAgarose melted while the cell and tissue samples are being prepared. ***When dealing with large number of samples, a convenient stopping point is to perform cell lysis overnight (step 5). In addition, cryopreservation allows experimental samples to be processed concurrently.***

1. Prepare Lysis Solution (see Section V, *Reagent Preparation*) and cool at 4 °C for at least 20 minutes before use:
  - 40 ml Lysis Solution
  - 4 ml DMSO (Optional -use for samples containing heme)
2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened. Place bottle in a 37 °C water bath for at least 20 minutes to cool. The temperature of the agarose is critical as the cells may undergo heat shock.
3. Combine cells at  $1 \times 10^5$ /ml with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipette 40 µl onto FLARE™ Slide. If necessary, use side of pipette tip to spread agarose/cells over sample

area to ensure complete coverage of the sample area. If sample is not spreading evenly warm the slide at 37 °C before application.

When working with many samples aliquot agarose into 37°C warmed tubes, add cells, mix gently by inversion, and spread 40 µl onto sample area.

- ❑ 400 µl Comet LMAgarose (molten and at 37 °C from step 2)
- ❑ 40 µl Cells in 1X PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free)

4. Place FLARE™ Slide flat at 4 °C in the dark (e.g. place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of sample area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.
5. Gently immerse slide in 4 °C Lysis Solution for 60 minutes. For added sensitivity or convenience incubate overnight at 4 °C.
6. Drain excess buffer from slide and immerse in freshly prepared 1X FLARE™ Buffer 1 at room temperature to equilibrate the slide. Change 50 ml 1X FLARE™ Buffer 1 three times over a 30 minute period. Gently tap to remove excess liquid.
  - ❑ 6 ml 25X FLARE™ Buffer 1
  - ❑ 144 ml deionized water
7. Add 75 µl of working Endo III Enzyme Solution (see Section V, item 8) to each sample area. Remember to include a buffer-only control, e.g. For sample area 1 of FLARE™ Slide, apply buffer only; sample areas 2, undiluted Endo III; and sample area 3, 1:2 dilution of Endo III. Alternatively, areas 2 and 3 can be combined as duplicate tests, and hydrophobic coverslips (cat# 4867-100) can be used to distribute the Endo III test dilutions evenly. Carefully place slides horizontally in humidity chamber (Appendix C) and incubate at 37 °C for 30-60 minutes. Allow coverslips to detach by placing slides back into 1X FLARE™ Buffer 1 used in step 6.
8. Transfer FLARE™ Slide to a Coplin jar containing 50 ml room temperature Alkali Solution pH >13 (see step 4, Section V) and incubate for 30 minutes at room temperature in the dark. Change Alkali Solution once. *Do not pour liquid over slides.*
9. For the CometAssay® ES unit, add ~850 ml 4 °C Alkali Solution, place slides in electrophoresis slide tray (slide label adjacent to black cathode) and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 30 minutes. (If not using an ES unit, see Appendix A).
10. Drain excess solution, gently immerse twice in dH<sub>2</sub>O, for 5 minutes each, then in 70% ethanol for 5 minutes. *Do not pour liquid over slides.*

11. Dry slides at 37 °C for 15-30 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature with desiccant prior to scoring at this stage.

**NOTE:** Trevigen offers **CometAssay® Silver Staining Kit** designed for comet staining (Cat# 4254-200-K). Silver staining allows visualization of comets on any transmission light microscope and permanently stains the samples for archiving and long term storage. It is recommended that samples be dried before silver staining.

12. Place 100 µl of diluted SYBR® Gold (see Section V: *Reagent Preparation*) onto each circle of dried agarose and stain 30 minutes (room temperature) in the dark. Gently tap slide to remove excess SYBR solution and rinse briefly in water. Allow slide to dry completely at 37 °C.
13. View slide by epifluorescence microscopy. (SYBR® Gold's maximum excitation/emission is 496 nm/522 nm. Fluorescein filter is adequate).

