TREVIGEN® Instructions

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

DASH™ CometAssay™

Rapid Assessment of Apoptosis

Catalog # 4255-050-K

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: 301-216-2801
e-mail: info@trevigen.com
www.trevigen.com

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DASH™ CometAssay™

Cat. # 4255-050-K

Table of Contents

I. Introduction

Trevigen's new comet assay kit, DASH™ (Diffusion Apoptosis Slide Halo), allows discrimination of healthy and damaged cells in just a few hours. Simply embed cells in low melting agarose on a pre-treated slide, lyse under alkaline conditions, and precipitate the DNA of the remaining nuclear region (i.e. nucleoid) in the agarose.

The precipitated DNA is visualized using SYBR® Green I nucleic acid stain. When excited (425 - 500 nm) the DNA-bound SYBR® Green I emits green light. In healthy cells, the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and thus does not diffuse very far, if at all, from the nucleoid. In cells that have accrued damage to the DNA, the alkali lysis treatment unwinds the DNA, releasing fragments that diffuse away from the center of the nucleoid, creating a "halo" surrounding a small compact origin.

This kit includes ready to use, pre-coated slides and optimized reagents for your convenience.

Advantages/Features:

- Identify apoptotic cells quickly and easily
- Ready to use slides and reagents
- Work with a small number of cells

II. Precautions and Limitations

1. For research use only. Not for use in diagnostic procedures.

2. The physical, chemical, and toxicological properties of the products contained within this kit have not yet been fully investigated. 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. MSDS sheets are available upon request.

3. Lysis Solution-DQ contains 0.01% sodium lauryl sarcosinate, an irritant. In case of eye or skin contact, wash thoroughly under running water. In case of ingestion, rinse mouth with water and seek medical advice. MSDS sheets are available upon request.

4. SYBR® Green I contains DMSO. MSDS sheets are available upon request.

III. Materials and Equipment

A. Materials Supplied

DASH Assay Kit Contents

Component	Catalog Number	Amount Provided	Storage
CometSlides™	4250-050-03	25 slides	RT
LMAgarose-DQ	4255-050-02	15 mL	RT
Lysis solution-DQ	4255-050-01	2 x 500 mL	RT
100X NP Buffer-DQ	4255-050-04	20 mL	RT
SYBR Green I	4250-050-05	5 µL	-20°C

B. Materials/Equipment Required But Not Supplied
1. Pipettors and tips
2. Reagent Alcohol
3. Cell culture media and supplies
4. Microfuge and/or 15 mL conical tubes
5. 1X PBS, Ca and Mg free (Trevigen sells 10X PBS for your convenience, see Section IX, Related Products page9)
6. Hot plate
7. Water bath

IV. Reagent Information and Preparation

1. Prepared Lysis Solution-DQ
If your samples, such as blood cells or tissue, contain heme, then the lysis solution should contain DMSO.
For up to 10 slides (2 samples per slide), prepare:
Lysis Solution (Cat#4250-050-01) 40 mL
DMSO (optional) 4 mL
Otherwise, the Lysis Solution-DQ is ready to use.

2. LMAgarose-DQ
Boil the bottle until the agarose is melted. Before adding cells to the molten agarose, cool it to 37°C.

3. CometSlides™
Comet slides are ready to use.

4. Prepared NP (Neutralization and Precipitation) Buffer-DQ
Stock solution is provided at 100X, and is mixed with autoclaved (i.e. nuclease-free) dH_2O and reagent alcohol.
For up to 10 slides (2 samples per slide), prepare:
100X NP Buffer-DQ (Cat#4255-050-04) 1 mL
Autoclaved dH_2O 24 mL
Reagent Alcohol 75 mL

5. SYBR® Green I Staining Solution
Prepare SYBR® Green I Staining Solution from the SYBR® Green I concentrate provided (10,000X concentrate in DMSO).
SYBR® Green I (Cat#4250-050-05) 1 µL
TE Buffer, pH 7.5* 10 mL
(*TE: 10 mM Tris-Cl pH 7.5, 1 mM EDTA, nuclease-free)
The diluted stock is stable for several weeks when stored at 4°C in the dark.

V. Assay Protocol

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 dim or yellow light to prevent DNA damage from ultraviolet light. Also, any PBS must be calcium and magnesium free to inhibit endonuclease activities. Remember to include appropriate controls (see below). Optimal results in the DASH™ Assay are usually obtained with 500-1000 cells per CometSlide™ sample area. Using 50 µL of a cell suspension at 1 x 10^5 cells per mL, combined with 500 µL of LMAgarose, will provide the correct agarose concentration and cell density for optimal results when plating 75 µL per sample.

