CometAssay® Silver Kit

Reagents for CometAssay and Staining with Silver

Catalog Number 4251-050-K

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The CometAssay®, or single cell gel electrophoresis assay, provides a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage. The Neutral CometAssay is typically used to detect double-stranded breaks, whereas the Alkaline CometAssay is more sensitive, and is used to detect smaller amounts of damage including single and double-stranded breaks.

The CometAssay uses the CometSlide™ that is specially treated to promote adherence of low melting point agarose. This eliminates the time consuming and unreliable traditional method of preparing base layers of agarose. The use of the CometSlide shortens assay time and allows the rapid and reliable analysis of large numbers of samples. The CometAssay Silver Kit provides all the reagents for silver staining of the processed CometSlide allowing visualization by standard light microscopy and providing permanent staining for sample archiving.

In comet assay, cells are immobilized in a bed of low melting point agarose, on a CometSlide. Following gentle cell lysis, and for the Alkaline CometAssay, samples are treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. For both assays, cells are lysed and the remaining nucleoids are subjected to electrophoresis and subsequent staining with a fluorescent DNA intercalating dye and/or silver stain.

CometAssay Alkaline Control Cells (R&D Systems, Catalog # 4256-010-CC) are recommended when performing alkaline electrophoresis, and CometAssay Neutral Control Cells (R&D Systems, Catalog # 4257-010-NC) are commended when performing the neutral comet assay, to monitor assay conditions and verify reproducibility between separate runs. SYBR® Gold for DNA visualization and quantitation by epifluorescence microscopy is recommended. Silver staining can replace or follow fluorescent analysis.

It is also recommended to use the CometAssay Electrophoresis System II (R&D Systems, Catalog # 4250-050-ES) which is designed to eliminate known causes of assay variability. The electrophoresis step is performed using an Alkaline Electrophoresis Solution pH>13, for the alkaline version, whereas a Neutral Electrophoresis Buffer is recommended for the neutral version. Quantitative and statistical data can readily be generated by fluorescence analysis of the results using CometAssay Analysis Software (R&D Systems, Catalog # 4260-000-CS) to calculate tail length, percent DNA in the tail, and tail moment.

The CometAssay Silver Kit may be coupled with the FLARE™ (Fragment Length Analysis using Repair Enzymes) Assay, providing the added ability to probe for specific types of DNA damage using DNA repair glycosylases.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between each sample and reagent additions and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The physical, chemical, and toxicological properties of the products contained within the CometAssay Silver Kit may not have been fully investigated. Therefore, the use of gloves, lab coats, and eye protection is recommended while using any of these chemical reagents.

Lysis Solution contains 1% sodium lauryl sarcosinate which is an irritant and precipitates with long term storage at 2-8 °C.

The Silver Staining reagents contains small quantities of hazardous materials:
2.8% Formaldehyde is found in 20X Staining Reagent #2 (R&D Systems, Catalog # 4254-200-02) and 10% tungstosilicic acid is found in 20X Staining Reagent #3 (R&D Systems, Catalog # 4254-200-03). Consult the SDS sheets for details.

The final Staining Solution is considered hazardous material. Disposal should be performed per local and state regulations. It is recommended to tap solution off the slide into a container for safe disposal.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SYBR Gold contains DMSO. Refer to manufacturer website.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

COMPONENTS	PART	PART #	AMOUNT PROVIDED	STORAGE OF OPENED MATERIAL
CometAssay	CometAssay LMAgarose	4250-050-02	15 mL	Store at 2-8 °C
	CometAssay Lysis Solution	4250-050-01	2 x 500 mL	Room temperature
	CometSlide	4250-050-03	25 slides	
	200 mM EDTA, pH 10	4250-050-04	12.5 mL	
Silver Staining	20X Staining Reagent #1	4254-200-01	1.2 mL	
	20X Staining Reagent #2	4254-200-02	1.2 mL	
	20X Staining Reagent #3	4254-200-03	1.2 mL	
	2X Staining Reagent #4	4254-200-04	1.2 g	
	10X Fixation Additive	4254-200-05	2.2 mL	

