Protocol for the Alkaline Comet Assay on GelBond film

Prepared by:

James McNamee, Ph.D. and Pascale Bellier, M.Sc.
Radiation Protection Bureau, Health Canada
775 Brookfield Road, Ottawa, ON, Canada, K1A 1C1
tel:(613) 954-7804 or 941-3667
email:james_mcnamee@hc-sc.gc.ca

Purpose: The following document describes an alternative sample preparation and handling methodology whereby comet assay samples are processed on a Gelbond film, rather than on microscope slides. The Gelbond film provides a stable surface to which the agarose adheres, thereby eliminated both the need to pre-coat the casting surface and the unexpected loss of samples (as often occurs with glass slides). In order to allow multiple samples to be processed on the same sheet (film) of Gelbond, Lab-Tek II chambers are adhered to create casting wells on the Gelbond film. One, 2, 4, 8, and 16 well chambers are available from multiple manufacturers, however the Lab Tek II chambers contain an adhesive tape on the bottom of the chambers which, when removed from their glass slide-mounts, allow the chambers to be re-affixed to the Gelbond film. Other brands of chambers have a built-in (plastic) locking assembly which does not allow re-affixing to Gelbond or possess slanted sides to the chamber wells, which results in a greater meniscus when the agarose gels are cast. Once agarose-embedded samples are solidified, the Lab-Tek II chambers are removed and the Gelbond film containing multiple samples are processed according to standard comet assay techniques. Our standard methodology is described as follows:

Preparation of Solutions

0.75% Agarose: Add 0.75% (750mg per 100ml) LMP agarose in PBS. Heat in a microwave, then dispense 2-4ml aliquots to scintillation vials and store at 4°C until used. On day of experiment, then warm in microwave until melted and maintain at 42°C in a water-bath.

Lysis Buffer A: To a 1L round-bottom flask, add:

2.5M NaCl (58.4g/mol)	=	146.1 g/L
100mM Tetra-sodium EDTA (416g/mol)	=	41.6 g/L
10mM Tris base (121.1g/mol)	=	1.2 g/L
1% N-Lauryl Sarcosine (293.4g/mol)	=	10.0 g/L

Add ddH20 while mixing to achieve a volume of 1L. Adjust pH to 10.0, store at room temperature.

Note: Add 1% Triton X-100 (v/v) to required volume of Lysis Buffer A on the day of experiment, 30 minutes prior to use.

Lysis Buffer B: To a 1L round-bottom flask, add:

2.5M NaCl (58.4g/mol)	=	146.0 g/L
100mM Tetra-sodium EDTA (416g/mol)	=	41.6 g/L
10mM Tris base (121.1g/mol)	=	1.2 g/L

Add ddH20 while mixing to achieve a volume of 1L. Adjust pH to 10.0, store at room temperature.

Note: Do not add N-lauryl sarcosine, or Triton-X100.

Five min. prior to use, add Proteinase-K to achieve a final concentration of 1mg/ml PK in Lysis Buffer B.

Unwinding/Electrophoresis Buffer:

To a 1 L round-bottom flask, add:

0.3M NaOH (40.0 g/mol)	=	12.0g/L
10mM Tetra-sodium EDTA (416g/mol)	=	4.2g/L
0.1% (w/v) 8-hydroxyquinoline	=	1.0g/L

Add 700ml ddH20 and 2% (v/v) DMSO (20ml) while mixing. Once dissolved, add additional ddH20 to achieve 1L. Adjust pH to 13.1 with concentrated NaOH or HCl. Unwinding/Electrophoresis Buffer must be made fresh on the day of experimentation.

Neutralization Buffer: To a 100mL round-bottom flask, add:

1M Ammonium acetate (77.1g/mol)	=	7.7g/100ml

Check to ensure that pH is approximately 7.0.

Staining Solution: SYBR Gold stain (Molecular Probes) was prepared by addition of 5ul stock to 50ml ddH20. Stain is prepared fresh daily, and stored at room temperature in the dark.

Sample preparation using Gelbond film and Lab-Tek II chambers

- Detach three Lab-Tek II, 2-well chambers (Nalge Nunc International, catalog#154461) from their glass slide-mounts and re-affix to a 10cm x 6.5cm sheet of GelBond film (FMC Bioproducts, catalog#53740).
  Note: Gelbond films can be pre-cut in advance of experimentation.

All subsequent steps are performed in subdued light!

- Add a 30µl aliquot of cell suspension (~5000 cells) to 270µl of liquified 0.75% agarose (42^{o}C), and gently mix by pipetting.

- Cast a 120µl aliquot of the cell/agarose mixture into an individual well of a 2-well chamber for each sample.

- Once the agarose has solidified, the chambers are carefully removed from the GelBond and the samples of agarose-embedded cells, now attached to the GelBond, are placed in Lysis Buffer.

- The remainder of the Comet Assay can be performed using standard procedures (see below for our method).

Note: Lab-Tek chambers can be bleached for 5-10 minutes, rinsed, allowed to dry and then re-used at a later date. Over time, the adherent tape on the bottom of the Lab-Tek chambers will loose their adhesiveness, however the chambers can be dipped in a thin layer of molten agarose and then affixed to the Gelbond film. The agarose, when solidified, will provide a seal.

Alkaline Comet Assay Protocol with Gelbond Film:

- Once the Lab Tek chambers are removed, the Gelbond film containing agarose-embedded samples are placed into a petri dish containing 50ml Lysis Buffer A (containing Triton X-100) overnight in the dark.

- **OPTIONAL STEP**: GelBond films are removed from Lysis Buffer A, rinsed with ddH20, then placed into a petri dish containing 40ml Lysis Buffer B (containing 1mg/ml proteinase-K), and maintained at 37^{o}C for 60 min.

- After lysis, the GelBond films are rinsed with ddH20, then placed in 50ml Unwinding/Electrophoresis Buffer (pH13.1) for 30 min at room temperature in the dark.

- The Gelbond films are removed from the Unwinding/Electrophoresis Buffer, then placed in a Hoefer Submarine Gel Electrophoresis Unit containing 220ml fresh Unwinding/Electrophoresis Buffer. The gels are then electrophoresed at 300mA (~1.5V/cm) for 20 min in the dark.

- After electrophoresis, the Gelbond films are transferred to a petri dish containing 40ml of 1M Ammonium Acetate solution for 30 min to neutralize the gels.

- Finally, the gels are placed in 100% ethanol for 2hrs, then allowed to air-dry overnight.

DNA STAINING AND FLUORESCENCE VISUALIZATION

- Dried agarose-Gelbond films are cut into 65 x 25mm strips, stained with SYBR Gold (1/10000 dilution of stock, Molecular Probes), rinsed with water, then placed on glass slides.

- A 50 x 22mm glass cover-slip is applied to the sample and firmly but gently pressed onto the gel, removing excess water and forming a tight seal.

- Fluorescence stained comet images are analysed on an Olympus BX-60 fluorescence microscope using a 'NB' filter cube, a programmable Hitachi KP-D8 digital camera and the Alkomet v3.3 image analysis system.

Note: It is important to use a nucleic acid stain which does not emit in the red spectrum where epifluorescence from the GelBond film occurs. We recommend SYBR Gold, which provides a high quantum yield with a relatively low background fluorescence.