Toxicology in Vitro 27 (2013) 768–773

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

A comparative performance test of standard, medium- and high-throughput comet assays

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ARTICLE INFO

Article history:
Received 4 April 2012
Accepted 7 December 2012
Available online 20 December 2012

Keywords:
Comet assay
Medium throughput
High throughput
Edge effect

ABSTRACT

A serious limitation of the conventional comet assay (single cell gel electrophoresis) is the restriction on the number of samples that can be processed in one experiment, imposed by the size of the electrophoresis platform. One approach to increasing throughput is to reduce the size of gels. We here compare the conventional system of two large gels on a microscope slide, with two recent developments, namely 12 minigels per slide, and a format with 96 minigels on GelBond® film. We used cells treated with X-rays or methylmethanesulphonate (MMS). The level of damage detected (% tail DNA) in X-irradiated or MMS-treated cells was not affected by the format used. Parallel experiments, using all three formats, were performed with MMS-treated cells in two independent laboratories; the difference in results between the two laboratories was of borderline significance. The potential problem of anomalous comets seen at the border of the gel, the so-called 'edge effects', has been addressed. A reliable, high throughput comet assay has applications in genotoxicity testing (particularly for *in vivo* studies with samples from different organs) as well as ecogenotoxicology and human biomonitoring, where the numbers of samples collected can be considerable.

1. Introduction

The alkaline comet assay (single cell gel electrophoresis) is a simple, economical and versatile assay for measuring DNA strand breaks (SBs) at the level of individual cells. After lysis of agarose-embedded cells the resulting 'nucleoids', comprising supercoiled DNA, are electrophoresed and DNA containing breaks extends towards the anode. On examination by fluorescence microscopy, the extended DNA forms a comet-like tail, the relative intensity of which reflects the break frequency. Digestion of nucleoids with enzymes that convert specific lesions to DNA breaks allows measurement of oxidised pyrimidines and purines, alkylated bases, and UV-induced pyrimidine dimers.

The number of samples that can be analysed in each experiment is limited by the size of the electrophoresis tank, which typically holds around 20 slides; with the conventional assay, with one or two gels per slide, the limit is therefore 20 or 40 gels. If each sample is to be analysed for SBs and for different kinds of damage using lesion-specific enzymes, the number of samples per experiment is further reduced. Biomonitoring and genotoxicology studies (both *in vivo* and *in vitro*) would greatly benefit from a high-throughput version of the comet assay, since the variability of the data will decrease if larger numbers of samples within a study are analysed in the same experiment. Initiatives such as the EU Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) legislation and the EU 7th Amendment Cosmetics Directive (which banned *in vivo* genotoxicity tests for cosmetics from 2009), have driven the need for high-throughput *in vitro* genotoxicity screens (Pfuhler et al., 2010).

During the last 15 years, several medium- and high-throughput approaches have been applied to the comet assay, using GelBond® films (McNamee et al., 2000), specific 96-well plates (Stang and Witte, 2009, 2010), glass microscope slides (Ritter and Knebel, 2009; Shaposhnikov et al., 2010) and micro cell arrays (Mercey et al., 2010; Wood et al., 2010). In this study we compare the results obtained with TK-6 lymphoblastoid cells treated with 0 and 250 μM methylmethanesulphonate (MMS) using the standard comet assay format (two large gels/slide; '2-gel format'), a medium throughput version (12 minigels/slide; '12-minigel format',

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http://dx.doi.org/10.1016/j.tiv.2012.12.006

Shaposhnikov et al., 2010), and the high throughput '96-minigel format' (minigels set within an 8 × 12 array on a GelBond® film; developed by K.B. Gutzkow and G. Brunborg and described in brief in Jackson et al., 2012), in two different laboratories participating in the EC-funded COMICS project. We also evaluate the three formats with X-irradiated cells (in one laboratory).

It is well known that comets close to the edge of the gel tend to be distorted, with tails differing in size, intensity, shape or even direction compared with comets located closer to the centre of the gel. With minigels the periphery occupies a large part of the whole area, and the importance of 'edge effects' and how to avoid them were therefore investigated.

2. Materials and methods

2.1. Cells

TK-6 cells (human-derived lymphoblastoid cell line) were grown in RPMI (Roswell Park Memorial Institute) medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum (Sigma), 0.2 mg/ml sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were maintained as a suspension culture at 37 °C in a humidified atmosphere with 5% CO_2. Each laboratory received an aliquot of the same original culture of TK-6 cells, cultured them routinely for several weeks, and carried out the treatment on the day of the experiment.

2.2. Treatment of TK-6 cells with X-rays

Five ml of TK-6 cells at 2.5×10^5 cells/ml in PBS were irradiated in Petri dishes with 10 Gy X-rays, or mock-irradiated. A Phillips Model MG300 X-ray unit was used at 260 keV and 10 mA. Radiation was filtered through 0.5 mm Cu and the dose rate was 10 Gy/min as determined by iron sulphate dosimetry. Cells were kept ice-cold to minimise DNA repair.