OTHER MATERIALS REQUIRED

Reagents:

- Distilled water
- 10X PBS (Ca^{2+} and Mg^{2+} free R&D Systems, Catalog # 4870-500)
- 70% and 95% Ethanol Buffer (10 mM Tris (pH 7.5), 1 mM EDTA)

Equipment:

- Pipettor and pipette tips
- Boiling water bath and 37 °C water bath
- CometAssay Electrophoresis System II (R&D Systems, Catalog #4250-050-ES)
- Epifluorescence microscope equipped with Fluorescein filter or light transmission microscope when using silver staining components
- 1 L graduated cylinder
- 2-8 °C refrigerator
- Staining Jars for 3" x 2" slides

For Alkaline Assays:

- NaOH Pellets
- 500 M EDTA (pH 8.0)
- CometAssay Alkaline Control Cells (R&D Systems, Catalog # 4256-010-CC)

For Neutral Assays:

- Tris Base (mot. wt. = 121.14)
- Ammonium Acetate and hydrous (mot wt.=77.08)
- Sodium Acetate Trihydrate (mot. wt. = 136.08)
- Glacial Acetic Acid
- CometAssay Neutral Control Cells (R&D Systems , Catalog # 4257-010-NC)

For Silver Staining:

- Methanol
- Glacial Acetic Acid

Optional reagent:

- 10,000X SYBR® Gold in DMSO
- Dimethylsulfoxide (DMSO)
- TE Buffer, pH 7.5
- Trypsin - EDTA 0.25% Trypsin, 1 mM EDTA

REAGENT PREPARATION

1X PBS - Dilute 10X PBS with deionized water to prepare 1X PBS and store at room temperature.

Lysis Solution - Cool to 2-8 °C for at least 20 minutes before use. Lay slide on flat and cover samples area with the Lysis Solution. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. For up to 10 slides (2 samples per slide) prepare:

Reaction Component	Volume
CometAssay Lysis Solution	40 mL
DMSO (optional)	4 mL

CometAssay LMAgarose - The CometAssay 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 at least 20 minutes to cool. The CometAssay LMAgarose will remain molten at 37 °C for sample preparation indefinitely.

For Alkaline CometAssay

Alkaline Unwinding Solution, pH>13 (200 mM NaOH, 1 mM EDTA) - Wear gloves when preparing and handling the Alkaline Unwinding Solution. Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use. Per 50 mL of Alkaline Unwinding Solution combine:

Reaction Component	Volume
NaOH Pellets	0.4 g
200 mM EDTA, pH 10	250 µL
Distilled water	49.75 mL

Alkaline Electrophoresis Solution pH >13 (200 mM NaOH, 1 mM EDTA) for the CometAssay Electrophoresis System II - Prepare a stock solution of 500 mM EDTA, pH 8. Use of freshly made solution is recommended. Cool to 2-8 °C.

Reaction Component	Volume
NaOH Pellets	8.0 g
500 mM EDTA	250 µL
Distilled water (after NaOH is dissolved)	1 liter

For Neutral CometAssay

1X Neutral Electrophoresis Buffer - Dissolve in 450 mL of distilled water. Adjust to pH 9.0 with Glacial Acetic Acid. Adjust volume to 500 mL and filter sterilize and store at room temperature. Dilute the 10X stock to 1X in dH2O to prepare 1 liter working strength buffer and cool to 2-8 °C. To prepare 10X Neutral Electrophoresis Buffer:

Reaction Component	Volume
Tris Base	60.57 g
Sodium Acetate	204.12 g

DNA Precipitation Solution - Prepare a 10 mL stock solution of 7.5 M Ammonium Acetate.