Suspension Cells
Harvest suspension cells by centrifugation at 250 x g. Resuspend cells at 1 x 10^5 cells/mL in ice cold 1X PBS (Ca^{++} and Mg^{++} free). Ensure complete removal of cell culture media, as it can reduce the adhesion of the agarose on the CometSlide™.

Adherent Cells
Gently scrape cells using a rubber policeman. Transfer cells and medium to a centrifuge tube, count cells, and then pellet cells at 250 x g. Wash once in ice cold 1X PBS (Ca^{++} and Mg^{++} free). Resuspend cells at 1 x 10^5 cells/mL in 1X PBS (Ca^{++} and Mg^{++} free).

Controls
A sample of untreated cells should always be processed to control for endogenous levels of damage within cells, and for 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 under low-level yellow light during processing. If you require a sample that will be positive for comet tails, treat cells with 100 µM H_2O_2 or 25 µM $KMnO_4$ for 20 minutes at 4°C. Treatment will generate significant oxidative damage in the majority of cells, thereby providing a positive control for each step in the assay. Note that the dimensions and characteristics of the halo, as a consequence of H_2O_2 or $KMnO_4$ treatment, may be different to those induced by the damage under investigation.

Method for Cryopreservation of Cells Prior to the DASH Assay™
Certain cells, e.g. lymphocytes, may be successfully cryopreserved prior to performing a comet assay (Visvardis et al.). A pilot study should be performed to determine if cryopreservation is appropriate for the cells in use.

1. Centrifuge cells at 200 x g for 5 minutes.
2. Resuspend cell pellet at 1 x 10^7 cells/ml in 10% (v/v) dimethylsulfoxide (DMSO), 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of 2 x 10^6 cells into freezing vials.
4. Freeze at -70°C with -1°C per minute freezing rate.
5. Recover cells by submerging in 37°C water bath until the last trace of ice has melted.
6. Transfer to 15 ml of prechilled 40% (v/v) medium, 10% (w/v) dextrose, 50% (v/v) fetal calf serum.
7. Centrifuge at 200 x g for 10 minutes at 4°C.
8. Resuspend in 1X PBS (Ca^{++} and Mg^{++} free) and proceed with the DASH™ assay.

DASH™ Assay

All steps are performed at room temperature unless otherwise specified. Work under dimmed or yellow light to prevent damage from UV.

1. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened. Place bottle in a 37°C water bath for at least 20 minutes to cool. When working with many samples, it may be convenient to place aliquots of the molten agarose into pre-warmed microcentrifuge tubes and place the tubes at 37°C.

Note: The temperature of the agarose is critical or the cells may undergo heat shock. Heat blocks are not recommended for regulating the temperature of the agarose.

2. Combine cells at 1 x 10^5/mL with molten LMAgarose (at 37°C) at a ratio of 1: 10 (v/v) and immediately pipette 75 µL onto CometSlide™. Use the side of pipette tip to spread agarose/cells over sample area to ensure complete coverage of the sample area. If the agarose was aliquoted, add cells to one tube, mix by gently pipetting once or twice, and then transfer 75 µL aliquots onto each sample area as required. Then proceed with the next sample of cells.

   For example, combine:

Comet LMAgarose (molten and at 37°C from step 1)	500 µL
Cells in 1X PBS (Ca^{++} and Mg^{++} free) at 1 x 10^5/mL	50 µL

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

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

4. Immerse slide in prepared Lysis Solution-DQ (see Section IV.1, page 3 for preparation) for 10 minutes at room temperature in a clean coplin jar (allows simultaneous incubation of up to 10 slides) or 50 mL tubes. WEAR GLOVES WHEN PREPARING OR HANDLING THIS SOLUTION.

5. Tap off excess buffer from slide and immerse in freshly prepared NP Buffer (see Section IV.4, page 3, for preparation). Incubate for 20 minutes at room temperature.

6. Immerse in a second aliquot of freshly prepared NP Buffer. Incubate for 15 minutes at room temperature.

7. Air-dry the slides. Alternatively, heating the slides to 35°C can accelerate this step. Drying brings all of the cell remnants into a single plane, facilitating observation. At this stage, samples may be stored at room temperature, with desiccant.

8. Pipet 50 µL of diluted SYBR® Green I (See Section IV.5, page 3, for preparation) onto each well of dried agarose.

9. View slide by epifluorescence microscopy. (The maximum excitation and emission of SYBR® Green I are 494nm and 521nm, respectively. A fluorescein filter works well.)