## VIII. Optimization of Assay Conditions

Under optimal conditions for FLARE™, there will be an increase in the comet tails of cells containing specific damage recognized by the target specific enzyme, compared to cells without enzyme treatment. Control cells that have been processed through the assay but have the enzyme treatment omitted, may still generate comets due to the presence of other types of damage detected directly by the comet assay, e.g. endogenous abasic sites. If the comet tails in the control cells are too large, it can be difficult to detect small changes as a consequence of enzyme treatment.

**For consistent results, the Endo III FLARE™ Kit requires optimization or consideration of the following parameters:**

### A) Degree of drug/agent exposure

The exposure of your particular cell line to the drug/agent under study must be such that comet damage parameters (% DNA in the tail or Tail Moment) in the absence of Endo III are significantly less than that in the cells exposed to Endo III. Too high an exposure will create comets that mask any incremental increase in comet size induced by the action of the enzyme. Conversely, too little exposure may require very high levels of Endo III for an observable effect on tail moment.

### B) Temperature

During the process of DNA repair, abasic sites, nicks, and gaps in the DNA can be introduced that may generate comets in the FLARE™ Assay. Repair is inhibited by cold temperatures, therefore, it is important to keep the cells and solutions used cold (4 °C or on ice) until cell lysis and deproteination are complete. Some repair has been reported even at 4 °C, therefore, cells should not be held at 4 °C for long periods of time before sample preparation. FLARE™ may be used to monitor the repair process in which case the cells would be incubated at physiological temperatures to allow repair, before analysis by FLARE™.

### C) Enzyme titration

We recommend an Endo III titration, using 1 µl of enzyme (undiluted, 1:2, 1:5 and 1:10 dilutions) in 75 µl Endo III FLARE™ Reaction Buffer per sample area, to optimize the differences in damage parameters between untreated and Endo III-treated cells. If necessary, up to 7.5 µl of undiluted enzyme can be applied in 75 µl of Endo III FLARE™ Reaction Buffer.

### D) Incubation times with Endo III

The optimum temperature for Endo III is 37 °C. Vary the incubation time at 37 °C up to 1 hour so as to optimize the differences in damage parameters between untreated and Endo III-treated cells.

### E) Health of your cells

The comet assay is designed for assay of DNA in viable cells and your cells should be at least 90% viable as measured by Trypan Blue exclusion. The comet assay should not be used for detection of DNA damage under conditions of high cytotoxicity when there is massive secondary DNA damage. Note that extensive trypsinization may induce non-specific DNA damage and repair and, therefore, high background in Comet and FLARE™ Assays. When dealing with adherent cells, always use the most gentle trypsinization conditions for your cell type.

## IX. Controls

Controls should be included in each experiment. Two samples of untreated cells, incubated in presence and absence of Endo III enzyme in Endo III FLARE™ Reaction Buffer, should always be processed to control for endogenous levels of damage within cells and damage that may occur during sample preparation. Each FLARE™ Slide has three sample areas providing a convenient format for comparing samples with and without Endo III treatment.

If you require a sample that will be positive for comet tails, treat cells with freshly prepared 10-100 µM hydrogen peroxide or 5-25 µM KMnO<sub>4</sub> for 20 minutes at 4 °C. Hydrogen peroxide or KMnO<sub>4</sub> treatment will generate significant oxidative damage/DNA adducts in the majority of cells. Note that the dimensions and characteristics of the comet tail, as a consequence of hydrogen peroxide or KMnO<sub>4</sub> treatment, may be different from those induced by the damage under investigation.

## X. Data Analysis

When excited (425-500 nm), DNA-bound SYBR® Gold emits green light. In healthy cells, the fluorescence is confined to the nucleoid (comprised of high molecular weight DNA): undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleoid under the influence of an electric current. Whereas in cells that have accrued DNA damage, migrating fragments (comet tail) from the nucleoid (comet head) are observed. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. Common descriptors of DNA damage for alkaline comet assays are Percent DNA in the Tail, and Tail Moment. Percent DNA in the Tail is a normalized measure of the percent of total cell DNA

found in the tail. Tail moment is a damage measure combining the amount of DNA in the tail with distance of migration.

### Qualitative Analysis

The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for a healthy cell. Scoring can then be made according to nominal, medium, or high intensity tail DNA content. At least 50 cells should be scored per sample.

