

# TREVIGEN® Instructions

*For Research Use Only. Not For Use In Diagnostic Procedures.*

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## cv-PDG FLARE™ Assay Kit

Reagent Kit for Analysis of DNA  
Damage in Single Cells Using the Trevigen  
CometAssay™ and cv-PDG

**Catalog # 4065-100-FK**

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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# cv-PDG FLARE™ Assay Kit

## Reagent Kit for Analysis of DNA Damage in Single Cells Using the Trevigen CometAssay™ and cv-PDG

Cat# 4065-100-FK

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### I. Introduction

Trevigen's cv-PDG FLARE™ (Fragment Length Analysis using Repair Enzymes) Assay Kit provides the ability to detect DNA damage in single cells using chlorella virus pyrimidine dimer glycosylase (cv-PDG) in conjunction with Trevigen's CometAssay™ single cell gel electrophoresis kit. To assess the type of DNA damage induced by a putative mutagen, drug, or treatment regimen, cells are harvested after treatment and immobilized in a layer of low melting point agarose on the FLARE™ Slide. The cells are gently lysed and then incubated with cv-PDG, which recognizes *cis-syn* and *trans-syn* cyclobutane pyrimidine dimers and AP sites with equal efficiency. cv-PDG also cleaves AP sites on both double and single-stranded DNA.

The slides are then immersed in an alkali solution to unwind the DNA strands and subjected to neutral gel electrophoresis. The 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 nuclear cell membrane. 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 cv-PDG.

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 with image analysis software that calculates tail length and tail moment (see Section X, Data Analysis, page10).

Trevigen's exclusive FLARE Slide, available as a separate order or as part of the FLARE Assay Kit, promotes adherence of low melting point agarose, 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. SYBR® Green I is included in the kit for DNA visualization with improved sensitivity compared to ethidium bromide.

## 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 the FLARE™ Assay Kit 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. 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® Green I contains DMSO. Please refer to Material Safety Data Sheets.

## III. Materials Supplied

### Module 3950-075-SP

<u>Component</u>	<u>Catalog #</u>	<u>Amount</u>	<u>Storage</u>
Lysis Solution	4250-050-01	2 x 500 ml	Room temp.
Comet LMAgarose	4250-050-02	15 ml	Room temp.
FLARE Slides	3950-075-02	25 each*	Room temp.
SYBR® Green I nucleic acid gel stain	4250-050-05	5 µl	-20°C

### Module 4065-100-FM

<u>Component</u>	<u>Catalog #</u>	<u>Amount</u>	<u>Storage</u>
cv-PDG	4065-100-01	1000 units	4°C
25X FLARE Buffer 1	3950-040-01	40 ml	Room temp.
REC™ Dilution Buffer	3950-010-03	10 ml	-20°C
100X BSA Additive	3950-100-04	100 µl	-20°C

\*Additional FLARE Slides (x100), Cat# 3950-300-02, available from Trevigen.

## IV. Additional Materials/Equipment Needed

### 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 (hot plate not recommended)
4. Horizontal electrophoresis apparatus
5. Epifluorescence microscope equipped with Fluorescein filter
6. 1 L beaker
7. 4°C refrigerator
8. Humidity chamber

### Reagents:

1. 10X PBS, Ca<sup>++</sup> and Mg<sup>++</sup> free<sup>1</sup>
2. NaOH pellets
3. Dimethylsulfoxide (optional)
4. Ethanol

<sup>1</sup> Available for purchase from Trevigen.

5. 3 M NaCl
6. 500 mM EDTA, pH 8.0
7. Tris HCl, pH 7.5
8. Glycerol
9. p-Phenylenediamine dihydrochloride
10. Deionized water

## V. Reagent Preparation

Reagents marked with an asterisk (\*) should be prepared immediately before use. Wear gloves, lab coat, and eye protection when handling any chemical reagents.

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

Dilute 10X PBS with deionized water to prepare 1X PBS. Store at room temperature. (10X PBS is available from Trevigen, see Related Products).

