DATA SHEET

Neutral CometAssay®
Control Cells

For Single Cell Gel Electrophoresis Assay

Catalog # 4257-010-NC

Sufficient materials for 10 assays.

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I. QUICK REFERENCE PROCEDURE (Assay Protocol)

The Assay Protocol described below is written as a Quick Reference Protocol for using Neutral Comet Control Cells (cat#4257-010-NC). Reagents and detailed instructions including reagent preparation are provided with Trevigen's CometAssay® Kits (Please See Section XI).

This page is designed to be photocopied and used as a checklist:

- ☐ 1. Chill Lysis Solution at 4°C for at least 20 minutes before use.
- ☐ 2. Melt LMAgarose and cool in a 37°C water bath for at least 20 minutes.
- ☐ 3. Combine 50 µl of NC0 (control cells) with 500 µl molten LMAgarose (at 37°C) and immediately spread 50 µl per well over a 2 well, or 30 µl per well for a 20 well CometSlide™.
- ☐ 4. Repeat step 3 for samples NC1, NC2, and NC3, respectively.
- ☐ 5. Place slides flat at 4°C for 10 minutes.
- ☐ 6. Immerse slides in prechilled Lysis Solution at 4°C, for 60 minutes.
- ☐ 7. Immerse slides in 50 ml of prechilled 1X Neutral Electrophoresis Buffer for 30 minutes at 4°C.
- ☐ 8. For CometAssay® ES tank, add 950 ml prechilled 1X Neutral Electrophoresis Buffer, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21V and apply voltage for 1 hour. For other horizontal electrophoresis units, set the voltage to 1 Volt/cm (~130 mA).

  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 from electrode to electrode).
- ☐ 9. Immerse slides in DNA Precipitation Solution for 30 minutes at room temperature followed by 30 minutes in 70% EtOH.
- ☐ 10. Dry samples at ≤ 45°C for 10-15 minutes.
- ☐ 11. Place 100 µl (2 well slide) or 50 µl (20 well slide) of diluted SYBR® Green I onto each sample for 30 minutes. Remove excess SYBR solution. Allow slide to dry completely at room temperature in the dark.
- ☐ 12. View slide by epifluorescence microscopy. (SYBR® Green I[1] has excitation and emission wavelengths of 425 nm and 521 nm, respectively. A fluorescein filter is adequate.)

[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.9 for complete licensing terms. Use of this reagent outside of the scope of these terms is not endorsed by Trevigen, Inc.

II. Background

Trevigen's CometAssay®, or single cell gel electrophoresis assay, provides a simple and effective method for evaluating DNA damage in cells. The principle of the neutral comet assay is based upon the ability of 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.

In this assay, cells are immobilized in a bed of low melting point agarose, on a Trevigen CometSlide®. Following gentle cell lysis, samples are neutralized, submitted to neutral electrophoresis and stained with a fluorescent DNA intercalating dye. The sample is then visualized by epifluorescence microscopy. 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 has developed a set of suspension cell preparations containing different levels of DNA damage to be used as controls with Trevigen's CometAssay® Kits. When performing the neutral CometAssay®, the four control cell populations show incremental increases in tail moment. The healthy control cell population (NC0) was treated with Bleomycin under various conditions to increase the amount of damage in populations NC1, NC2 and NC3, respectively. Bleomycin complexed with metal ions induces double-stranded and single-stranded DNA breaks by a mechanism involving free radicals. These cryo-preserved controls are designed to act as controls to standardize and compare comet assay results under neutral electrophoresis conditions between individual users and laboratories.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the CometAssay® Control Cells 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.

IV. Materials Supplied

Neutral CometAssay® Control Cells should be stored in liquid nitrogen.

Components	Description	Cap Color	Catalog #	Amount
NC0	Healthy Cells	Pink	4257-010-NC0	500 µl
NC1	Treated Cells	Orange	4257-010-NC1	500 µl
NC2	Treated Cells	Blue	4257-010-NC2	500 µl
NC3	Treated Cells	Violet	4257-010-NC3	500 µl
Data Sheet	Lot specific			

V. Materials/Equipment Required But Not Supplied

Equipment:

1. 20–200 µl, 200–1,000 µl pipettors, and tips
2. Table Top Centrifuge (vertical rotor)
3. Water Bath
4. -80°C Freezer
5. Liquid Nitrogen Storage System
6. CometAssay® ES (cat# 4250-050-ES) or other horizontal electrophoresis unit.
7. Power Supply

Reagents:

1. Ice cold 1X PBS pH 7.4, Ca^{++} and Mg^{++} free
2. Isopropanol
3. CometAssay® Kit[1] (required)
4. 10X Neutral Electrophoresis Buffer
5. Ammonium Acetate
6. Sodium Acetate
7. Glacial Acetic Acid

[1] Available from Trevigen; refer to Section XI for ordering information.