### Quantitative Analysis

There are several image analysis systems that are suitable for quantitation of CometAssay® data. The more sophisticated systems include the microscope, camera and computer analysis package. These systems can be set up to measure the length of DNA migration, image length, nuclear size, and calculate the DNA damage parameters. At least 50 randomly selected cells should be analyzed per sample.

A list of commercially available software packages is available from Trevigen.

## XI. References

1. Nia, A.B., F.J. Van Schooten, P.A. Schilderman, T.M. De Kok, G.R. Haenen, M.H. Van Herwijnen, E. Van Agen, D. Pachen, and J.C. Kleinjans. 2001. A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms. *Carcinogenesis*. **22**:395-401.
2. Lemay, M. and K.A. Wood. 1999. Detection of DNA damage and identification of UV-induced photoproducts using the CometAssay™ kit. *BioTechniques* **27**:846-851.
3. Angelis, K.J., M. Dusinska and A.R. Collins. 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20**:2133-2138.
4. Morris, E.J., J.C. Dreixler, K-Y. Cheng, P.M. Wilson, R.M. Gin and H.M. Geller. 1999. Optimization of single-cell gel electrophoresis (SCGE) for quantitative analysis of neuronal DNA damage. *BioTechniques* **26**:282-289.
5. Malyapa, R.S., C. Bi, E.W. Ahern, and J.L. Roti Roti. 1998. Detection of DNA damage by the alkali comet assay after exposure to low dose gamma radiation. *Radiation Res* **149**:396-400.
6. Henderson, L., A. Wolfreys, J. Fedyk, C. Bourner, S. Windeback. 1998. The ability for the comet assay to discriminate between genotoxins and cytotoxins. *Mutagenesis* **13**:89-94.
7. Visvardis, E.E., A.M. Tassiou, and S.M. Piperakis. 1997. Study of DNA damage induction and repair capacity of fresh cryopreserved lymphocytes exposed to H<sub>2</sub>O<sub>2</sub> and γ-irradiation with the alkaline comet assay. *Mutation Res* **383**:71-80.
8. Fairbairn, D.W., P.L. Olive, K.L. O'Neill. 1995. The comet assay: a comprehensive review. *Mutation Res.* **339**:37-59.
9. Collins, A.R., A.G. Ma, and S.J. Duthie. 1995. The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidine dimers) in human cells. *Mutation Res.* **336**:69-77.

10. Singh, N.P., M.T. McCoy, R.R. Tice, and E.L. Schneider. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175**:184 -191.

11. Östling, O. and K. J. Johanson. 1984. Microelectrophoretic study of radiation-induced DNA damage in individual cells. *Biochem Biophys Res Commun* **123**:291-298.

12. Cosa, G, Focsaneanu, K.-S, McLean, J.R.N., McNamee, J.P., Scaiano, J.C., 2001. Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. *Photochemistry and Photobiology* **73**(6):585-599.

## XII. Related Products Available From Trevigen

Contact Trevigen for details of our unique product line for studying DNA damage and repair. All of Trevigen's kits include highly qualified enzymes, substrates, buffers, full instructions for use, and a synopsis specific for your kit.

### FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples
4040-100-FM			100 samples
4130-100-FK	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples
4130-100-FM			100 samples
4100-100-FK	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples
4100-100-FM			100 samples
4045-01K-FK	Endonuclease III Kit	Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydroxy-mine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydroxy-uracil, uracil glycol, 5-hydroxy-5-methylthio-dantoin, 5-hydroxycytosine, 5-hydroxy-uracil, methyl-taronylurea, thymine ring saturated or fragmentation product	75 samples
4045-01K-FM			100 samples

### CometAssay® Kits:

Catalog #	Description	Size
4250-050-ESK	CometAssay® Starter Kit	each
4250-050-ES	CometAssay® ES	each
4251-050-K	CometAssay® Silver Kit	50 samples
4254-200-K	CometAssay® Silver Staining Kit	200 samples
4252-040-K	CometAssay® Higher Throughput Kit	40 samples
4253-096-K	CometAssay® Kit 96 Wells	96 samples