### 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)

Chill at 4°C, or on ice, 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.

### 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 10 minutes to cool. The LMAgarose will remain molten at 37°C for sample preparation indefinitely.

### 4. Alkali Solution, pH 12.1

Prepare on day of use. Wear gloves and eye protection when preparing and handling the Alkali Solution. Standardizing pH meter with pH 10.0 standard buffer immediately before making the Alkali Solution is recommended. In a 2 L beaker, add:

500 mM EDTA, pH 8.0	4 ml
3 M NaCl	200 ml
deionized water	1700 ml

Add dropwise with stirring, 5 M NaOH until the pH of the solution reaches 12.1. Add dH<sub>2</sub>O to a final volume of 2 liters. Store 100 ml (if using 1 coplin jar) at room temperature and chill the rest at 4°C.

### 5. cv-PDG Enzyme Dilutions\*

The final concentration of cv-PDG to be used in FLARE must be optimized to maximize the difference in comet size between UV-exposed cells treated with cv-PDG and those exposed to reaction buffer only. Typical final dilutions for

cv-PDG are 1:100, 1:500, and 1:1,000. We recommend making initial dilutions (eg. 1:5, and 1:10) in **REC™ Dilution Buffer**, which is provided in Trevigen's cv-PDG FLARE Kit. Take 1 µl of undiluted enzyme or 1 µl of the initial dilutions and dilute 1:100 in FLARE Reaction Buffer (see step 8 below) for direct application to the FLARE Slide sample areas. The long term stability of diluted forms of cv-PDG has not been established, therefore, immediate use of diluted enzyme is recommended.

#### 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. cv-PDG FLARE™ Reaction Buffer

Prepare the following cv-PDG 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 cv-PDG Enzyme Solutions\*

Each sample area on the FLARE Slide requires 100 µl of working cv-PDG Enzyme Solution prepared as follows (for 1 sample area):

cv-PDG FLARE™ Reaction Buffer (prepared in Step 7)	100 µl
cv-PDG Enzyme (undiluted or diluted) (dilutions prepared in Step 5)	1 µl

Place on ice and use immediately.

#### 9. SYBR® Green I Staining Solution

Prepare SYBR® Green I Staining Solution from the SYBR® Green I nucleic acid gel stain provided (10,000X concentrate in DMSO).

SYBR® Green I nucleic acid gel stain (Cat# 4250-050-05)	1 µl
TE Buffer, pH 7.5 (TE: 10 mM Tris-Cl (pH 7.5), 1 mM EDTA)	10 ml

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

#### 10. Anti-fade Solution

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. Make volume up to 5 ml with 1X PBS, add 45 ml of glycerol, and vortex thoroughly. Apply 10 µl per sample area, apply cover slip and seal with enamel nail polish. Re-staining of slides is not recommended. Store anti-fade solution at -20°C for up to one month. Darkening of solution may occur.

## 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 chilled to 4°C or on ice to inhibit endogenous damage occurring during sample preparation and to inhibit repair in the unfixed 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 500-1000 cells per FLARE Slide sample area. Using 50 µl of a cell suspension at 1 x 10<sup>5</sup> cells per ml combined with 500 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 75 µl per sample.

### Suspension Cells

Cell suspensions are harvested by centrifugation. Resuspend cells at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free). The media used for cell culture can reduce the adhesion of the agarose on the CometSlides.

### Adherent Cells

Trypsinize cells with Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA). First, wash monolayer of cells with sterile PBS, pre-warmed to 37°C. Add enough Trypsin-EDTA to coat entire monolayer of cells. Incubate flask at 37°C for 2 minutes or when cells easily become detached upon tapping of flask. Then add 10 ml of complete media (containing fetal bovine serum) to inactivate trypsin. Transfer cells to centrifuge tube. Perform cell count and then pellet cells. Wash once in ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free). Resuspend at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free).

