

# **TREVIGEN<sup>®</sup> Instructions**

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

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## **FLARE<sup>™</sup> Sample Prep**

**Reagents for FLARE<sup>™</sup> slide preparation**

**Catalog #: 3950-075-SP**

# **FLARE™ Sample Prep**

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**Cat#: 3950-075-SP**

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## I. Background

Trevigen's **FLARE™**(Fragment Length Analysis using Repair Enzymes) **Assay Kits** provide the ability to detect specific DNA damage in single cells using DNA repair enzymes 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 a DNA specific repair enzyme that catalyzes the removal of moieties of interest to assess the amount of damage present. 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 the added DNA repair enzyme.

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. 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™ Sample Prep** 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

## 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. FLARE™ Assay Module (XII. Related Products)
2. Deionized water
3. 10X PBS, Ca<sup>2+</sup> and Mg<sup>2+</sup> free (cat# 4870-500)\*
4. NaOH Pellets
5. 0.5M EDTA (pH 8.0)
6. 95% Ethanol (reagent grade)
7. TE buffer (10 mM Tris (pH 7.5), 1 mM EDTA)
8. 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

**FLARE™ Sample Prep does not describe enzyme preparations. Please refer to specific FLARE™ Assay Kit or Module instructions for use.**

**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. 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*.

## 6. 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of a cell suspension at  $1 \times 10^5$  cells per ml combined with 400  $\mu$ l of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 40  $\mu$ l 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 (Ca<sup>2+</sup> and Mg<sup>2+</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 suspend at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>2+</sup> and Mg<sup>2+</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>2+</sup> and Mg<sup>2+</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 suspend at 1 x 10<sup>5</sup> cells/ml in ice cold 1X PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> 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 x 10<sup>5</sup> 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 (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) to tube.
8. Centrifuge at 200 x g for 10 minutes at 4 °C.
9. Suspend in 100 µl ice cold 1X PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) at ~1 x 10<sup>5</sup> cells/ml and proceed with FLARE™ Assay.

## **VII. FLARE™ Assay Protocol**

**FLARE™ Sample Prep provides a general enzyme protocol. Please refer to specific FLARE™ Assay Kit or Module instructions for use.**

Modifications may be necessary for use of the FLARE™ Assay 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  $\mu$ l 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  $\mu$ l onto sample area.

- 400  $\mu$ l Comet LMAgarose (molten and at 37 °C from step 2)
  - 40  $\mu$ l 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 50 ml freshly prepared 1X FLARE™ Buffer (enzyme specific) at room temperature to equilibrate the slide. Change 1X FLARE™ Buffer three times over a 30 minute period. Gently tap to remove excess liquid.
  7. Add 75  $\mu$ l of working Enzyme Solution 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 enzyme; and sample area 3, dilution of enzyme. Alternatively, areas 2 and 3 can be combined as duplicate tests, and hydrophobic coverslips (cat# 4867-100) can be used to distribute the enzyme 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 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 FLARE™ Assay 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 enzyme are significantly less than that in the cells exposed to enzyme. 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 enzyme 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 enzyme titration, using 1 µl of enzyme (undiluted or diluted) in 75 µl FLARE™ Reaction Buffer (enzyme specific) per sample area, to optimize the differences in damage parameters between untreated and enzyme-treated cells. If necessary, up to 7.5 µl of undiluted enzyme can be applied in 75 µl of FLARE™ Reaction Buffer.

## **D) Incubation times with Enzyme**

The optimum enzyme temperature 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 enzyme-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 enzyme in FLARE™ Reaction Buffer (enzyme specific), 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 enzyme 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

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## 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-dihydrothymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhy-dantoin, 5-hydroxycytosine,5-hydroxy-uracil, methyl-tartonylurea, 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

Catalog #	Description	Size
4251-050-K	CometAssay <sup>®</sup> Silver Kit	50 samples
4254-200-K	CometAssay <sup>®</sup> Silver Staining Kit	200 samples
4252-040-K	CometAssay <sup>®</sup> Higher Throughput Kit	40 samples
4253-096-K	CometAssay <sup>®</sup> 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
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 <sup>™</sup> Slides	100 slides
3950-075-SP	FLARE <sup>™</sup> Sample Prep	>100 samples
4250-050-03	CometSlide <sup>™</sup> (2 well)	25 slides
4252-200-01	CometAssay <sup>®</sup> HT Slide (20 well)	10 slides
4253-960-03	96 Well CometSlide <sup>™</sup>	10 slides
4256-010-CC	CometAssay <sup>®</sup> Control Cells (alkaline assay)	1 set
4257-010-NC	Neutral CometAssay <sup>®</sup> 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.*)

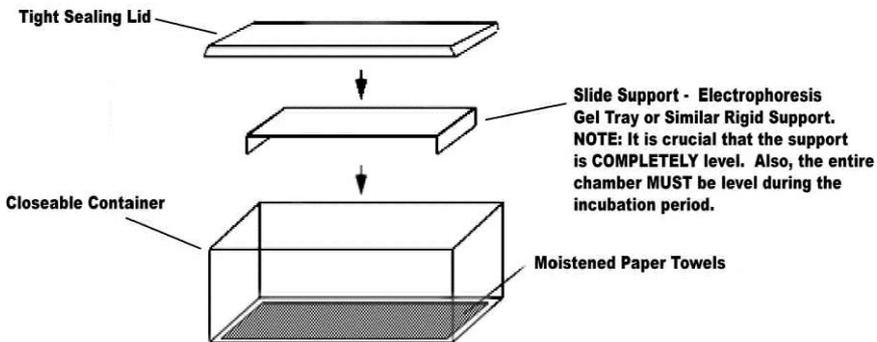
Dye	Abs/Em (nm)	ss:dsDNA fluorescence	ss:dsDNA decay	Signal:Bkgrd
EtBr	520/608	1.0	0.89	~10
DAPI	356/455	0.55	0.85	~20
Propidium Iodide	536/624	0.93	0.93	~20
SYBR Gold	496/540	0.84	0.74	>1000
SYBR Green	496/522	0.57	0.47	>1000
YoYo-1	490/507	0.66	0.73	~400

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

PROBLEM	CAUSE	ACTION
<p>Cells in LMAgarose did not remain attached to FLARE™ Slide.</p>	<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>
<p>Majority of cells in <b>untreated</b> samples have large comet tails with obliterated nuclei after exposure to enzyme.</p>	<p>The concentration of enzyme 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 enzyme 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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