Reaction Component	Volume
NH_4Ac	5.78 g
Distilled water (after NH4Ac is dissolved)	10 mL

For 50 mL of DNA precipitation solution combine:

Reaction Component	Volume
7.5 M NH_4Ac	6.7 mL
95% Ethanol	43.3 mL

Fluorescent Staining (optional)

SYBR Gold Staining Solution - The diluted stock is stable for several weeks stored at 2-8 °C **in the dark.**

Reaction Component	Volume
10,000 SYBR® Gold in DMSO	1.0 µL
TE Buffer, pH 7.5	30 mL

Silver Staining

Fixation Solution - Prepare immediately before fixation (100 µL per sample). Mix per sample:

Reaction Component	Volume
10X Fixation Additive	10 µL
Distilled water	30 µL
Methanol	50 µL
Glacial Acetic Acid	10 µL

2X Staining Reagent #4 - Before first use, add 12 mL of distilled water to bottle, stir until dissolved and store at 2-8 °C. Stable for 3 months after suspension. Before each use, warm to room temperature.

Staining Solution - Prepare the Staining Solution (100 µL/sample) immediately before staining following the instructions below. After adding the reagents, mix by tapping the tube.

Component	1 sample	10 samples
Distilled water	35 µL	350 µL
20X Staining Reagent #1	5 µL	50 µL
20X Staining Reagent #2	5 µL	50 µL
20X Staining Reagent #3	5 µL	50 µL
2X Staining Reagent #4	50 µL	500 µL

Stop Solution - Prepare a 5% Glacial Acetic Acid solution. 100 µL per sample area is required.

SAMPLE PREPARATION

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 2-8 °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 CometAssay Silver Kit are usually obtained with 500-1000 cells per CometSlide sample area. Using 50 µL of a cell suspension at 1 x 10^5 cells/mL combined with 500 µL of CometAssay LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 50 µL per sample.

Suspension Cells - Cell suspensions are harvested by centrifugation. Suspend cells at 1 x 10^5 cells/mL in ice cold 1X PBS. Media used for cell culture can reduce the adhesion of CometAssay LMAgarose to the CometSlide.

Adherent Cells - Gently detach cells from flask surface using Trypsin-EDTA by first washing the monolayer of cells with sterile PBS, warmed to 37 °C. Add enough Trypsin-EDTA (0.25% Trypsin, 1 mM 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. Suspend at 1 x 10^5 cells/mL in ice cold 1X PBS.

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.

Tissue Preparation - Place a small piece of tissue into 1-2 mL of ice cold 1X PBS with 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 10^5 cells/mL in ice cold 1X PBS.

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, 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 10^5 cells/mL in ice cold 1X PBS.

Controls - A sample of untreated cells should always be processed to control for assay variability, endogenous levels of damage within cells, and for additional damage that may occur during sample preparation. Control cells and treated cells should be handled in an identical manner. If UV damage is being studied; the cells should be kept in low level yellow light during processing. Two sets of suspension cell preparations are offered containing different levels of DNA damage to standardize methods between individual users, different runs and laboratories. For alkaline electrophoresis, use the CometAssay Alkaline Control Cells (R&D Systems, Catalog # 4256-010-CC) and for neutral electrophoresis, use the CometAssay Neutral Control Cells (R&D Systems, Catalog # 4257-010-NC).

Note, optional: *To generate samples positive for comet tails, treat cells with 100 µM hydrogen peroxide or 25 µM $KMnO_4$ for 20 minutes at 2-8 °C. Treatment will generate significant oxidative damage in most cells, thereby providing a positive control for each step in the alkaline comet assay.*

METHOD FOR CRYOPRESERVATION OF CELLS PRIOR TO COMETASSAY

Certain cells (*e.g.* lymphocytes) may be successfully cryopreserved prior to performing CometAssay (1). 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 10^5 cells/mL in 10% (v/v) DMSO, 40% (v/v) medium, 50% (v/v) fetal bovine 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 to tube.
8. Centrifuge at 200 x g for 10 minutes at 2-8 °C.
9. Suspend in 100 µL ice cold 1X PBS at ~1x10^5 cells/mL and proceed with CometAssay.