### Tissue Preparation

Place a small piece of tissue into 1-2 ml of ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> 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 resuspend at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free). For blood rich organs (e.g., liver, spleen), chop tissue into large pieces (1-2 mm<sup>3</sup>), let settle for 5 minutes then aspirate and discard medium. Add 1-2 ml of ice cold 20 mM EDTA in 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> 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 resuspend at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free).

### Method for Cryopreservation of Cells Prior to FLARE™/CometAssay

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. Resuspend cell pellet at  $1 \times 10^7$  cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of  $2 \times 10^6$  cells into freezing vials.
4. Freeze at  $-70^\circ\text{C}$  with  $-1^\circ\text{C}$  per minute freezing rate.
5. Recover cells by submerging in  $37^\circ\text{C}$  water bath until the last trace of ice has melted.
6. Transfer to 15 ml of prechilled 40% (v/v) medium, 10% (w/v) dextrose, 50% (v/v) fetal calf serum.
7. Centrifuge at 200 x g for 10 minutes at  $4^\circ\text{C}$ .
8. Resuspend in ice cold 1X PBS ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free) and proceed with FLARE Assay.

## VII. FLARE Assay Protocol

Modifications may be necessary for use of the cv-PDG FLARE Assay Kit with other types of slides. The protocol utilizes neutral electrophoresis conditions, which detects single-stranded DNA breaks, double-stranded DNA breaks, and apurinic and apyrimidinic sites. Alkali electrophoresis may be performed if higher sensitivity is desired. Prior to performing the FLARE Assay, a cell viability assay should be performed to determine the dose of the test substance or treatment that gives at least 75% viability. False positives may occur when high doses of cytotoxic agents are used.

The FLARE assay requires approximately 3 hours to complete. Once the cells or tissues have been prepared, the procedure is not labor intensive. The Lysis Solution can be chilled and the LMAgarose melted while the cell and tissue samples are being prepared. The FLARE Assay involves the use of hazardous reagents including DMSO, strong alkali, and solutions containing detergents. Please exercise caution when handling any of these reagents. The use of a lab coat, eye protection, and gloves is recommended when preparing for and performing the FLARE Assay.

**Note: All steps are performed at room temperature unless otherwise specified. Work under dimmed or yellow light to prevent damage from UV.**

1. Prepare Lysis Solution (see Section V, Reagent Preparation, p.4) and chill at  $4^\circ\text{C}$  or on ice 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  $37^\circ\text{C}$  water bath for 10 minutes.
3. Refer to Section VI for sample preparation. Combine cells at  $1 \times 10^5$ /ml with molten LMAgarose (at  $37^\circ\text{C}$ ) at a ratio of 1: 10 (v/v) and immediately pipette 75  $\mu\text{l}$  onto FLARE™ Slide. Use side of pipette tip to spread agarose/cells over sample area.

- 500  $\mu\text{l}$  Comet LMAgarose, molten and at  $37^\circ\text{C}$  (Step 2).
- 50  $\mu\text{l}$  cells in PBS ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free)

**Note:** If sample is not spreading evenly on the slide, warm the slide at  $37^\circ\text{C}$  before application.

4. Place FLARE™ Slide flat at  $4^\circ\text{C}$  in the dark (*e.g.* place in refrigerator) for 10 minutes or until 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. Immerse slide in prechilled Lysis Solution and leave on ice or at  $4^\circ\text{C}$  for at least 30 minutes. Overnight lysis at  $4^\circ\text{C}$  is recommended for maximum sensitivity.
6. Tap off excess buffer from slide and immerse in freshly prepared 1X FLARE Buffer 1 at room temperature to equilibrate the slide.
  - 6 ml 25X FLARE Buffer 1
  - 144 ml deionized water

Change the 1X FLARE Buffer 1 three times over a 15 minute period.

