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TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CometChip® Electrophoresis Starter Kit

Catalog# 4260-096-ESK

High-throughput platform to treat and measure DNA damage on a single slide

Instructions applicable to 4260-096-CSK and 4260-096-K

See http://trevigen.com/cometchip-protocol-demonstration-videos/ for videos 1 to 5 demonstrating steps.

CometChip® Electrophoresis Starter Kit

Cat# 4260-096-ESK

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I. Quick Reference Procedure (Alkaline Assay Protocol)

The Assay Protocol described below is written as a Quick Reference for one CometChip®. Reagents and detailed instructions including reagent preparation are provided in Sections VI-VIII.

Day 1:

1. Equilibrate CometChip® in 100 ml PBS at room temperature.

2. Cool 100 ml Lysis Solution and 1.5L Alkaline Solution to 4°C. Melt 5 ml LMAgarose and cool in a 45°C water bath.

3. Prepare 12 ml single cell suspension at >1.0 x 10^5 cells/ml.

4. Place CometChip® into the 96-Well CometChip® System and aspirate wells.

5. Aliquot 100 µl cells per well, cover with lid and place in tissue culture incubator for 20 minutes. Half way through the incubation period, gently rock E-W and N-S.

6. Aspirate wells.

7. Aliquot 100 µl treatment per well, cover with lid and place in tissue culture incubator for 30 minutes.

8. Aspirate wells.

9. Remove CometChip® from the 96-Well CometChip® System and gently rinse with 5 ml PBS.

10. Overlay CometChip® with 5 ml LMAgarose. Allow to set for 3 min then transfer to 4°C for 12 minutes to completely solidify.

11. Place CometChip® in 100 ml Lysis Solution for 1 hour at 4°C.

12. Equilibrate CometChip® in 250 ml Alkaline Solution for 20 min at 4°C then repeat for additional 20 minutes in fresh solution.

13. Electrophorese at 4°C for 50 min at 22 V using 700 ml Alkaline Solution. Note: Slide Tray Overlay is not used with CometChip®.

14. Neutralize CometChip® for 15 min at 4°C in 100 ml 0.4M Tris pH 7.4. Repeat with fresh solution.

15. Equilibrate CometChip® for 30 min at 4°C in 100 ml 20 mM Tris pH 7.4.

16. Stain overnight at 4°C in 100 ml 0.2X SYBR® Gold.

Day 2:

1. Destain ~1 hour at room temperature in 100 ml 20 mM Tris pH 7.4.

2. Score well images at 4-10X using fluorescence microscope with fluorescein filter.

II. Introduction

Trevigen's 96-Well CometChip® System (Figure 1) is a high through-put 96 well platform to treat, electrophorese and measure DNA damage, in one or more cell types, on a single slide using the comet assay. The CometChip® is an array of spatially encoded micropores patterned on agarose. 96 separate macrowells, each containing ~400 micropores, are created by inserting the CometChip® into a magnetically sealable cassette. Cells added to each macrowell are deposited by gravity into the micropores - creating a pattern of non-overlapping cells (Figure 3A, Section IX Data Interpretation).

Multiple experimental conditions are performed and evaluated in parallel by the addition of different chemicals to respective wells. Once treatment is complete and the CometChip® removed from the magnetic cassette, an LMAgarose overlay is added to immobilize the cells within the micropores. When performing the Alkaline CometAssay®, the CometChip® is immersed in Lysis Buffer for gentle cellular lysis, followed by Alkaline Solution to unwind and denature the DNA and hydrolyze sites of damage. Comets are developed by alkaline electrophoresis using Trevigen's CometAssay® Electrophoresis System. Subsequent staining with SYBR® Gold, a fluorescent DNA intercalating dye, allows visualization of the comet tail shape for assessment of DNA damage by fluorescence microscopy.

