TREVIGEN® Instructions

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UVDE FLARE™ Assay Kit

Reagent Kit for Analysis of DNA Damage in Single Cells Using the Trevigen CometAssay™ and *S. pombe UVDE*

Catalog # 4100-100-FK

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I. Background

Trevigen's **UVDE FLARE™** (**F**ragment **L**ength **A**nalysis using **R**epair **E**nzymes) **Assay Kit** provides the ability to detect and quantitate UV-induced DNA damage in single cells using *S. pombe* GST-Δ228-Ultraviolet Damage Endonuclease (UVDE) in conjunction with Trevigen's **CometAssay™** single cell gel electrophoresis kits. To assess the type of DNA damage induced by UV irradiation, 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 UVDE, which repairs bipyrimidine DNA photoproducts, such as cyclobutane pyrimidine dimers and (6-4) photoproducts, and cleaves immediately 5' to the photoproduct site.

The slides are then immersed in an alkali solution, to unwind the DNA strands, and subjected 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 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 UVDE.

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, page 9). 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. SYBR® Green I[1] 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 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. 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.

[1]SYBR Green I is a registered product of Molecular Probes, Eugene OR, and is sold under license from Molecular Probes, Inc. Please see p. 14 for complete licensing terms. Use of this reagent outside the scope of these terms is not endorsed by Trevigen, Inc.

III. Materials Supplied

Module 3950-075-SP

Component	Amount Provided	Storage	Catalog #
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
SYBR® Green I nucleic acid gel stain	5 µl*	-20 °C	4250-050-05

Module 4100-100-FM

Component	Amount Provided	Storage	Catalog #
S. pombe UVDE GST-Δ228	100 units	-80 °C*	4100-100-01
25X FLARE™ Buffer 2	40 ml	Room temp.	3951-040-01
REC™ Dilution Buffer	10 ml	-20 °C	3950-010-03
100X BSA Additive	100 µl	-20 °C	3950-100-04
100X Cation Solution	100 µl	-20 °C	3950-100-05

♦Additional FLARE™ Slides (x100), Cat# 3950-300-02, available from Trevigen.
*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 pipettes
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
9. Hydrophobic Coverslips (cat# 4867-100)

Reagents:

1. 10X PBS, Ca^{2+} and Mg^{2+} free[1]
2. 10N NaOH
3. Dimethylsulfoxide (optional)
4. Ethanol
5. 500 mM EDTA, pH 8.0
6. Tris HCl, pH 7.5
7. Glycerol
8. p-Phenylenediamine dihydrochloride (pH 7.5-8)
9. Deionized water
10. NaOH pellets

[1]Sold separately (Cat# 4870-500-6)

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^{2+} and Mg^{2+} free (sold separately)**
   Dilute 10X PBS with deionized water to prepare 1X PBS. Store at room temperature. (10X PBS is available from Trevigen, Cat# 4870-500-6).

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 > 13 (200 mM NaOH; 1 mM EDTA)**
   Prepare on day of use. Wear gloves and eye protection when preparing and handling the Alkali Solution. Prepare a 0.5 M EDTA stock (pH = 8.0)

0.5 M EDTA	2 ml
NaOH pellets	8 gm
dH_2O	to 1 L

   Adjust the volume prepared based on the electrophoresis apparatus to be used. Use of freshly prepared solution is recommended. Store 50 ml (if using 1 Coplin jar) at room temperature and chill the rest at 4 °C.

5. **UVDE Enzyme Dilutions**
   The final concentration of UVDE 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 UV-exposed cells treated with UVDE and those exposed to reaction buffer only. Typical final dilutions may include 1:100, 1:500, and 1:1,000, depending upon the unit activity of the enzyme. Initial dilutions of UVDE are prepared in **REC™ Dilution Buffer**, which is provided in the UVDE FLARE™ Kit. Take 1 µl of undiluted enzyme or 1 µl of initial dilutions and dilute 1:75 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 UVDE has not been established, therefore, immediate use of diluted enzyme is recommended.

6. **1X FLARE™ Buffer 2**
   Prepare the 1X FLARE Buffer 2 (sufficient for 10 slides):

25X FLARE Buffer 2, Cat# 3951-040-01	6 ml
Deionized water	144 ml

7. **UVDE FLARE™ Reaction Buffer**
   Prepare the following UVDE FLARE Reaction Buffer 2 (sufficient for 10 samples):

25X FLARE Buffer 2, Cat# 3951-040-01	40 µl
100X BSA Additive, Cat# 3950-100-04	10 µl
100X Cation Solution, Cat# 3950-100-05	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 UVDE Enzyme Solution**
   Each sample area on the FLARE™ Slide requires 75 µl of working UVDE Enzyme Solution prepared as follows (for 1 sample area):

UVDE FLARE™ Reaction Buffer (prepared in Step 7)	75 µl
UVDE Enzyme or diluted Enzyme (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
10X 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 10X PBS, add glycerol for a final volume of 50 ml, 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 antifade 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 50-150 cells per FLARE™ Slide sample area. Using 40 µl of a cell suspension at 1 x 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. Resuspend cells at 1 x 10^5 cells/ ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free). Media used for cell culture can reduce the adhesion of the agarose to the CometSlides.