VI. Preparation of Control Cells

Control cells should be prepared immediately before starting the CometAssay® protocol.

Storage

Neutral CometAssay® Control Cells are stored using a Liquid Nitrogen Storage System. To avoid the accumulation of damage due to freeze thaw, the control cells should be aliquotted and cryopreserved as described below.

1. Recover cells by submerging in 37°C water bath to quickly thaw cells, and place on ice.
2. Gently invert to mix and transfer 50 µl aliquots into freezing vials.
3. Freeze at −80°C with −1°C per minute freezing rate. This can be done by placing the vials in a Styrofoam box containing room temperature Isopropanol and placing in a -80°C freezer overnight. Vials are placed in a plastic box or rack then placed in room temperature isopropanol. The lid of the Styrofoam container is put in place then the box is placed in the -80°C freezer.
4. Transfer to Liquid Nitrogen System for long-term storage.

Assay Preparation Protocol:

1. Remove 50 µl aliquots of NC0, NC1, NC2 and NC3 Neutral CometAssay® Control Cells from Liquid Nitrogen Storage.
2. Quickly thaw cells by submerging in 37°C water bath, and add 600 µl of ice cold 1X PBS (Ca^{++} and Mg^{++} free).

3. Centrifuge cells at 150 x g for 5 minutes and gently remove supernatant, except for about 50 µl.
   NOTES: 1) A cell pellet will not be visible after centrifugation.
   2) Removing supernatant completely will result in cell loss.

4. Gently resuspend cell pellet in 50 µl of ice cold 1X PBS.

5. Immediately use the cells in the CometAssay® protocol described for Neutral Comet.

VII. Assay Protocol

The assay protocol is the same as listed in the checklist on page 1. Additional reagents are required. For information regarding preparation of all needed reagents, please see the instructions for use for Trevigen's CometAssay® (cat# 4250-050-K).

VIII. Data Interpretation

When excited (425–521 nm) the DNA-bound SYBR® Green I 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 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.

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 DNA damage parameters. At least 50 randomly selected cells should be analyzed per sample.

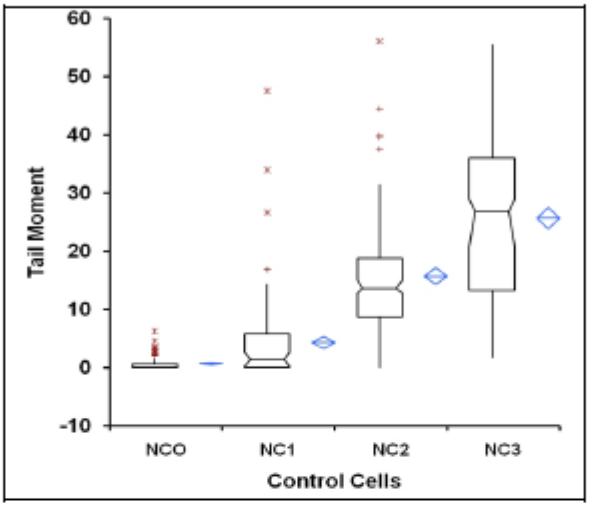
A list of commercially available software package is available from Trevigen.

Featured Data:

To evaluate the degree of damage, the Neutral Control Cells were processed using the CometAssay® System under defined electrophoresis conditions. In the example below, neutral electrophoresis was performed on twenty-well slides (4252-040-01) using CometAssay® HT Kit (4252-040-K). Images were captured and analyzed using Loats Associates, Inc Comet Analysis System. Data was exported into Analyze-it™ (www.analyse-it.com) for Microsoft Excel® to graphically represent the spread of data.

In Figure 1a, data collected from 75 cells for each Neutral Control Cell population are 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. For each lot of Control Cells, population values are provided in a data sheet. An example is provided below.