ASSAY PROTOCOL

The electrophoresis conditions used will determine the sensitivity of the assay. Neutral CometAssay will detect double-stranded DNA breaks, whereas Alkaline CometAssay will detect single and double-stranded DNA breaks, and the majority of abasic sites as well as alkali-labile DNA adducts (*e.g.* phosphoglycols, phosphotriesters). The comet assay has been reported to detect DNA damage associated with low doses (0.6 cGy) of gamma irradiation, providing a simple technique for quantitation of low levels of DNA damage. Prior to performing the comet assay, a viability assay should be performed to determine the dose of the test substance that gives at least 90% viability. False positives may occur when high doses of cytotoxic agents are used.

The Alkaline CometAssay requires approximately 2-3 hours to complete, whereas the Neutral CometAssay requires 4 hours, including the incubations and electrophoresis. Once the cells or tissues have been prepared the procedure is not labor intensive. The Lysis Solution may be cooled and the CometAssay LMAgarose melted while the cell and tissue samples are being prepared.

Note: *When dealing with large number of samples, a convenient stopping point is to perform cell lysis overnight. In addition, cryopreservation allows experimental samples to be processed concurrently.*

ALKALINE COMETASSAY

1. Prepare Lysis Solution and cool at 2-8 °C for at least 20 minutes before use.
2. Melt CometAssay 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 or the cells may undergo heat shock.
3. Combine cells at 1 x 10^5/mL with molten CometAssay LMAgarose (at 37 °C) at a ratio of 1: 10 (v/v) and immediately pipette 50 µL onto CometSlide. 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 on the slide, 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 50 µL onto sample area

Comet LMAgarose (molten and at 37 °C from Step 2)	500 µL
Cells in 1X PBS at 1 x 10^5/mL	50 µL

4. Place slide flat at 2-8 °C **in the dark** (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.
5. Immerse slide in 2-8 °C Lysis Solution for 30- 60 minutes. For added sensitivity or convenience incubate overnight at 2-8 °C.

6. Drain excess buffer from slides and immerse in freshly prepared Alkaline Unwinding Solution, pH>13.
   Note: *Wear gloves when preparing or handling this solution.*

7. Immerse CometSlide in Alkaline Unwinding Solution for 20 minutes at room temperature or 1 hour at 2-8 °C, **in the dark**.

8. For the CometAssay Electrophoresis System II, add ~850 mL 2-8 °C Alkaline Electrophoresis 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.

9. Drain excess electrophoresis solution from slides and gently immerse twice in distilled water for 5 minutes each, then in 70% ethanol for 5 minutes. Do not pour liquid over slides.

10. Dry samples at 37 °C for 10-15 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.

11. Proceed to Fluorescent Staining section (optional) before silver staining or directly to Silver Staining section.

NEUTRAL COMETASSAY

1. Prepare Lysis Solution and cool at 2-8 °C for at least 20 minutes before use.

2. Melt CometAssay LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened, and then cool in a 37 °C water bath for at least 20 minutes.

3. Combine cells at 1 x 10^5/mL with molten CometAssay LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipette 50 µL onto CometSlide. Use side of pipette tip to spread agarose/cells over sample area

Comet LMAgarose (molten and at 37 °C from Step 2)	500 µL
Cells in 1X PBS at 1 x 10^5/mL	50 µL

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

4. Place slides flat at 2-8 °C **in the dark** (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.

5. Immerse slides in 2-8 °C (Step 1) Lysis Solution for 1 hour or overnight for added sensitivity.

6. Remove slides from Lysis Buffer, drain excess buffer from slide and gently immerse in 50 mL of 2-8 °C 1X Neutral Electrophoresis Buffer for 30 minutes.

7. For the CometAssay Electrophoresis System II, add ~850 mL 2-8 °C 1X Neutral Electrophoresis Buffer, 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 1 hour at 2-8 °C.

   For other electrophoresis units, align slides equidistant from electrodes, add 1X Neutral Electrophoresis Buffer not to exceed 0.5 cm above slides, and apply voltage at 1 volt/cm (measured electrode to electrode).

8. Drain excess Neutral Electrophoresis Buffer and immerse slides in DNA Precipitation Solution for 30 minutes at room temperature.