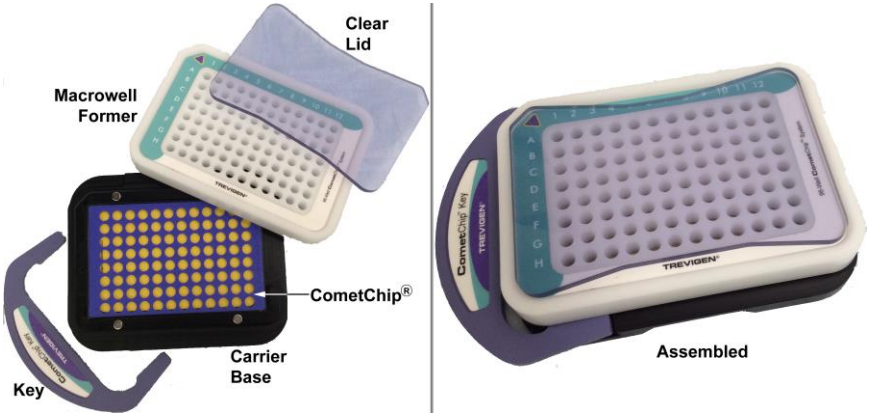


Figure 1: Trevigen's 96-Well CometChip® System
The 96-Well CometChip® System (4260-096-CS) contains a carrier base (black), a macrowell former (white), a lid (clear) and a Key (purple). The CometChip® (4260-096-01) is inserted into the black carrier base. When the macrowell former is placed over the CometChip® it magnetically snaps to the carrier base and forms 96 sealed macrowells on top of the CometChip®. The circumference of each macrowell encompasses ~400 micropores on the CometChip®. The clear lid sets on top of the white macroformer preventing media evaporation from the wells and allowing placement in CO_2 incubator. The purple Key acts as a lever to gently lower or release the white macroformer to and from the black carrier base. The 96-Well CometChip® System is designed to work with the CometAssay® Electrophoresis System II (4250-050-ES) which accommodates three CometChips®.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these products. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. **Do not freeze the CometChip® or store at room temperature.** Freezing will destroy the micropore array on the agarose and elevated temperatures may promote bacterial growth. The CometChip® contains an antibiotic preservative and recommended storage is at 4°C.
4. **Do not freeze the 96-Well CometChip® System**. The unit is designed for use from 4°C to 37°C. Do not expose the unit to excessive heat such as autoclaving.
5. **Clean the 96-Well CometChip® System with deionized water and carefully wipe dry.** Avoid abrasive cleaners and rough cloths or brushes.
6. Lysis solution contains 1% sodium lauroyl sarcosinate which is an irritant and precipitates with long-term storage at 4°C.
7. SYBR® Gold contains DMSO. Please refer to manufacturer for Safety Data Sheet.
8. Insert the CometChip® at an angle into a body of liquid. **Never pour a large volume of liquid on the CometChip®.**

IV. Materials Supplied

CometChip® Electrophoresis Starter Kit[1] cat# 4260-096-ESK

Catalog Number	Component	Amount Provided	Storage Temperature
4260-096-CSK	CometChip® Starter Kit	1	4°C and Room Temp
4250-050-ES	CometAssay® Electrophoresis System II	1	Room Temp

[1]Available from Trevigen with Power Supply.

CometChip® Starter Kit cat# 4260-096-CSK

Catalog Number	Component	Amount Provided	Storage Temperature
4260-096-K	CometChip® Kit	1	4°C and Room Temp
4260-096-CS	96-Well CometChip® System	1	Room Temp

CometChip® Kit cat# 4260-096-K

Catalog Number	Component	Amount Provided	Storage Temperature
4260-096-01	CometChip®, 30 micron	1	4°C 8-week stability
4250-010-01	Lysis Solution[2]	100 ml	Room Temp
4250-050-02	Comet LMAgarose[2]	15 ml	4°C

[2]Available from Trevigen in larger size.