7. Add 100  $\mu\text{l}$  of working cv-PDG Enzyme Solution (see Section V, step 8, p. 5) to each sample area. Remember to include a buffer-only control, *e.g.* For sample area 1 of FLARE Slide, apply buffer only; sample area 2, 1:500 dilution of cv-PDG; and sample area 3, 1:1000 dilution of cv-PDG. Carefully place slides horizontally in a humidity chamber and incubate at  $37^\circ\text{C}$  for 30-60 minutes.
8. Transfer FLARE Slide to a Coplin jar containing Alkali Solution at pH 12.1 (see step 4, Section V) and incubate for 30 minutes at room temperature in the dark. Change Alkali Solution once.
9. Transfer slide from Alkali Solution to a horizontal electrophoresis apparatus. Place slides flat onto a gel tray and align equidistant from the electrodes. Carefully pour the Alkaline Solution until level just covers slides. Set the voltage to about 1 Volt/cm. Perform electrophoresis for 20-40 minutes.

### Tips:

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 (25-30 cm between electrodes) is recommended along with recirculation of the electrophoresis solution. Alternatively, performing the electrophoresis at cooler temperatures (*e.g.*  $16^\circ\text{C}$  or  $4^\circ\text{C}$ ) will diminish background damage, ensure sample adherence at high pHs, and significantly improves reproducibility. Choose the method that is most convenient for your laboratory and always use the same conditions, power supply, and electrophoresis chamber for comparative analysis.

10. Gently tap off excess solution, and dip slide in 70% ethanol for 5 minutes.
  11. Air dry samples. 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 (see Appendix B, p. 16).
- NOTE:** Trevigen offers the 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 50 µl of diluted SYBR® Green I (see Section V, Step 9, p.5: Reagent Preparation) onto each circle of dried agarose for 1-5 minutes.
  13. View slide by epifluorescence microscopy. (SYBR® Green I's maximum excitation and emission are respectively 494 nm/521nm. Fluorescein filter is adequate).  
If fading occurs, tap off excess SYBR® Green I and apply 50 µl of anti-fade solution (see Section V, Step 10, p. 5) onto each circle.

## VIII. Optimization of Assay Conditions

Under optimal conditions for cv-PDG FLARE™, there will be an increase in the comet tail moment of cells containing thymine dimers incubated with cv-PDG, compared to cells without cv-PDG treatment. Control cells that have been processed through the assay but have the cv-PDG 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 cv-PDG treatment. Parameters to consider for optimization include:

### A) 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.

### B) Enzyme selection and titration

cv-PDG recognizes *cys-sin* and *trans-syn* thymine dimers and has an associated AP-lyase activity. Other FLARE Kits are available that may be more appropriate for the type of damage under investigation. We recommend that you titrate cv-PDG to optimize the differences in tail moment between untreated and cv-PDG-treated cells. Typical final dilutions of cv-PDG used are 1:100, 1:500, and 1:1000. If neces-

sary, up to 10 µl of undiluted enzyme can be applied in 100 µl of cv-PDG FLARE Reaction Buffer (*i.e.* a final dilution of 1:10).

### C) Incubation times with cv-PDG

Optimum temperature for cv-PDG is 37°C. Vary the incubation time at 37°C up to 1 hour with cv-PDG so as to optimize the differences in tail moment between untreated and cv-PDG-treated cells.

### D) Health of your cells

The comet assay is designed for assay of DNA in viable cells and your cells should be at least 95% 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 with cv-PDG and cv-PDG FLARE Reaction Buffer 1 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 cv-PDG 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), the DNA-bound SYBR® Green emits green light. In healthy cells, the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and thus does not migrate very far from the nucleoid under the influence of an electric current. In cells that have accrued damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the cell when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. When using TBE as the electrophoresis buffer, the length of the comet tail may be used to correlate with DNA damage. When using alkaline electrophoresis conditions, the distribution of DNA between the tail and the head of the comet is used to evaluate the degree of DNA damage. The characteristics of the comet tail including length, width, and DNA content may also be useful in assessing qualitative differences in the type of DNA damage

### 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 75 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 establish the length of DNA migration, image length, nuclear size, and calculate the tail moment. At least 75 randomly selected cells should be analyzed per sample.