9. Immerse slides in 70% Ethanol for 30 minutes at room temperature.

10. Dry samples at 37 °C for 10-15 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.

11. Proceed to Fluorescent Staining (optional) before Silver staining or directly to Silver Staining.

FLUORESCENT STAINING (optional)

1. Place 100 µL of SYBR Gold Staining Solution 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 slides to dry completely at 37 °C.

2. View slides by epifluorescence microscopy. (SYBR Gold's maximum excitation/emission is 496 nm/540 nm. Fluorescein filter is adequate).

3. Proceed to Silver Staining section.

SILVER STAINING

1. Cover the sample area with 100 µL of Fixation Solution.

2. Incubate for 20 minutes at room temperature.

3. Rinse in distilled water for 30 minutes. Removal of all residual acetic acid is essential.

4. Cover sample area with 100 µL of Staining Solution.

5. Incubate at room temperature for 5-20 minutes. (Intensity of staining can be visualized under the microscope using 10X objective, and reaction stopped when comets are easily visible.)

6. Stop reaction by covering samples with 100 µL of Stop Solution and incubate for 15 minutes.

7. Rinse in distilled water.

8. Air dry and store **in the dark.**

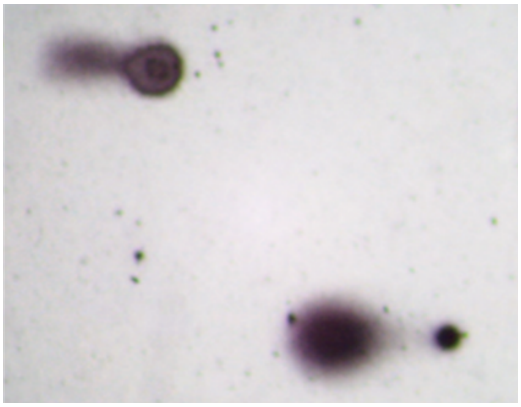
DATA ANALYSIS

Silver Staining of DNA generates a brown to black stain easily detectable by microscopy. In healthy cells, the stain 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. In neutral comet assays, Tail Moment is primarily used, since tail length continues to increase in contrast to alkaline comet tails which have finite lengths.

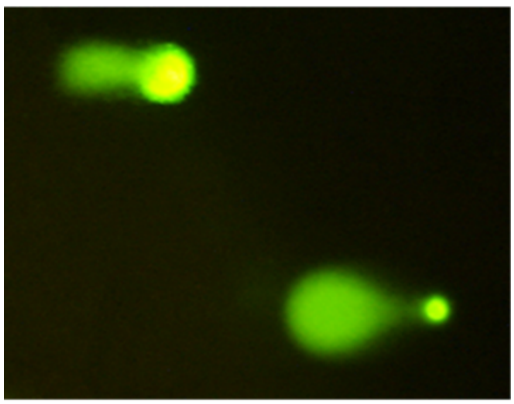
Qualitative Analysis (Alkaline CometAssay) - The comet tail can be scored per DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for a healthy cell. Scoring can then be made per nominal, medium or high intensity tail DNA content. At least 50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

Quantitative Analysis (Alkaline and Neutral CometAssay) - The CometAssay Analysis Software (R&D Systems, Catalog # 4260-000-CS) provides the computational power to efficiently analyze digital images presenting fields of cells run in the CometAssay. The Software applies state of the art image processing methods to automatically locate scorable cells, and generate powerful analytic measures on each, to characterize and quantify the degree of DNA damage revealed by the CometAssay. The Software can rapidly evaluate large numbers of cells, related to each treatment group or screening target in a study, and generate summary statistics based on the corresponding numeric results. The Software is specially engineered to complement and operate as the computational component of the unique standardized CometAssay System, which offers an array of products designed to facilitate sensitive, easy and consistent performance of the comet assay.