Adherent Cells

Trypsinize cells with Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA). First, wash the monolayer of cells with sterile PBS, pre-warmed to 37 °C. Add enough Trypsin-EDTA to coat entire monolayer. 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^{2+} and Mg^{2+} free). Resuspend at 1 x 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free).

Tissue Preparation

Place a small piece of tissue into 1-2 ml of ice cold 1X PBS (Ca^{2+} and 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 resuspend at 1 x 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free). For blood rich organs (e.g., liver, spleen), chop tissue into large pieces (1-2 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 (Ca^{2+} and 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 resuspend at 1 x 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} 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 x 10^7 cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of 2 x 10^6 cells into freezing vials.
4. Freeze at -70 °C with -1 °C per minute freezing rate.
5. Recover cells by submerging in 37 °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 °C.

8. Resuspend in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free) and proceed with FLARE™ Assay.

VII. FLARE™ Assay Protocol

Modifications may be necessary for use of the UVDE 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 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 3-4 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) and chill at 4 °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 °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. Refer to Section VI for sample preparation. Combine cells at 1 x 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. Use side of pipette tip to spread agarose/cells over sample area.

   - 400 µl Comet LMAgarose, molten and at 37 °C (Step 2).
   - 40 µl cells in PBS (Ca^{2+} and Mg^{2+} free)

   Note: If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.

4. Place FLARE™ Slide flat at 4 °C in the dark (e.g. place in refrigerator) for 15 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 °C for 60 minutes. Lysis times can range from 30 minutes to overnight lat 4 °C depending on cell type.

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 2
   - 144 ml deionized water

   Change the 1X FLARE™ Buffer 2 three times over a 30 minute period. Tap off gently to remove excess liquid.

7. Add 100 µl of working UVDE 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 area 2, 1:75 dilution of UVDE; and sample area 3, 1:450 dilution of UVDE. Alternatively, areas 2 and 3 can be combined as duplicate tests, and hydrophobic coverslips (cat# 4867-100) can be used to distribute the UVDE test dilutions evenly. Carefully place slides horizontally in a humidity chamber and incubate at 37 °C for 30-60 minutes. Allow coverslips to detach by placing slides back into 1X FLARE™ Buffer 2 used in step 6.

8. Transfer FLARE™ Slide to a Coplin jar containing Alkali Solution at pH >13 (see Section V, item 4) and incubate for 20 minutes at room temperature in the dark. Change Alkali Solution once.

9. Transfer slide from Alkali Solution to a horizontal electrophoresis apparatus.

10. Place slides flat onto a gel tray and align equidistant from the electrodes.

11. Carefully pour the chilled Alkaline Solution until level just covers slides. We recommend adding the slides to the chamber with the running solution present, if possible. 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 °C or 4 °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.

12. Gently tap off excess solution, immerse 2 times in dH_2O, for 10 minutes each, and immerse in 70% ethanol for 5 minutes.

13. Dry slides at 45 °C for ~10 minutes or 37 °C for ~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 (see Appendix B).

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

14. Place 100 µl of diluted SYBR® Green I (see Section V, item 9) onto each circle of dried agarose and place in refrigerator for 15-30 minutes. Allow slide to dry completely at room temperature in the dark.

15. View slide by epifluorescence microscopy. (SYBR® Green I's maximum excitation and emission are respectively 494 nm/521nm. A fluorescein filter is adequate). If fading occurs, tap off excess SYBR® Green I and apply 50 µl of anti-fade solution (see Section V, item 10) onto each circle.

VIII. Optimization of Assay Conditions

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

A) Degree of UV exposure

The dose and wavelength for your particular cell line must be such that the tail moment of the comets in the cells in the absence of UVDE are significantly less than that in the cells exposed to UVDE. 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 UV exposure may require very high levels of UVDE 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 that you titrate UVDE, using 1:75, 1:450 and 1:900 dilutions of enzyme per sample area, to optimize the differences in tail moment between untreated and UVDE-treated cells. If necessary, up to 10 µl of undiluted enzyme can be applied in 100 µl of UVDE FLARE™ Buffer.

D) Incubation times with UVDE
The optimum temperature for UVDE is **30 °C**. Vary the incubation time at 30 °C up to 1 hour with UVDE so as to optimize the differences in tail moment between untreated and UVDE-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 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.

F) Other Activities of UVDE
In addition to UV-induced DNA damage, UVDE recognizes base mispairs and may function in a general excision repair pathway. These other activities of UVDE should be considered in your analysis.

