TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CometAssay® Control Cells

For Single Cell Gel Electrophoresis Assay

Catalog # 4256-010-CC

Sufficient materials for 10 assays.

interchim

CometAssay® Control Cells

For Single Cell Gel Electrophoresis Assay

Catalog # 4256-010-CC

Table of Contents

© 2010 Trevigen, Inc. All rights reserved. Trevigen, CometAssay, and CometSlide are registered trademarks of Trevigen, Inc. SYBR Green is a registered trademark of Molecular Probes, Inc. Excel is a registered trademark of Microsoft, Inc. Analyze-it is a registered trademark of Analyze-it software LTD.

I. QUICK REFERENCE PROCEDURE (Assay Protocol)

The Assay Protocol described below is written as a Quick Reference using alkaline Comet Control Cells (cat#4256-010-CC). 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 CCO (control cells) with 500 µl molten LMAgarose (at 37˚C) and immediately spread 50 µl per well over a 2 well, or 30 ul per well for a 20 well CometSlide™.
- ☐ 4. Repeat step 3 for samples CC1, CC2, and CC3, respectively.
- ☐ 5. Place slides flat at 4°C in the dark for 10 minutes.
- ☐ 6. Immerse slides in prechilled Lysis Solution at 4°C, for 30 minutes.
- ☐ 7. Immerse slides in 50 ml freshly prepared Alkaline Unwinding Solution, pH>13 for 20 minutes at room temperature, in the dark.
- ☐ 8. For the CometAssay® ES tank, add 950 ml prechilled Alkaline Electrophoresis Solution, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 30 minutes.

  For other horizontal electrophoresis units, carefully pour cold Alkaline Solution until level just covers samples (not to exceed 0.5 cm). Set the voltage to 1 Volt/cm. Add or remove buffer to adjust current to ~220 mA.
- ☐ 9. Immerse slides twice in dH_2O for 5 minutes each, then in 70% ethanol for 5 minutes.
- ☐ 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 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 Alkaline CometAssay® allows for sensitive detection of both single and 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 treated with alkali to unwind and denature the DNA and hydrolyze sites of DNA damage. The samples are then submitted to electrophoresis and staining 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 alkaline electrophoresis, the four control cell populations show incremental increases in percent DNA in the tail. The healthy control cell population (CC0) was treated with Etoposide under various conditions to increase the amount of damage in populations CC1, CC2 and CC3, respectively. Etoposide is a DNA synthesis inhibitor that induces double-stranded and single-stranded DNA breaks. These cryopreserved controls are designed to act as controls to standardize and compare alkaline electrophoresis methods 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

CometAssay® Control Cells should be stored in liquid nitrogen.

Components	Description	Cap Color	Catalog #	Amount
CC0	Healthy Cells	White	4256-010-CC0	500 µl
CC1	Treated Cells	Yellow	4256-010-CC1	500 µl
CC2	Treated Cells	Green	4256-010-CC2	500 µl
CC3	Treated Cells	Red	4256-010-CC3	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.

Reagents:

1. Ice cold 1X PBS pH 7.4, Ca^{++} and Mg^{++} free
2. Isopropanol
3. NaOH Pellets
4. 0.5 M EDTA (pH 8.0)
5. CometAssay® Kit[1] (required)

[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

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 CC0, CC1, CC2 and CC3 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 Alkaline Electrophoresis.

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 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 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 (Alkaline **CometAssay®**)

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.