A: Silver Stain

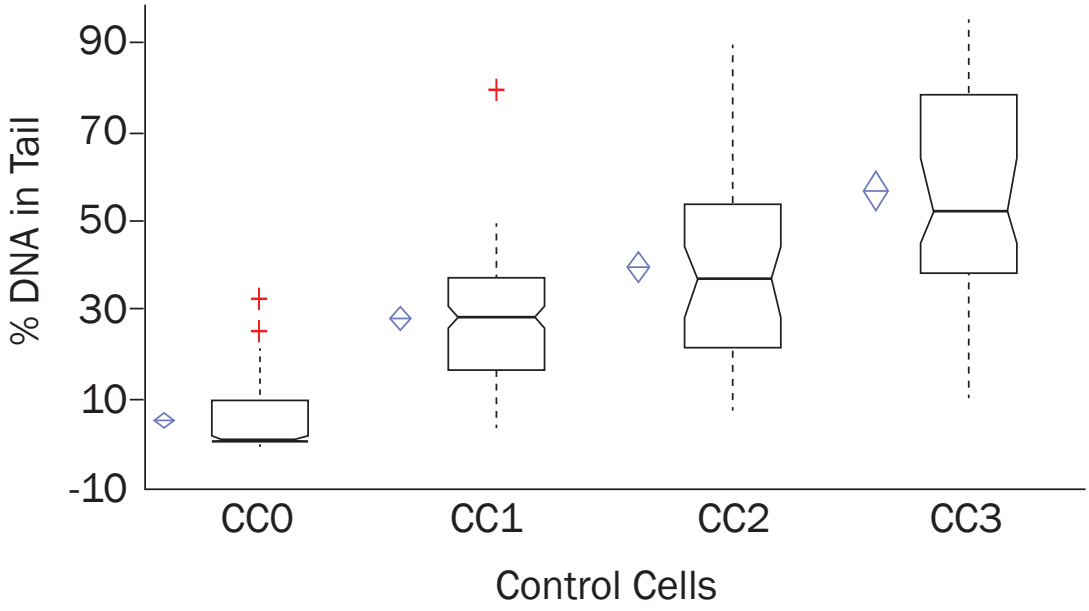


B: Fluorescent stain



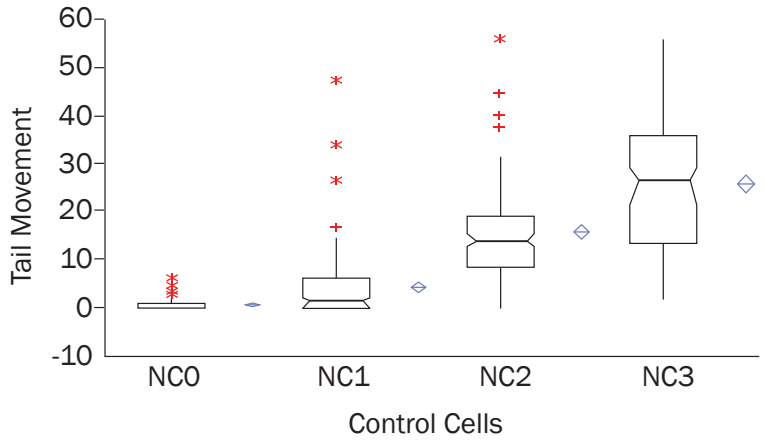
Examples of Silver and Fluorescent Stained Comets. Potassium permanganate-treated WEHI 7.1 cells were processed using the CometAssay Kit. Comets were visualized using (Figure A) Silver Stain or (Figure B) Fluorescent (SYBR) Stain.

Quantitative data is shown as side-by side vertical box plots for comparison. The diamond shows the mean and confidence interval around the mean. The notched box shows the median, lower and upper quartiles, and the 75% confidence interval around the median. Examples are provided for both the Alkaline and Neutral CometAssay protocols.



% DNA BY ETOPOSIDE	N	MEAN	SD	SE	75% CI OF MEAN	MEDIAN	IQR	75% CI OF MEDIAN
CC0	50	5.757	7.2720	1.0928	4.485-7.029	1.640	8.925	1.290-2.230
CC1	50	28.374	14.0080	1.9810	26.068-30.680	28.990	20.313	25.180-31.840
CC2	50	39.736	21.8164	3.0853	36.144-43.328	37.050	32.183	27.790-44.630
CC3	50	56.800	23.5896	3.3360	52.916-60.683	51.905	40.240	45.460-64.390

Box-Whisker Plots of Comet Tail Analysis of Alkaline Control Cells. A) Data collected for each CometAssay Alkaline Control Cell population (R&D Systems, Catalog # 4256-010-CC) is shown as side-by side vertical box plots for comparison.