V. Materials/Equipment Required But Not Supplied

Reagents/Disposables:

1. Tissue Culture Grade 1X PBS, Ca^{++} and Mg^{++} free
2. NaOH Pellets
3. 0.5M EDTA (pH 8.0)
4. 20% Triton X-100
5. 10,000X SYBR® Gold in DMSO (Life Technologies cat# S-11494)
6. 25 ml reservoir
7. 70 micron filter (for single cell suspension of adherent cells)
8. 15 and 50 ml screw cap conical

Equipment:

1. Power Supply
2. Fluorescence microscope with fluorescein filter and imaging system
3. Comet Analysis Software (Trevigen, Inc. cat# 4260-000-CS)
4. Incubation trays with lids (Ted Pella, Inc. cat# 139-26)
5. Cell counter
6. Tissue culture incubator
7. 45°C and 85°C water bath
8. Low vacuum aspirator
9. 4°C refrigerator/cold room
10. Multichannel pipettor 30 µl - 300 µl

VI. Reagent Preparation

1. 1X PBS, Ca^{++} and Mg^{++} free

Store at room temperature.

2. CometChip®, 30 micron

The CometChip® storage buffer is 1X PBS with antibiotic/mycotic. Before use, remove CometChip® from packaging and equilibrate in 100 ml tissue grade 1X PBS for 30 minutes at room temperature. The micropores are visible with a light microscope only if the CometChip® surface is wet.

3. Lysis Solution

Cool 100 ml Lysis Solution per CometChip® to 4°C before use.

4. Comet LMAgarose

Each CometChip® requires 5 ml LMAgarose. Loosen the bottle cap to allow for expansion then heat the bottle in 85°C water bath until the agarose is molten. Prepare 5 ml aliquots in 15 ml conical and store long term at 4°C. (Caution: Microwaving is not recommended.)

Before use, place aliquot in 85°C water bath until the agarose is molten then transfer or cool water bath to 45°C. The LMAgarose will remain molten at 45°C until needed to overlay CometChip®.

5. Treatment Media

A. Single Treatment Time: Dilute test reagent in complete media to the final concentration. Prepare sufficient volume to add test reagent to triplicate wells in 100 µl aliquots. Always run untreated control in triplicate using complete media.

B. Multiple Treatment Times: Dilute test reagent in complete media to two-times (2X) the final concentration. Prepare sufficient volume to add test reagent to triplicate wells in 50 µl aliquots. Always run untreated control in triplicate using complete media.

Note: Prior to the comet assay, a separate cell viability assay using different doses of the test reagent should be performed. This assay will determine the dose of the test substance that results in less than 10% cell death at the end of the treatment period. Higher doses of cytotoxic agents may induce apoptotic DNA fragmentation resulting in a false positive comet result.

6. Alkaline Solution, pH>13 for Equilibration and Electrophoresis

Prepare a stock solution of 500 mM EDTA, pH 8.
Prepare a stock solution of 20% Triton X-100 in ddH_2O.
Recommend storing 2L ddH_2O at 4°C for routine use.

Prepare Alkaline Solution same day and cool to 4°C. Scale as needed:
Equilibration requires 500 ml for each CometChip®.
Alkaline Electrophoresis requires 700 ml for 1-3 CometChips®.
The addition of Triton X-100 improves comet analysis.

For 1 liter Alkaline Solution (200 mM NaOH/1 mM EDTA/0.1% Triton X-100):

NaOH pellets	8 g
500 mM EDTA, pH 8	2 ml
ddH_2O (after NaOH is dissolved) add to:	1 liter
20% Triton X-100	5 ml

7. CometAssay® Electrophoresis System for CometChip®

Cool electrophoresis tank, lid, and 96 well slide tray to 4° by placing in a cold room or refrigerator (see Figure 2). **Note:** Slide Tray Overlay is not used.