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

## XI. Troubleshooting Guide

Problem	Cause	Action
Majority of cells in untreated control sample without cv-PDG treatment have large comet tails.	Unwanted damage to cells occurred in culture or in sample preparations.	Check morphology of cells to ensure healthy appearance.  Handle cells or tissues gently to avoid physical damage.
	Intracellular endonuclease activity.	Keep cells on ice and prepare cell samples immediately before combination with molten LMAgarose.
	LMAgarose too hot.	Cool LMAgarose to 37°C in water bath before adding cells.
Majority of cells in untreated control sample without cv-PDG treatment have small to medium comet tails.	Endogenous oxidative damage or endonuclease activity is damaging DNA during sample preparation.	Ensure lysis solution was thoroughly 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.

## XI. Troubleshooting Guide (continued)

Problem	Cause	Action
In positive control (e.g. 100 µM hydrogen peroxide for 20 minutes on ice) no evidence of comet tail.	No damage to DNA.	Use freshly prepared reagents to induce damage.
	Sample was not processed correctly.	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.	Increase time in Alkali Solution up to one hour.
	Insufficient electrophoresis time.	Increase time of electrophoresis up to 20 minutes. Maintain voltage at 1 volt/cm.
	Buffer level during electrophoresis too high.	Remove some buffer from electrophoresis chamber. If too much alkali buffer is present, migration will not occur.
Cells in LMAgarose did not remain attached to FLARE™ Slide.	Cells were not washed to remove medium before combining with LMAgarose.	The pH of medium and carry over of serum proteins etc. can reduce the adhesion of the agarose. Resuspend cells in 1X PBS.
	Agarose percentage was too low.	Do not increase ratio of cells to molten agarose by more than 1 to 10 e.g. add 50 µl of cell suspension to 500 µl of molten LMAgarose.
	LMAgarose not fully set before samples processed.	Ensure 0.5 mm dried ring due to agarose disc retraction is seen at edge of sample area. Extend gelling time to 30 minutes at 4°C.
	LMAgarose unevenly set on slide.	Spread the agarose with the side of a pipet tip to ensure uniformity of agarose disc and better adherence.
Majority of cells in <b>untreated</b> samples have large comet tails with obliterated nuclei after exposure to cv-PDG.	The concentration of cv-PDG is too high.	Titrate down the cv-PDG to reduce or eliminate the comet in the untreated cells. Altering the enzyme concentration and incubation times are necessary to <b>maximize</b> the difference in comet tail size between treated and untreated cells.
	Incubation time with the DNA repair enzyme is too long.	Reduce incubation times.
	Endogenous DNA damage due to handling conditions.	Refer to first troubleshooting section on previous page.

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## XIII. Related Products Available From Trevigen

The DNA repair enzymes listed below are available to study specific types of damages. cv-PDG, T4 Endo V, Fpg, Endo III, and hOGG1 have been used successfully in combination with the comet assay (see FLARE modules on p.15).