TM By Bleomycin	N	MEAN	SD	SE	75% CI OF MEAN	MEDIAN	IQR	75% CI OF MEDIAN
NC0	75	0.677	1.2410	0.1433	0.511-0.843	0.000	0.637	0.000-0.140
NC1	75	4.316	7.7817	0.8986	3.274-5.358	1.360	5.748	0.240-2.510
NC2	75	15.711	10.7829	1.2451	14.268-17.155	13.600	10.117	12.830-14.950
NC3	75	25.730	13.7918	1.5925	23.884-27.577	26.780	22.750	20.810-28.930

Box-Whisker Plots of Comet Tail Analysis of Neutral Control Cells. A) Data collected for each CometAssay Neutral Control Cell population (R&D Systems, Catalog # 4257-010-NC) is shown as side-by side vertical box plots for comparison.

APPENDICES

APPENDIX A

Neutral CometAssay

The CometAssay may be performed using neutral conditions that employ 1X TBE. Without treatment with Alkaline Buffer, this Neutral CometAssay will also detect mainly double-stranded breaks.

1. Prepare Lysis Solution and cool to 2-8 °C for at least 20 minutes before use.
2. Melt CometAssay LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened, and then cool in a 37 °C water bath for at least 20 minutes.
3. Combine cells at 1 x 10^5/mL with molten CometAssay LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipette 50 µL onto CometSlide. Use side of pipette tip to spread agarose/cells over sample area.

Comet LMAgarose (molten and at 37 °C from Step 2)	500 µL
Cells in 1X PBS at 1 x 10^5/mL	50 µL

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

4. Place slides flat at 2-8 °C *in the dark* (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.

5. Immerse slides in 2-8 °C Lysis Solution for 1 hour or overnight for added sensitivity.

6. Remove slides from Lysis Buffer, drain excess buffer from slide and wash slide by immersing in 50 mL of 2-8 °C 1X TBE buffer for 15 minutes.

   a. To prepare 10X TBE, dissolve in 900 mL distilled water:

Reaction Component	Volume
Tris Base	108 g
Boric Acid	55 g
EDTA	9.3 g

   b. Adjust volume to 1 liter and filter sterilize, and store at room temperature. Dilute the 10X TBE to 1X in distilled water to prepare 1 liter working strength buffer and cool to 2-8 °C.

7. For the CometAssay Electrophoresis System II, add 2-8 °C ~850 mL 2-8 °C 1X TBE Buffer, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 40 minutes.
   Note: *For other electrophoresis units, align slides equidistant from electrodes, add 1X TBE Buffer not to exceed 0.5 cm above slides, and apply voltage at 1 volt per cm (measured electrode to electrode).*

8. Drain excess TBE, immerse slides in distilled water for 5 minutes.

9. Immerse slides in 70% Ethanol for 5 minutes.

10. Dry samples at 37 °C for 10-15 minutes. Drying brings all the cells into a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.

11. Proceed to Fluorescent Staining (optional) before silver staining or directly to Silver Staining section.

APPENDIX B

Instructions for Alkaline CometAssay 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.* 2-8 °C) will diminish background damage, increase sample adherence at high pH and significantly improves reproducibility. Choose the method that is most convenient for your laboratory and always use the same conditions, CometAssay Alkaline Control Cells (R&D Systems, Catalog # 4256-010-CC), power supplies and electrophoresis chambers for comparative analysis.