8. Neutralization Buffers, pH 7.4

Prepare 2 M Tris-HCl pH 7.4 stock solution. Each CometChip® requires 20 mM and 400 mM Tris-HCl, pH 7.4 (~200 ml of each). Cool to 4°C before use.

Scale as needed:

For 250 ml	20 mM	400 mM
2 M Tris-HCl, pH 7.4	2.5 ml	50 ml
ddH_2O	247.5 ml	200 ml

9. SYBR® Gold Staining Solution

i. Prepare 1X SYBR® Gold Staining Solution. The diluted stock is stable for several weeks when stored at 4°C in the dark.

10,000X SYBR® Gold in DMSO	6 µl
20 mM Tris-HCl (pH 7.4)	60 ml

ii. Prepare 100 ml 0.2X SYBR® Gold Staining Solution for each CometChip® and cool to 4°C just before use.

1X SYBR® Gold Staining Solution	20 ml
20 mM Tris-HCl (pH 7.4)	80 ml

VII. Cell Preparation

1. Suspension and Adherent Cells:
   a. Verify 90% cell viability.
   b. Minimize centrifugation to avoid cell clumping which decreases cell loading efficiency into micropores on the CometChip®.
   c. Prepare 12 ml of single cell suspension at >1.0 x 10^5 cells/ml in complete media for each CometChip®. Verify cell count.

2. Adherent Cells:
   a. Gently detach cells from flask surface using standard methods being careful to avoid excessive trypsin treatment.
   b. To avoid centrifugation, recommend suspending the detached cells directly in complete media. Verify 90% cell viability.
   c. Adherent cells may require filtration through 70 micron filter to generate single cell suspension. Count cells after filtering and adjust to >1.0 x 10^5 cells/ml. Prepare 12 ml for each CometChip®.

VIII. Assay Protocols

See http://trevigen.com/cometchip-protocol-demonstration-videos/ for videos 1 to 5 demonstrating steps.

Day 1:

1. Equilibrate CometChip® in 100 ml tissue culture grade 1X PBS for 30 minutes at room temperature.

2. Insert CometChip® into the 96-Well CometChip® System (see **video 1**).

a. Separate the white macrowell former from the black base by inserting the purple Key into the indentations located on the carrier base. The magnetic seal is released by lifting the purple key which raises one end for easy removal of the white macrowell former.
b. Insert the purple Key into the indentations located on the black carrier base and place the CometChip® into the carrier base with column 1 of the CometChip® positioned adjacent to the purple Key.
c. Gently lower the white macrowell former at an angle onto the black carrier base using the purple Key. Recommend fitting the right edge of the white macrowell former (column 12) onto the black carrier base and lower the left edge (column 1) using the Purple Key.
d. Ensure the macrowell former is seated flush against black base. Remove the purple Key until step 9.

Note: ***Always hold 96-Well CometChip® System by the black base and avoid touching the white macrowell former once magnetically attached to prevent well distortion.***

3. Carefully aspirate residual PBS from all wells (see **video 2**).

a. Hold the 96-Well CometChip® System at a slight angle by the black base.
b. Attach a fine-gel loading tip to a low vacuum aspiration system.
c. Carefully aspirate liquid from the edge of each well.

4. Load 100 µl cell suspension (>1.0 x 10^5 cells/ml) per well (>10,000 cells/well) using multichannel pipette and 25 ml reservoir (see **video 2**).

Note: CometChip® loading efficiency depends on cell size, concentration, single cell suspension efficiency, and loading time.

5. Cover 96-Well CometChip® System with clear lid and place in tissue culture incubator for at least 10 minutes to allow cells to settle. After 10 minutes, gently rock East-West 3-4 times then rock North-South 3-4 times (see **video 2**). Place back in incubator for an additional 10 minutes.

6. Carefully aspirate media from all wells as described in Step 3 to avoid cell loss from micropores.

7. TREATMENT

Single Treatment Time

a. Aliquot 100 µl media into triplicate wells for untreated controls.
b. Aliquot 100 µl "Treatment" media into triplicate wells.
c. Cover with lid and return to the incubator for treatment time (ex. 30 minutes).