### PARP Assay Kits:

Catalog #	Description	Size
4520-096-K	HT PARP in vivo Pharmacodynamic Assay II	96 tests
4676-096-K	HT Universal Chemiluminescent PARP Assay	96 tests

Catalog #	Description	Size
4677-096-K	HT Universal Colorimetric PARP Assay	96 tests
4684-096-K	HT Colorimetric PARP/Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP /Apoptosis Assay	96 tests
4690-096-K	HT Homogeneous PARP Inhibition Assay	96 tests

### Accessories:

Catalog #	Description	Size
3950-300-02	FLARE™ Slides	100 slides
3950-075-SP	FLARE™ Sample Prep	>100 samples
4250-050-03	CometSlide™ (2 well)	25 slides
4252-200-01	CometAssay® HT Slide (20 well)	10 slides
4253-960-03	96 Well CometSlide™	10 slides
4256-010-CC	CometAssay® Control Cells (alkaline assay)	1 set
4257-010-NC	Neutral CometAssay® Control Cells	1 set
4380-096-K	HT 8-oxo-dG ELISA Kit II	96 wells
4866-20	Fluorescence Mounting Medium	20 ml
4867-100	Hydrophobic Coverslips 22 x 40 mm	100 each
4870-500	10X PBS, Ca <sup>++</sup> and Mg <sup>++</sup> free	500 ml

## XIII. Appendices

### Appendix A

#### Instructions for alkaline comet assay with other electrophoresis units.

Since the Alkaline Electrophoresis Solution is a non-buffered system, **temperature control is highly recommended**. In-house testing has shown great temperature fluctuations when conducting the alkaline electrophoresis at ambient temperature. To improve temperature control, the use of a large electrophoresis apparatus (20–30 cm between electrodes) is recommended. Performing the electrophoresis at cooler temperatures (*e.g.* 4°C) will diminish background damage, increase sample adherence at high pH and significantly improve reproducibility. Choose the method that is most convenient for your laboratory and always use the same conditions, power supplies and electrophoresis chambers for comparative analysis.

Align slides equidistant from electrodes and carefully add 200 mM NaOH (1 mM EDTA) Alkaline Solution until level just covers samples. Set the voltage to about 1 Volt/cm and perform electrophoresis for 20–40 minutes.

Continue at step 10 on page 7.

### Appendix B

#### DNA Stains

1. Important parameters to consider in choosing a DNA stain for the alkaline comet assay are similar fluorescence and decay rates for single- and double-strand DNA.

**Table 1: DNA Stains Parameters** (Cosa et al.)

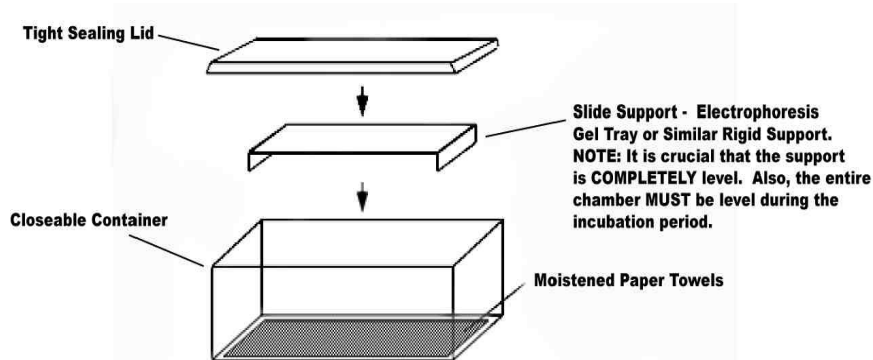
Dye	Abs/Em (nm)	ss:dsDNA fluorescence	ss:dsDNA decay	Signal:Bkgnd
EtBr	520/608	1.0	0.89	~10
DAPI	356/455	0.55	0.85	~20
Propidium Iodide	536/624	0.93	0.93	~20
SYBR Gold	496/540	0.84	0.74	>1000
SYBR Green	496/522	0.57	0.47	>1000
YoYo-1	490/507	0.66	0.73	~400

2. To use SYBR Green instead of SYBR Gold, simply prepare 1:10,000X SYBR® Green I Staining Solution. The diluted stock is stable for several weeks when stored at 4 °C in the dark.