ENZYME (OTHER NAMES)	SUBSTRATE	3' END PRODUCED	DAMAGE ASSAYED
Fpg protein (FaPy-DNA glycosylase)	AP sites, DNA containing formamidopyrimidine moieties, 8 oxoguanine	3' phosphate group	Radiation damage, oxidation damage
Endonuclease III (nth protein, thymine glycol DNA glycosylase, TG DNA glycosylase)	AP sites, thymine glycol, 5,6 dihydrothymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6 hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxy-cytosine, 5-hydroxyuracil, methyltartonylurea thymine ring saturated or fragmentation product, cytosine hydrate	3' $\alpha$ , $\beta$ unsaturated sugar aldehyde	Radiation, oxidation, UV damage
Endonuclease VIII	Modified T and C, $\beta$ -ureidoisobutyric acid	3' $\alpha$ , $\beta$ unsaturated sugar aldehyde	Radiation damage, oxidation damage
Exonuclease III	AP sites	3' OH group	AP sites, oxidizing agents (bleomycin, tertbutyl hydroperoxide), alkylating agents (MMS, mitomycin)
Endonuclease IV (nfo protein)	AP sites, urea	3' OH groups	AP sites, oxidizing agents (bleomycin, tertbutyl hydroperoxide), alkylating agents (MMS, mitomycin)
Uracil-N-glycosylase (Ura-DNA glycosylase, UNGase)	DNA containing uracil	None - AP site formed	Deamination of cytosine
Human DNA Polymerase $\beta$ (Human $\beta$ polymerase)	Double-stranded DNA with recessed 3' OH group	3' OH group	Gaps in DNA
MutY DNA glycosylase	G/A mismatches	3' $\alpha$ , $\beta$ unsaturated sugar aldehyde	G/A mismatches, oxidative damage
TDG enzyme (Thymine mismatch DNA glycosylase)	G/T mismatches	None - AP site formed	G/T mismatches, deamination of 5-methylcytosine
Endonuclease V (dl-3'-endonuclease)	A/C mismatches, deoxyinosine, urea, AP sites, A/A mismatches	3' OH group	Oxidative damage, A/C & A/A mismatch
Aag protein (3-mA-DNA glycosylase II)	3-methyladenine, 7-methylguanaine,	None - AP site formed	Alkylation damage, methylated DNA
UVDE	DNA containing pyrimidine dimers	?	UV damage
T4 endonuclease V (T4-PDG, DEN V)	DNA containing pyrimidine dimers	None - AP site formed	UV damage
AP endonuclease (APE/ref-1)	AP sites, phosphoglycolate ends, urea, phosphate blocked 3' ends	3' OH-group	AP sites, oxidizing agents (bleomycin, tertbutyl hydroperoxide), alkylating agents (MMS, mitomycin)
Mismatch Uracil DNA Glycosylase (Mug)	3, N <sup>4</sup> -ethenocytosine, Uracil in duplex DNA	3' $\alpha$ , $\beta$ unsaturated sugar aldehyde	Uracil in U/G mismatch, 3, N <sup>4</sup> -ethenocytosine in C/G mismatches
Human 7,8-dihydro-8-oxoguanine DNA glycosylase (hOGG1)	DNA containing formamidopyrimidine moieties, 8 oxoguanine	3' phosphate group	Radiation damage, Oxidation damage
Chlorella Virus Pyrimidine Dimer Glycosylase (cv-PDG)	DNA containing pyrimidine dimers	None - AP site formed	UV damage

### XIII. Related Products Available From Trevigen (continued)

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 an enzyme synopsis specific for your kit. The previous table is a listing of enzymes available from Trevigen useful for the study of DNA damage and repair and in the FLARE™ Assay.

Catalog #	Description	Size
4250-050-03	CometSlides™	25 slides
4250-200-03	CometSlides	100 slides
4252-200-03	CometSlide HT (20 sample slide)	10 slides
3950-075-02	FLARE™ Slides	25 slides
3950-300-02	FLARE Slides	100 slides
3950-075-SP	FLARE Sample Prep	>100 samples
4254-200-K	CometAssay™ Silver Staining Kit	200 samples
4870-500-6	10X PBS, pH 7.4	6 X 500 ml

#### DNA Repair Enzyme Kits Complete with CometAssay™ Components

4040-100-FK	Fpg FLARE Kit	>100 samples
4045-100-FK	Endonuclease III FLARE Kit	>100 samples
4100-050-FK	UVDE FLARE Kit	>100 samples
4065-100-FK	cv-PDG FLARE Kit	>100 samples
4055-100-FK	T4-Endo V FLARE Kit	>100 samples
4130-100-FK	hOGG1 FLARE Kit	>100 samples

#### DNA Repair Enzyme Modules for Use with CometAssay™

4040-100-FM	Fpg FLARE Module	>100 samples
4045-100-FM	Endonuclease III FLARE Module	>100 samples
4100-050-FM	UVDE FLARE Module	>100 samples
4065-100-FM	cv-PDG FLARE Module	>100 samples
4055-100-FM	T4-Endo V FLARE Module	>100 samples
4130-100-FM	hOGG1 FLARE Module	>100 samples