Alternative Reagents:

Alkaline Unwinding Solution, pH>13 (300 mM NaOH, 1 mM EDTA) - Wear gloves when preparing and handling the Alkaline Unwinding Solution. Per 50 mL of Alkaline Solution combine:

Reaction Component	Volume
NaOH Pellets	0.6 g
500 mM EDTA	250 µL
distilled water	49.75 mL

Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

Alkaline Electrophoresis Solution pH >13 (300 mM NaOH, 1 mM EDTA) for other electrophoresis systems: - Prepare a stock solution of 500 mM EDTA, pH 8. For 1 liter of electrophoresis solution:

Reaction Component	Volume
NaOH Pellets	12 g
200 mM EDTA	2 mL
distilled water	1 Liter

1. Adjust the volume prepared based on the dimensions of your electrophoresis apparatus. Use of freshly made solution is recommended. Cool to 2-8 °C.
2. Align slides equidistant from electrodes and carefully add the Alkaline Solution until level just covers samples. Set the voltage to about 1 Volt/cm. Add or remove buffer until the current is approximately 300 mA and perform electrophoresis for 20-40 minutes.
3. Proceed to Step 9 on of the Alkaline CometAssay Protocol.

APPENDIX C

DNA Stains

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.

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

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 2-8 °C **in the dark.**

Reaction Component	Volume
SYBR Green I (10,000X concentrate in DMSO)	1 µL
TE Buffer, pH 7.5	10 mL

TROUBLESHOOTING

PROBLEM	CAUSE	SOLUTION
Unexpected and/or variety of tail shape.	CometAssay LMAgarose too hot.	Cool CometAssay LMAgarose to 37 °C before adding cells.
Cells in CometAssay LMAgarose did not remain attached to the CometSlide.	Electrophoresis solution too hot.	Control temperature performing electrophoresis at 2-8 °C.
	Cells were not washed to remove medium before combining with CometAssay LMAgarose.	The pH of medium and carry over serum proteins, etc., can reduce the adherence of the agarose. Suspend cells in 1X PBS.
	Agarose percentage was too low.	Do not increase ratio of cells to molten agarose by more than 1-10.
	CometAssay LMAgarose was not fully set before samples were processed.	Ensure 0.5 mm dried ring due to agarose disc retraction is seen at the edge of the CometSlide area.
	CometAssay LMAgarose unevenly set on the slide.	Spread the agarose with the side of a pipette tip to ensure uniformity of agarose disc and better adherence.
	Rinsing steps to harsh.	Gently place slides into solutions. Do not pour solutions over slides

Specific to Alkaline CometAssay

PROBLEM	CAUSE	SOLUTION
Most cells in untreated control sample 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.
	Electrophoresis solution too hot.	Control temperature by performing electrophoresis at 2-8 °C.
	Intracellular activity.	Keep cells on ice and prepare cell samples immediately before combining with molten CometAssay LMAgarose.
Most cells in untreated control sample have small to medium comet tails.	Endogenous oxidative damage or endonuclease activity after sample preparation is damaging DNA.	Ensure Lysis Solution was chilled before use.
		Add DMSO to any cell sample that may contain heme groups.
		Ensure PBS used is calcium and magnesium free.
		Work under dimmed light conditions or under yellow light.
In positive control (*e.g.* 100 µM hydrogen peroxide for 30 minutes on ice) no evidence of comet tail.	No damage to DNA.	Use fresh hydrogen peroxide to induce damage.
	Sample was not processed correctly	Ensure each protocol step 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 Alkaline Solution.	Increase time in Alkaline Solution up to 1 hour.
	Insufficient electrophoresis time.	Increase time of electrophoresis up to 1 hour for alkaline electrophoresis. Increase time of electrophoresis when running at cold temperatures.

Specific to Neutral CometAssay

PROBLEM	CAUSE	SOLUTION
In positive control, no evidence of comet tail.	Damaging agent doesn't cause double-strand breaks.	Confirm damage by Alkaline Comet.
		Run Neutral Control Cells to confirm electrophoresis conditions.
		Increase treatment with damaging agent.
In positive control, comet tails are too long and do not fit analysis window.	Cells are necrotic or apoptotic.	Verify 90% viability.
	Electrophoresis time too long.	Decrease treatment with damaging agent.
		Decrease electrophoresis time to 15-30 minutes.

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