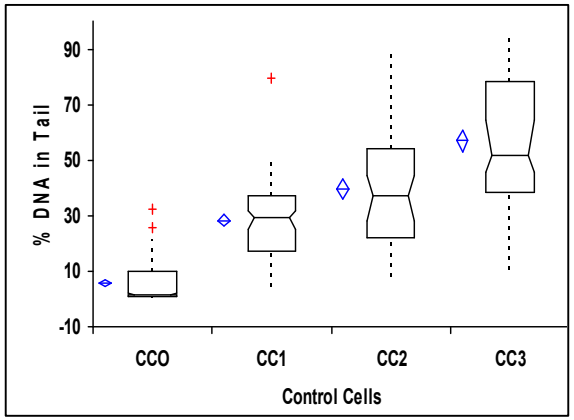
Featured Data:

To evaluate the degree of damage, the Control Cells were processed using the CometAssay® System under defined electrophoresis conditions. In the example below, alkaline electrophoresis was performed on two-well slides (4250-050-03) using CometAssay® Kit (4250-050-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 50 cells for each Control Cell population (lot# 12161M6) 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 Control Cells: Percent DNA in Comet Tail



% DNA by Etoposide	n	Mean	SD	SE	75% CI of Mean	Median	IQR	75% CI of Median
CCO	50	5.757	7.7270	1.0928	4.485 to 7.029	1.640	8.925	1.290 to 2.230
CC1	50	28.374	14.0080	1.9810	26.068 to 30.680	28.990	20.313	25.180 to 31.840
CC2	50	39.736	21.8164	3.0853	36.144 to 43.328	37.050	32.183	27.790 to 44.630
CC3	50	56.800	23.5893	3.3360	52.916 to 60.683	51.905	40.240	45.460 to 64.390

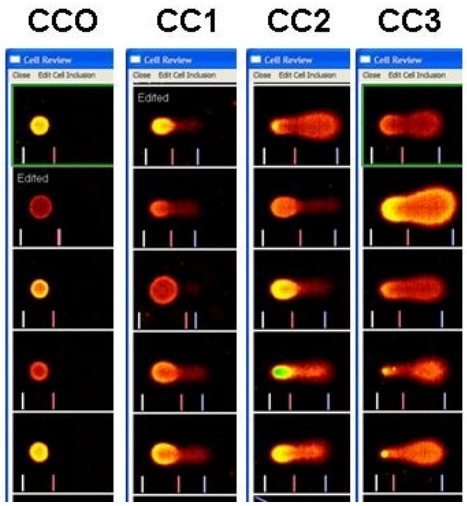


Figure 1b: Examples of comet tail shapes for each population.

In Table 2, a one-way Analysis of Variance (ANOVA) was used to test for a difference between means of the treated control cells (CC1, CC2 and CC3). 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 treated Control Cells

n	150				
% DNA by Etoposide	n	Mean	SD	SE	
CC1	50	28.374	14.008	1.9810	
CC2	50	39.736	21.816	3.0853	
CC3	50	56.800	23.589	3.3360	
Source of variation	SSq	DF	MSq	F	p
Etoposide	20471.839	2	10235.919	24.99	<0.0001
Within cells	60203.173	147	409.545		
Total	80675.012	149			
Contrast	Difference	Tukey 95% CI			
CC1 v CC2	-11.362	-20.945 to -1.779	(significant)		
CC1 v CC3	-28.426	-38.009 to -18.843	(significant)		
CC2 v CC3	-17.064	-26.647 to -7.481	(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 150x g is used.
	Electrophoresis solution too hot	Control temperature by performing the assay at 4° C. Pre-chill the electrophoresis solution.
	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.
	LMAgarose too hot	Cool LMAgarose to 37° C before adding cells.

PROBLEM	CAUSE	ACTION
Treated Control Cells show no evidence of comet tail.	Sample was not processed correctly/ Insufficient denaturation in Alkaline Solution.	Ensure each step in protocol was performed correctly. Failure to lyse, denature in alkali, or to properly perform electrophoresis may generate poor results. Increase time in Alkaline Solution up to 1 hour.
Comet tails present but not significant in Treated Control Cells.	Insufficient electrophoresis time.	Increase time of electrophoresis up to 1 hour for alkaline electrophoresis. Increase time of electrophoresis when running at 4° C.
Cells in LMAgarose did not remain attached to the CometSlide™.	Electrophoresis solution too hot.	Control temperature by performing the assay at 4° C.
	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 after alkali treatment 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