Multiple Treatment Times

a. Aliquot 50 µl media into all test wells and 100 µl media for untreated controls.
b. Add 50 µl 2X "Treatment" media into triplicate wells at designated time.
c. Cover with lid and return to the incubator for designated time (ex. 2 hour treatment).
d. Repeat steps b and c until time course is completed (ex. 1 hour treatment followed by 30 minute treatment).

Note: 96-Well CometChip® System is not designed for overnight treatment.

8. Carefully aspirate media from all wells as described in Step 3 to avoid cell loss from micropores.

9. Remove the CometChip® from 96-Well CometChip® System as described in Step 2a.

10. Using 5 ml serological pipette, gently wash CometChip® with ~5 ml 1X PBS (see **video 3**). Recommend holding the slide at an angle and adding drops of PBS above row A and along slide edges to avoid cell loss from micropores. If necessary, add PBS above non-rinsed wells. While still holding CometChip® at an angle, remove excess PBS from slide corner with Kim Wipe being careful to avoid touching wells.

11. Place CometChip® on a flat surface and overlay with 5 ml LMAgarose using 5 ml serological pipette (see **video 4**). Recommend adding LMAgarose in a single continuous serpentine fashion between rows, avoiding the wells. This

will allow the LMAgarose to spread slowly over the wells and solidify as a level surface.

12. Keep CometChip® at room temperature for 3 minutes before moving to 4°C for 12 minutes to completely solidify LMAgarose.

13. Place CometChip® in a horizontal position in 100 ml Lysis Buffer for 1 hour at 4°C.

14. Equilibrate CometChip® horizontally in 250 ml Alkaline Solution for 20 min at 4°C then repeat for additional 20 minutes in fresh solution.

15. **Perform Electrophoresis at 4°C for 50 min at 22 V** (see Figure 2 and **video 5**).

a. Insert CometChip® into slide tray with "Trevigen logo" adjacent to negative (black) electrode (Figure 2).

Figure 2: Assembling CometAssay® ES for CometChip®. Trevigen logo on CometChip® adjacent to negative (black) electrode.

b. Verify CometChip® is flat in tray and carefully add 700 ml Alkaline Solution to electrophoresis tank. **Do not pour liquid over CometChips®.**
c. Attach lid to electrophoresis tank.
d. Set power supply to 22V and electrophorese for 50 minutes.

Note: Do not use Slide Tray Overlay with CometChip®. Amperage is ~280 mA +/- 40 mA for 1-3 CometChips®.

16. Neutralize CometChip® in a horizontal position for 15 min at 4°C in 100 ml 0.4M Tris pH 7.4. Repeat with fresh solution.

17. Equilibrate CometChip® in a horizontal position for 30 min at 4°C in 100 ml 20 mM Tris pH 7.4.

18. Stain overnight at 4°C in 100 ml 0.2X SYBR® Gold.

Day 2:

1. Destain ~ 1 hr at room temperature in 100 ml 20 mM Tris pH 7.4.

2. Score well images using fluorescence microscope equipped with fluorescein filter at 4-10X magnification.

IX. Data Interpretation

Background

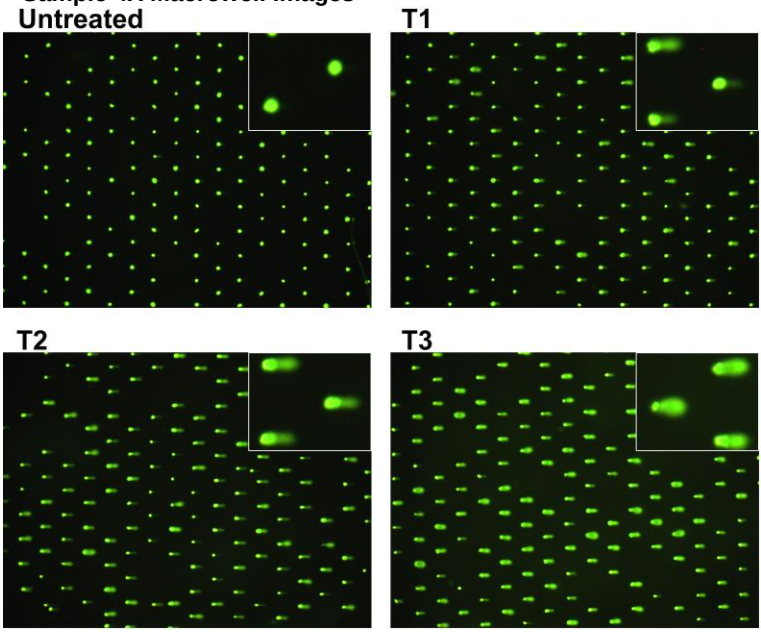
When excited (425-500 nm) the DNA-bound SYBR® Gold emits green light. In healthy cells the fluorescence is confined to the nucleoid (comprised of high molecular weight DNA): undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleoid under the influence of an electric current. 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. The most reliable descriptor of DNA damage for the alkaline comet assay is Percent DNA in the Tail. The comet tail is scored according to DNA content (fluorescent intensity) using quantitative image analysis system which includes a fluorescent microscope, camera, and analysis package.

Quantitative Analysis using Comet Analysis Software (4260-000-CS)

1. Image capture.
    a. Auto set the camera on untreated cells to avoid saturation of undamaged heads.
    b. Position comet head to the left and comet tail to the right.
    c. Take all images at same magnification (4X to 10X) and camera settings.
    d. Save images as TIFF, JPEG or PNG.
2. Edit well images to remove comets not located within pattern of non-overlapping micropores (Figure 3A).
3. Determine median % DNA in the Tail for each of the 96 macrowells (Figure 3B).
4. Untreated controls serve as a baseline for comparison to treated cells. Generally, <10% DNA in the tail is indicative of a healthy population.
5. Use boxplots to summarize data graphically (Figure 3C).

Figure 3. CometChip® analysis of lymphocyte cell line (T0) treated for 30 minutes with increasing levels of Etoposide (T1 to T3). Alkaline electrophoresis was performed using Trevigen's CometAssay® Electrophoresis System II. Images were captured and analyzed using Comet Analysis Software (cat# 4260-000-CS). Sample 4X macrowell images (A), well medians for % DNA in Tail (B), and box plot (C) are provided below.