Figure 1a: Box-Whisker plot of Neutral Control Cells: Tail Moment



TM by Bleomycin	n	Mean	SD	SE	75% CI	Median	IQR	75% CI
NCO	75	0.677	1.2410	0.1433	0.511 to 0.843	0.000	0.637	0.000 to 0.140
NC1	75	4.316	7.7817	0.8986	3.274 to 5.358	1.360	5.748	0.240 to 2.510
NC2	75	15.711	10.7829	1.2451	14.268 to 17.155	13.600	10.117	12.830 to 14.950
NC3	75	25.730	13.7918	1.5925	23.884 to 27.577	26.780	22.750	20.810 to 28.930

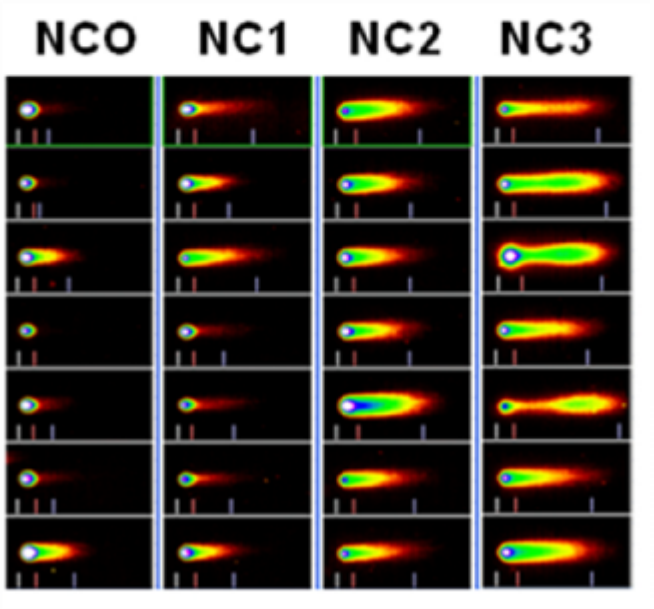


Figure 1b: Examples of comet tail shapes for each population

In Table 2, a one-way Analysis of Variance (ANOVA) was used to test for a difference between means of the treated control cells (NC1, NC2 and NC3). A $p<0.0001$ was obtained indicating a significant difference. In addition, contrasts between the three treated control cells indicated that they belonged to different populations.

Table 2: One-way ANOVA and contrasts between Neutral Control Cells

n	225				
TM by Bleomycin	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
NC1	75	4.316	0.8986	1.2772	7.782
NC2	75	15.711	1.2451	1.2772	10.783
NC3	75	25.730	1.5925	1.2772	13.792

Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Bleomycin	17219.649	2	8609.825	70.37	<0.0001
Residual	27160.985	222	122.347		
Total	44380.634	224			

Tukey Contrast	**Difference**	**95% CI**	
NC1 v NC2	-11.395	-15.657 to -7.133	(significant)
NC1 v NC3	-21.414	-25.676 to -17.152	(significant)
NC2 v NC3	-10.019	-14.281 to -5.757	(significant)

IX. Troubleshooting Guide

PROBLEM	CAUSE	ACTION
No visible Control Cells.	Loss of cells when pipetting off the supernatant.	Recommend using a vertical rotor when pelleting cells. Gently remove supernatant except for 50 µl to avoid cell loss.
Majority of cells in Untreated Control Cells have large comet tails.	Unwanted damage to cells occurred during preparation of Control Cells	Handle cells gently to avoid physical damage. Avoid excessive mixing with pipet. Be sure that no more than 200x g is used.
	Intracellular activity	Keep cells on ice and prepare Control Cells immediately before combining with molten LMAgarose. Ensure Lysis Solution was chilled before use. Ensure PBS used is calcium and magnesium free.

PROBLEM	CAUSE	ACTION
Majority of cells in Untreated Control Cells have large comet tails (cont.).	Performed electrophoresis too long	Shorten electrophoresis time.
	LMAgarose too hot	Cool LMAgarose to 37°C before adding cells.
Treated Control Cells show no evidence of comet tail or present but not significant.	Insufficient electrophoresis time.	Increase time of electrophoresis.
Comet tails present but too long to fit into analysis software window.	Excessive electrophoresis time.	Reduce time of electrophoresis.
Cells in LMAgarose did not remain attached to the CometSlide™.	Cells were not washed to remove medium before combining with LMAgarose.	The pH of medium and carry over serum proteins, etc., can reduce the adherence 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.
	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.
	LMAgarose unevenly solidified on the slide.	Spread the agarose with the side of a pipette tip to ensure uniformity of agarose disc and better adherence.
	LMAgarose was not completely melted	Completely solubilize the agarose before transferring to a 37 °C water bath.
	Rinse steps were too harsh. LMAgarose may detach when rinse solutions (e.g. dH_2O, EtOH) are poured over slides.	Gently submerge slides into pipette box lids or other suitable tray to perform rinses.

X. References

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