1. Lemay, M. and K.A. Wood, 1999. Detection of DNA damage and identification of UV-induced photoproducts using the CometAssay® kit. *BioTechniques* **27**(4):846-51.
2. Angelis, K.J., M. Dusinska and A.R. Collins. 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20:**2133-8.
3. Morris, E.J., J.C. Dreixler, K-Y. Cheng, P.M. Wilson, R.M. Gin and H.M. Geller. 1999. Optimization of single-cell gel electrophoresis (SCGE) for quantitative analysis of neuronal DNA damage. *BioTechniques* **26:**282-9.
4. Malyapa, R.S., C. Bi, E.W. Ahern, and J.L. Roti, 1998. Detection of DNA damage by the alkali comet assay after exposure to low dose gamma radiation. *Radiation Res* 1**49:**396-400.
5. Henderson, L., A. Wolfreys, J. Fedyk, C. Bourner and S. Windeback, 1998. The ability for the comet assay to discriminate between genotoxins and cytotoxins. *Mutagenesis* **13:**89-94.
6. Visvardis, E.E., A.M. Tassiou and S.M. Piperakis,1997. Study of DNA damage induction and repair capacity of fresh cryopreserved lymphocytes exposed to H_2O_2 and γ-irradiation with the alkaline comet assay. *Mutation Res* **383:**71-80.
7. Fairbairn, D.W., P.L. Olive and K.L. O'Neill. 1995. The comet assay: a comprehensive review. *Mutation Res* **339:**37-59.
8. Collins, A.R., A.G. Ma and S.J. Duthie, 1995. The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidine dimers) in human cells. *Mutation Res* **336:**69-77.

9. Singh, N.P., M.T. McCoy, R.R. Tice and E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175:**184-91.
10. Östling, O. and K. J. Johanson, 1984. Microelectrophoretic study of radiation- induced DNA damage in individual cells. *Biochem Biophys Res Commun* **123:**291-8.

XI. Related Products Available From Trevigen

Trevigen's CometAssay® kits include Lysis Solution (Cat#: 4250-050-01), LMAgarose (Cat#: 4250-050-02), CometSlide®, SYBR® Green (4250-050-05), 200 mM EDTA (Cat# 4250-050-04) and full instructions.

CometAssay® Kits:

Catalog #	Description	Size
4250-050-ESK	CometAssay® Starter Kit	each
4250-050-ES	CometAssay® ES	each
4250-050-K	CometAssay® Kit	50 samples
4251-050-K	CometAssay® Silver Staining Kit	50 samples
4252-040-K	CometAssay® Higher Throughput Kit	40 samples
4253-096-K	CometAssay® Kit 96 Wells	96 samples
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

Control Cells:

Catalog #	Description	Size
4257-010-NC	Neutral CometAssay® Control Cells	1 set

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK 4040-100-FM	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4045-01K-FK 4045-01K-FM	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-methylhydan-toin, 5-hydroxycytosine,5-hydroxyuracil, methyl-tartonylurea, thymine ring saturated or fragmentation product	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 cyclobutane pyrimidine dimers	75 samples 100 samples
4065-100-FK 4065-100-FM	cv-PDG Kit	Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	75 samples 100 samples
4100-100-FK 4100-100-FM	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples 100 samples

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.

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

Trevigen, Inc.
8405 Helgerman Ct. Gaithersburg, MD 20877
Tel: 1-800-873-8443 • 301-216-2800
Fax: 301-560-4973
e-mail: info@trevigen.com
www.trevigen.com

Please Recycle

E2/25/10v1

www.interchim.com

211 bis Avenue Kennedy - BP 1140
03103 Montluçon - France
33 (0) 4 70 03 88 55
Fax 33 (0) 4 70 03 82 60
e-mail interchim@interchim.com

Agence Paris - Normandie
33 (0) 1 41 32 34 40
Fax 33 (0) 1 47 91 23 90
e-mail interchim.paris@interchim.com