2.3. Treatment of TK-6 cells with MMS

Five ml of TK-6 cells at 2.5×10^5 cells/ml were treated with 250 μM MMS (Chiron AS, Trondheim, Norway) or the vehicle (water) for 3 h at 37 °C. After the treatment, cells were centrifuged and washed with fresh medium first, then in phosphate-buffered saline (PBS), and ultimately suspended in PBS at a cell concentration of 1.0×10^6/ml. Cells were kept ice-cold to prevent DNA repair.

2.4. Preparation of different formats

Three different formats were used: (i) two large gels per slide as the standard format; (ii) 12 minigels per slide as a medium-throughput format; and (iii) 24 minigels per GelBond® film arranged within a standard 8 × 12 array as a high throughput format (Fig. 1). Minigels typically contained two to four hundred comets, compared with a few thousand in standard gels. Two laboratories (A and B) were involved in the trial and both A and B used the three formats.

Cells in PBS were mixed with 0.5% low melting point agarose at 37 °C in the proportion 1:9. Treated and untreated cells were placed on the same slide/GelBond® film following the pattern of Fig. 1. Two 40 μl aliquots (one treated and one untreated) were placed on the slides for the standard format, and a glass cover-slip placed on each gel. Gels were allowed to set and cover-slips were removed. Twelve drops of 4 μl (six treated and six untreated) were placed on a slide for the medium-throughput format. Twenty-four drops of 4 μl (12 treated and 12 untreated) were applied to a GelBond® film for the 96-minigel format. (Cover-slips were not used on the minigels.)

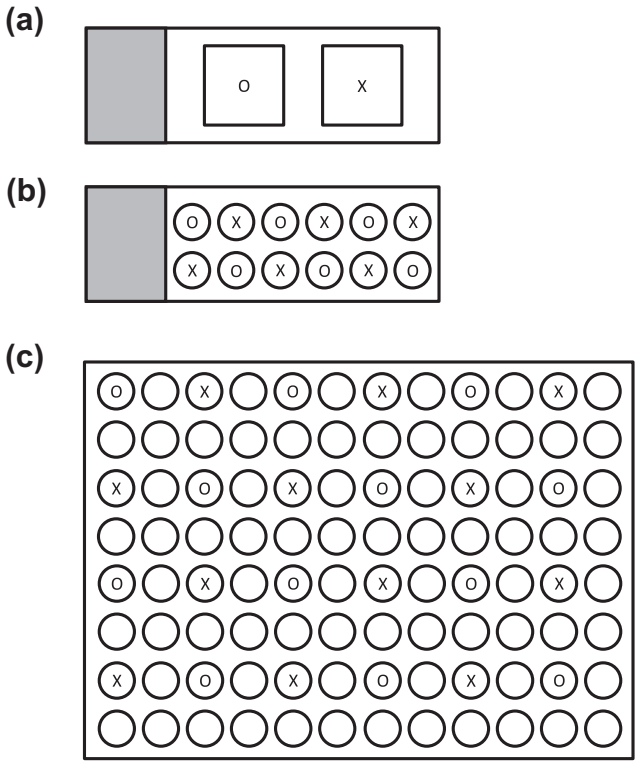

Fig. 1. The pattern followed when setting gels in the different formats: (a) 2 gels/slide, (b) 12 minigels/slide, (c) 24 minigels/GelBond film. X: cells treated with MMS or X-rays, O: cells treated with vehicle (water) or mock-irradiated.

One slide with two gels, one slide with 12 minigels, and a 96-minigel format GelBond® film with 24 minigels, were prepared per experiment in laboratory A. In laboratory B, four slides with two gels, two slides with 12 minigels and a GelBond® film with 24 minigels were prepared per experiment. Three independent experiments were performed in each laboratory. X-ray experiments were performed only in laboratory A.

2.5. Comet assay

Cells were lysed overnight by placing slides/GelBond® films in 2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 M Tris base, pH 10, 10% DMSO and 1% Triton X-100 (lysis solution) at 4 °C. Slides were then placed in a horizontal gel electrophoresis tank for 20 min in 0.3 M NaOH and 1 mM Na_2EDTA, pH >13, before the electrophoresis was carried out for 20 min at 0.7–1 V/cm in a 4 °C cold room. Laboratory A used a pump to recirculate buffer (75 ml/min) during the electrophoresis of the GelBond® films. The slides were neutralised by washing them three times for 5 min in 0.4 M Tris base (pH 7.5) at 4 °C and then rinsing in distilled water. Then slides were fixed in absolute ethanol for 1.5 h, air-dried overnight and stored at room temperature. For scoring, DNA was stained by immersing the slides in SYBR Gold® (Invitrogen), diluted according to the manufacturer's instructions in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0) for 30 min at 4 °C, followed by washing in water, drying, placing a drop of water on each gel and covering with a coverslip. A semi-automated image analysis system (Comet Assay IV; Perceptive Instruments) was used to evaluate 50 nucleoids per gel (or 30 per gel in the 96-minigel format, laboratory A). Percentage of DNA in tail was used to evaluate each comet.