VI. Sample Data and Data Analysis

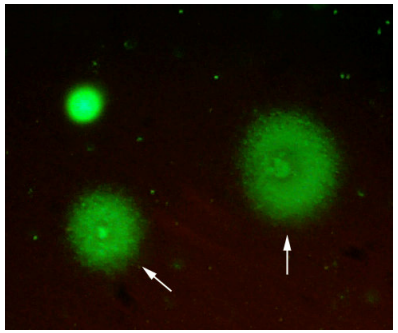

Figure 1. Results of a typical DASH™ Assay.
Raji cells were treated with 100 µM H_2O_2 for 10 minutes at 4°C, subjected to the standard DASH protocol, and visualized using fluorescein filters (450-490 ex./520 em.) with a Nikon E400 fluorescent microscope. The undamaged cell shows a compact, homogenous nucleoid, while the other two cells, indicated by arrows, show nucleoids with typical diffuse halo patterns.

VII. Troubleshooting Guide

PROBLEM	CAUSE	ACTION
Majority of cells in untreated control sample have halos	Cell damage occurred during culturing and/or in sample handling	Culture cells at proper density; most cells should never exceed 5 x 10^5 cells/mL or reach confluence during passaging. Check morphology of cells to ensure healthy appearance. Handle cells and/or tissues gently, minimizing pipetting and physical damage.
	LMAgarose-DQ too hot	Cool and maintain LMAgarose-DQ at 37°C before adding cells. Heat blocks are not recommended.
Very little or no halos in positive control (100 µM H_2O_2, 30 minutes)	Hydrogen peroxide no longer active	Purchase new solution or use solution that has been properly stored (e.g. 4ºC, tightly capped).
	Lysis Solution-DQ is no longer active	Use new Lysis Solution-DQ and store tightly capped.
LMAgarose-DQ disc contains bubbles	Air introduced to sample during preparation and/or LMAgarose too cool during handling	Hold LMAgarose-DQ at 37°C and immediately add to slide. Minimize pipetting and mixing of sample with LMAgarose-DQ. Warm slide to 37°C to ensure even coating of well.
LMAgarose-DQ disc detaches from slide	Incomplete removal of media from sample prep	Wash cells in 1X PBS (Ca and Mg free) before adding to LMAgarose-DQ.
	LMAgarose-DQ too dilute	Do not use a cell sample volume to LMAgarose-DQ ratio higher than 1:10.
	LMAgarose-DQ not fully solidified	Ensure 0.5 mm dried ring due to agarose disc retraction is seen at the edge of the CometSlide area.
	Rough handling of CometSlides™	Do not pour solutions directly over slides or drop slides (i.e. into coplin jars or containers). Instead, remove slides and then slowly immerse slides in pre-filled container or jar.

VIII. References

1. Singh NP. (2000) A simple method for accurate estimation of apoptotic cells. *Exp Cell Res*. 256(1): 328-37.

2. Singh NP, Muller CH, Berger RE. (2003) Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil. Steril.* 80(6): 1420-30.

3. Nigro M, Frenzilli G, Scarcelli V, Gorbi S, Regoli F. (2002) Induction of DNA strand breakage and apoptosis in the eel Anguilla anguilla. *Mar Environ Res.* 54(3-5): 517-20.

4. Barbisan LP, Scolastici C, Miyamoto M, Salvodori DM, Ribeiro LR, Ferreira da Eira A, Viana de Camargo JL. (2003) Effects of crude extracts of Agaricus Blazei on DNA damage and on rat liver carcinogenesis induced by diethynitrosomine. *Genet. & Mol. Res.* 2(3): 295-308.

5. Godard T, Deslandes E, Lebailly P, Vigreux C, Sichel F, Poul JM, Gauduchon P (1999) Early detection of staurosporine-induced apoptosis by comet and annexin V assays. *Histochem. Cell Biol.* 112: 155–161.

6. Sestili P and Cantoni O. (1999) Osmotically driven radial diffusion of single-stranded DNA fragments on an agarose bed as a convenient measure of DNA strand scission. *Free Rad. Biol. & Med*., 26(7/8): 1019–1026.

IX. Related Products Available From Trevigen

CometAssay™ Kits and Slides

Catalog #	Description	Size
4250-050-K	CometAssay Kit [25 slides (2 wells ea.) + reagents]	50 samples
4250-050-03	CometSlides™25	25 slides
4250-200-03	CometSlides-100	100 slides
4254-200-K	CometAssay™ Silver Staining Kit	200 samples
4870-500-6	10X PBS, pH 7.4	6 X 500 mL

DNA Repair Enzyme Modules for comet assay

4040-100-FM	Fpg FLARE Module	>100 samples
4045-100-FM	Endonuclease III FLARE Module	>100 samples
4055-100-FM	T4-Endonuclease V FLARE Module	>100 samples
4065-100-FM	cv-PDG FLARE Module	>100 samples
4100-100-FM	UVDE FLARE Module	>100 samples
4130-100-FM	hOgg1 FLARE Module	>100 samples