SYBR® Green I (10,000X concentrate in DMSO)                      1 µl  
 TE Buffer, pH 7.5    10 ml  
 (TE: 10 mM Tris-HCl pH 7.5, 1 mM EDTA)

**Appendix C. Humidity Chamber**

To prevent evaporation it is recommended that incubations at 37°C are carried out in a humidity chamber. A humidity chamber can be made using a plastic box with a tight fitting lid and two glass rods or other support. Place paper towel on the bottom of the box and wet thoroughly with water. Lay the glass rods parallel to each other and less than one slide length apart on the wet tissue. Position the slides on the glass rods and place the plastic box, with lid, in a 37 °C incubator. Ensure that the slides are horizontal.



**XIV. Troubleshooting Guide**

PROBLEM	CAUSE	ACTION
Majority of cells in untreated control sample without Endo III treatment have large comet tails.	Unwanted damage to cells occurred in culture or in sample preparations.  Intracellular endonuclease activity.  LMAgarose too hot.  Electrophoresis Solution too hot.	Check morphology of cells to ensure healthy appearance.  Handle cells or tissues gently to avoid physical damage.  Keep cells on ice and prepare cell samples immediately before combining with molten LMAgarose.  Cool LMAgarose to 37 °C in water bath before adding cells.  Control temperature by performing electrophoresis at 4 °C.
Majority of cells in untreated control sample with out Endo III treatment have small to medium comet tails.	Endogenous oxidative damage or endonuclease activity is damaging DNA during sample preparation.	Ensure lysis solution was chilled before use.  Add DMSO to any cell sample that may contain heme groups.  Ensure PBS used is calcium and magnesium free.  Work under dimmed light conditions or under yellow light.
In positive control (e.g. 100 µM hydrogen peroxide for 20 minutes on ice) no evidence of comet tail.	No damage to DNA.  Sample was not processed correctly.	Use freshly prepared reagents to induce damage.  Ensure each step in protocol was performed correctly. Failure to lyse, denature in alkali, or to properly perform electrophoresis may generate poor results.
Comet tails present but not significant in positive control.	Insufficient denaturation in Alkali Solution.  Insufficient electrophoresis time.  Buffer level during electrophoresis too high.	Increase time in Alkali Solution up to one hour.  Increase time of electrophoresis up to 40 minutes. Maintain voltage at 1 volt/cm.  Remove some buffer from electrophoresis chamber. If too much alkali buffer is present, migration will not occur.



PROBLEM	CAUSE	ACTION
Cells in LMAgarose did not remain attached to FLARE™ Slide.	<p>Electrophoresis solution is too hot.</p> <p>Cells were not washed to remove medium before combining with LMAgarose.</p> <p>Agarose percentage was too low.</p> <p>LMAgarose not fully set before samples processed.</p> <p>LMAgarose unevenly set on slide.</p> <p>Rinsing steps too harsh.</p>	<p>Control temperature performing electrophoresis at 4°C.</p> <p>The pH of medium and carry over serum proteins etc. can reduce adherence of the agarose. Suspend cells in 1X PBS.</p> <p>Do not increase ratio of cells to molten agarose by more than 1 to 10.</p> <p>Ensure 0.5 mm dried ring due to agarose disc retraction is seen at edge of sample area.</p> <p>Spread the agarose with the side of a pipette tip to ensure uniformity of agarose disc and better adherence.</p> <p>Gently place slides into solutions. Do not pour solutions over slides.</p>
Majority of cells in <b>untreated</b> samples have large comet tails with obliterated nuclei after exposure to Endo III.	<p>The concentration of Endo III is too high.</p> <p>Incubation time with the DNA repair enzyme is too long.</p> <p>Endogenous DNA damage due to handling conditions.</p>	<p>Titrate down on Endo III to reduce or eliminate the comet in the untreated cells. Altering the enzyme concentration and incubation times is necessary to <b>maximize</b> the difference in comet tail size between treated and untreated cells.</p> <p>Reduce incubation times.</p> <p>Refer to first troubleshooting section.</p>

**-NOTES-**

**The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.**



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