#### Antibodies to Damaged DNA

4350-MC-100	Anti-UVssDNA Monoclonal antibody	100 µg
4355-MC-100	Anti-8-oxo-dG Monoclonal antibody (Clone 1F7)	100 µg
4360-MC-100	Anti-BPDE Monoclonal antibody (Clone 8E11)	100 µg
4365-MC-100	Anti-BPDE Monoclonal antibody (Clone 5D11)	100 µg
4411-MC-100	Anti-phosphorylated histone H2AX antibody	100 µl

#### Poly(ADP-ribose) polymerase related products

4335-MC-100	Anti-poly(ADP-ribose), monoclonal	100 µg
4336-BPC-100	Anti-poly(ADP-ribose), polyclonal	100 µl
4338-MC-50	Anti-poly(ADP-ribose) polymerase (PARP)	50 µg
4667-050-K	PARP DNA Damage Assay Kit	50 reactions
4500-050-P	Poly(ADP-ribosylated) protein	50 µl
4667-050-01	Recombinant Human PARP Enzyme	100 U
4667-250-01	Recombinant Human PARP Enzyme	500 U
4667-1K-01	Recombinant Human PARP Enzyme	2000 U
4668-100-01	Recombinant Human PARP Enzyme (HSA)	100 U
4669-96-K	PARP Inhibition Assay	96 samples

### XIV. Appendices

#### Appendix A

##### SYBR® Green I nucleic acid gel stain licensing terms:

This product is sold under license from Molecular Probes, Inc. under US Patents Nos. 5,436,134 and 5,658,751 for use in a comet assay for internal research and development only, where research and development use expressly excludes the use of this product for providing medical, diagnostic or any other testing analysis or screening services or providing clinical information or clinical analysis, in return for compensation on a per-test basis, and research and development use expressly excludes incorporation of this product into another product for commercialization even if such other product would be commercialized for research and/or development use.

#### Appendix B

##### Reagents and Buffer Composition:

##### **Chlorella Virus Pyrimidine Dimer Glycosylase (Cat# 4065-100-01)**

cv-PDG is a DNA glycosylase with an associated AP lyase activity that is equally specific for *cis-syn* and *trans-syn* isomers of cyclobutane pyrimidine dimers and AP sites. Please contact Trevigen for lot specific activity.

##### **25X FLARE Buffer 1 (Cat# 3950-040-01)**

250 mM HEPES-KOH, pH 7.4  
250 mM EDTA  
2.5 M KCl

##### **REC™ Dilution Buffer (Cat# 3950-010-03)**

10 mM HEPES-KOH, pH 7.4  
100 mM KCl  
0.1 mg/ml BSA  
50% glycerol

##### **100X BSA Additive (Cat# 3950-100-04)**

Proprietary stabilizer reagent

##### **Lysis Solution (Cat# 4250-050-01)**

2.5 M sodium chloride  
100 mM EDTA, pH 10  
10 mM Tris base  
1% sodium lauryl sarcosinate  
1% Triton X-100

##### **Comet LMAgarose (Cat# 4250-050-02)**

1% low melting point agarose  
1X PBS

#### Appendix C

##### Storing FLARE™ Slides/CometSlides™:

Slides may be stored for several months in a low humidity atmosphere prior to staining with SYBR® Green I by fixing the samples in 70% ethanol and air-drying:

1. After electrophoresis, immerse CometSlide in 70% ethanol for 5 minutes.
2. Drain excess alcohol and lay CometSlide flat to dry at room temperature.
3. Store at room temperature with desiccant.
4. For scoring, cover sample areas with diluted SYBR® Green I and proceed with viewing.

#### Appendix D

##### Silver Staining of FLARE™ Slides/CometSlides™:

Silver staining offers the opportunity to visualize comets on any transmission light microscope and allows for long term storage. Trevigen recommends the use of our Silver Staining Kit, Cat# 4254-100-K.