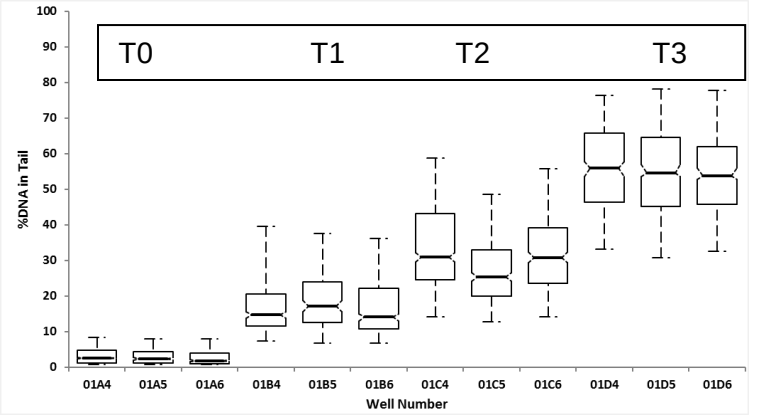
A) Sample 4X Macrowell Images



B) Plate Setup: Well Medians for % DNA in Tail, n~ 165 comets/well

row-treatment	2	3	4	5	6	7	8	9	10	11
A-T0	2.450	2.025	2.570	2.430	1.700	1.110	1.525	0.860	1.360	1.310
B-T1	16.070	16.110	14.650	17.220	14.140	15.220	15.455	12.790	15.290	13.170
C-T2	28.240	34.430	31.020	25.430	30.770	28.640	31.465	26.770	25.550	25.770
D-T3	56.495	58.135	55.990	54.490	53.700	54.790	56.880	52.990	49.930	47.440
E-T0	3.440	4.975	4.515	5.090	4.885	3.660	4.240	3.970	2.525	3.560
F-T1	18.040	17.250	16.210	18.710	16.915	16.340	16.940	17.530	17.200	17.250
G-T2	34.710	31.355	27.645	27.830	31.170	31.585	30.500	31.785	27.910	30.540
H-T3	54.115	50.250	52.145	52.760	49.760	53.860	51.790	50.980	52.350	49.560

C) Boxplot



X. References

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4. Watson C. *et al*. High-throughput screening platform for engineered nanoparticle-mediated genotoxicity using CometChip technology. *ACS Nano.* **2014**, *8*, 2118-33.
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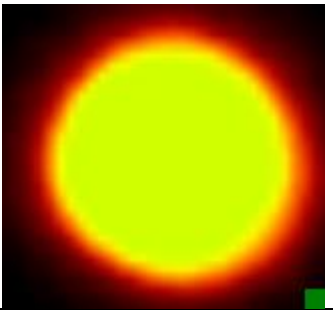
XI. Troubleshooting

PROBLEM	CAUSE	ACTION
Poor loading	Aggregated cells	Use 70 micron filter and perform cell count after filtering.
	Excessive washing	Reduce force of PBS wash before overlaying with LMAgarose.
	Macrowell Former is wet	Assure macrowell former is completely dry before placing over CometChip® to prevent media from adhering to macrowell sides.

PROBLEM	CAUSE	ACTION
Poor loading	Low cell concentration	Increase loading time and cell number.
	Harsh aspiration	Aspirate at well edge with fine tip.
High background between micropores	Insufficient washing	Wash with PBS along slide edges before overlaying with LMAgarose.
	Cell concentration	Reduce loading time and cell number.
	Poor aspiration	Ensure complete aspiration after cell loading.
	Pushing cells out of micropores	Add LMAgarose from slide edge and between rows so agarose slowly spreads to cover wells.
Unexpected tail shape or DNA diffusion artifacts	LMAgarose too hot	Cool LMAgarose to 45°C before overlaying cells.
	LMAgarose uneven	Add LMAgarose in a single continuous serpentine fashion without going back over.
Gel detached from slide	Alkaline treatment	Perform steps at 4°C for indicated times.
	Past expiration date	Store gels at 4°C and use within 8 weeks.
Untreated control has large comet tails.	Low viability	Verify 90% viability.
Positive control comet tails are diffuse with little or no head.	Cells are necrotic or apoptotic	Decrease treatment with the damaging agent. Verify viability after treatment is 90%.

PROBLEM	CAUSE	ACTION
Software is not calling end of head properly.	Incomplete DNA staining of internal region of comet head appears as donut. 	Stain overnight at 4°C at 0.2X SYBR Gold. 

XII. Related Products Available From Trevigen

CometChip®

Catalog #	*Description*	*Size*
4250-010-01	CometAssay® Lysis Solution	100 ml
4250-050-01	CometAssay® Lysis Solution	2 x 500 ml
4250-050-02	CometAssay® LMAgarose	15 ml
4250-500-02	CometAssay® LMAgarose	100 ml
4250-050-ES	CometAssay® Electrophoresis System II	1 each
4260-096-01	CometChip®, 30 micron	1 each
4260-096-CS	96-Well CometChip® System	1 each
4260-096-ESK-PS1	CometChip® Electrophoresis Starter Kit with Power Supply for North America	1 each
4260-000-CS	Comet Analysis Software	license

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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