IX. Controls

Controls should be included in each experiment. Two samples of untreated cells incubated with UVDE and UVDE FLARE™ Reaction Buffer should always be processed to control for endogenous levels of damage within cells and damage that may occur during sample preparation. Cells should be kept in low level yellow light during processing. Each FLARE™ Slide has three sample areas providing a convenient format for comparing samples with and without UVDE 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 $KMnO_4$ for 20 minutes at 4 °C. Hydrogen peroxide or $KMnO_4$ 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 $KMnO_4$ treatment, may be different from those induced by the damage under investigation.

X. Data Analysis

When excited (425--500 nm), 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, 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 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.

NIH Image, free software for image analysis, is available at http://rsb.info.nih.gov/nih-image. A useful macro for calculating the tail moment from digital images is provided by Professor Herbert M. Geller, Ph.D. and can be obtained at http://www2.umdnj.edu/~geller/lab/comet.htm.

XI. Troubleshooting Guide

Problem	Cause	Action
Majority of cells in untreated control sample without UVDE 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
	Electrophoresis solution is too hot	Control temperature by recirculating the electrophoresis solution or performing the assay in a cold room
Majority of cells in untreated control sample without UVDE 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.

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 40 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.	Electrophoresis solution is too hot	Control temperature by recirculating the electrophoresis solution or performing the assay in a cold room
	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 pipette tip to ensure uniformity of agarose disc and better adherence
Majority of cells in **untreated** samples have large comet tails with obliterated nuclei after exposure to UVDE	The concentration of UVDE is too high	Titrate down the UVDE to reduce or eliminate the comet in the untreated cells. Altering the enzyme concentration and incubation times are necessary to **maximize** 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

XII. References

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XIII. 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 **4040-100-FM**	**Fpg**	**8-oxoguanine, DNA containing formamidopyrimidine moieties**	**75 samples** **100 samples**
4045-010-FK **4045-010-FM**	**Endo III**	**Thymine glycole, 5,6-dihydrothymine, urea, 5-hydrozy-6-hydrothymine, 5,6-dihydrouracil, 5-hydrozyuracil, 5-hydroxycytosine**	**75 samples** **100 samples**

Catalog #	Description	Damage Recognized	Size
4050-100-FK 4050-100-FM	Endo IV	AP sites	75 samples 100 samples
4065-100-FK 4065-100-FM	cv-PDG	Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	75 samples 100 samples
4025-100-FK 4025-100-FM	UNGase	Uracil	75 samples 100 samples
4130-100-FK 4130-100-FM	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4055-100-FK 4055-100-FM	T4-PDG Kit	Cis-syn isomers of cyclo-butane pyrimidine dimers	75 samples 100 samples

CometAssay™ Kits:

Catalog #	Description	Size
4250-050-K	CometAssay™	50 samples
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

Control Cells:

Catalog #	Description	Size
4256-010-CC	CometAssay™ Control Cells	10 assays

PARP Assay Kits:

Catalog #	Description	Size
4677-096-K	HT Universal Colorimetric PARP Assay w/ Histone Coated Strip Wells	96 samples
4676-096-K	Universal Chemiluminescent PARP Assay w/Histone Coated Strip Wells	96 samples
4667-250-01	Recombinant Human PARP Enzyme	250 µl
4668-100-1	Recombinant Human PARP (High Specific Activity)	1000 Units

DNA Damage Antibodies:

Catalog #	Description	Size
4410-PC-100	Fen-1	100 µl
4411-PC-100	γ-H2AX	100 µl
4350-MC-100	UVssDNA	100 µg
4431-MC-100	XPF	100 µg
4421-MC-100	XRCC1	100 µg
4354-MC-50	anti-8-oxo-dG monoclonal	50 µl

Accessories:

Catalog #	Description	Size
4867-100	Hydrophobic Coverslips 22 x 40 mm	100 each
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
3950-300-02	FLARE™ Slides	100 slides
4040-100-FM	Fpg FLARE™ Module	>100 samples
4130-100-FM	hOGG1 FLARE™ Module	>100 samples
4045-100-FM	Endonuclease III FLARE™ Module	>100 samples
4055-100-FM	T4-PDG FLARE™ Module	>100 samples
4065-100-FM	cv-PDG FLARE™ Module	>100 samples
4100-050-FM	UVDE FLARE™ Module	>100 samples
3950-075-SP	FLARE™ Sample Prep	>100 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:

***S. pombe* GST-Δ228 UVDE (Cat# 4100-050-01)**
Please contact Trevigen for lot specific data.

25X FLARE™ Buffer 2 (Cat# 3951-040-01)
250 mM HEPES-KOH, pH 6.5
2.5 M NaCl

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 Cation Solution (Cat# 3950-500-05)
1.0 M $MgCl_2$
100 mM $MnCl_2$

100X BSA Additive (Cat# 3950-100-04)
Proprietary stabilizer reagent

Comet LMAgarose (Cat# 4250-050-02)
1% low melting point agarose
1X PBS

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

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 drying:

1. After electrophoresis, immerse slides 2X in dH_2O for 10 minutes each and 70% ethanol for 5 minutes.
2. Drain excess alcohol and lay slide flat to dry at 45 ^{o}C for ~10 minutes.
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.

Appendix E. 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.