

An improved non-enzymatic “DNA ladder assay” for more sensitive and early detection of apoptosis

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Abstract Conventional DNA ladder assay has certain shortcomings such as loss of DNA fragments during sample processing, involvement of multiple steps and requirement of expensive reagents. The present study demonstrates a rapid, easy-to-perform cost-effective method for detection of apoptotic DNA fragments with considerable improvement in the sensitivity by avoiding loss of DNA fragments. It involves a few minutes of procedure involving direct lysis of cells with dimethyl sulphoxide (DMSO), brief vortexing, addition of 2% SDS–TE buffer, and a single step of centrifugation. This cost- and time-efficient method reduces the assay time considerably and can be used for a large number of samples with excellent sensitivity.

Keywords Cell death · Apoptosis · Sf9 · DNA ladder · DNA fragmentation

Introduction

Apoptosis is a highly regulated cellular process that involves the activation of distinct biochemical/

molecular cascades and can manifest into morphological features characterized by cell shrinkage, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. Cellular/molecular assays for detecting externalization of phosphatidylserine moieties (using annexin-V staining), cytosolic cytochrome-c release from mitochondria (using western blotting or immunofluorescence microscopy) and caspase activity are used routinely for monitoring apoptosis, besides routine morphological assessment. Fragmentation of DNA and chromatin is an integral process during apoptosis, and has been reported to occur in more than one distinct stage. During initial stages, high molecular weight DNA fragments of 50 kbp or longer size have been observed in morphologically normal cells committed to undergo apoptosis. The low molecular weight DNA fragments are associated with late events such as formation of apoptotic bodies (Czene et al. 2002), although the phenomenon of nucleosome excision (ladder formation) is also reported to initiate before any obvious apoptotic changes in cell morphology (Tanuma et al. 1993).

The extensive DNA fragmentation induced during apoptosis can be detected using techniques such as DNA ladder assay (agarose gel electrophoresis), terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL assay) and comet assay (Otsuki 2000; Chandna 2004). Out of these methods, DNA fragmentation assay using agarose gel electrophoresis is the most frequent technique used for the detection of apoptosis and can easily

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discriminate between apoptotic and non-apoptotic (necrotic) modes of cell death, as in most cases the inter-nucleosomal cleavage of genomic DNA yielding the characteristic DNA ladder is a molecular hallmark of apoptotic cells (Kerr et al. 1972; Pandey et al. 1994). In the typical DNA fragment ladder obtained, molecular weights of the genome fragments are integer multiples of 180 base-pairs length associated with a nucleosome subunit. On the other hand, genomic fragments of irregular sizes are generally induced during necrotic cells, and a DNA smear is obtained during agarose gel electrophoresis.

The conventionally used DNA fragmentation assay involves separation of DNA fragments following “phenol–chloroform” method of DNA isolation, which has been the method of choice. However, it is associated with certain drawbacks such as requirement of relatively large amount of starting material (e.g., in vitro cultured cells or tissue pieces), loss of smaller fragments during the precipitation step and longer time-consumption. In addition, it is cumbersome to use for large number of samples due to the long processing time involved. In recent past extensive efforts has been made to develop simpler assay methodology for detection of apoptosis (Singh 2000; Rosl 1992; Basnakian and James 1994; Lamm et al. 1997; Willingham 1999; Saraste and Pulkki 2000; Sgonc and Guber 1998; Compton 1992; Nicoletti et al. 1991; Umansky et al. 1981). A simple DNA fragmentation method developed by Gong et al. (1994) is although rapid and has few advantages as well but it may have reduced sensitivity because of ethanol fixation, which is known to remove smaller DNA fragments during processing (Telford et al. 1991, 1992) and also require enzymatic removal of proteins and RNA (Gong et al. 1994). In addition to these modifications in DNA ladder assay other methods of apoptosis detections such as radioisometric, fluorimetric, flow cytometric and comet assay based methods were also tried for rapid detection of apoptosis (Willingham 1999), but despite these efforts the conventional DNA ladder method is still popular and is being widely used indicating the need of simplification, which can be easily adapted by the researchers.

In this study, we report a much simpler and shorter alternative method for isolating DNA with increased sensitivity for DNA fragment detection. We used Sf9 insect cells that display classical apoptotic morphology when treated with apoptogenic agents such as

Actinomycin-D (a transcriptional inhibitor), Etoposide (topoisomerase inhibitor) and ionizing radiation (Chandna et al. 2004; Suman et al. 2009; Kumarswamy et al. 2009). In order to demonstrate the advantages of this new method, we validated the results with conventional phenol–chloroform method, and monitored morphological apoptotic alterations using the differential image contrast (DIC) microscopy. Our results show that the proposed method not only detects DNA fragments with improved sensitivity, it can also facilitate assessment of apoptosis in a large number of samples due to ease of processing.

Materials and methods

Cell-culture

Sf9 cells were maintained as monolayers in 25 cm² culture flasks (T-25) at 28 °C in Grace’s insect cell medium (Sigma, St. Louis, MO, USA) supplemented with 3.33 g/L lactalbumin hydrolysate, 3.33 g/L yeastolate, 0.35 g/L NaHCO₃ and antibiotics (Penicillin-sodium salt 50,000 units/L, Streptomycin sulphate 50,000 µg/L, Nystatin 2,000 µg/L from 500,000 USP units/mg; all Sigma USA). Growth medium (pH 6.2) was prepared by adding 10% heat inactivated FBS (Fetal Bovine Serum, Sigma, USA) and stored at 2–8 °C. Sf9 cells were regularly sub-cultured twice a week in exponential phase by seeding at a density of 40,000–50,000 cells/cm² area.

Irradiation and treatment with apoptotic agents

Cells were irradiated in exponential growth phase at dose-rate of 36.66 Gy/min in a ⁶⁰Co gamma chamber (Gamma Chamber 5000, Board of Radiation and Isotope Technology, Department of Atomic Energy, Mumbai, India). Actinomycin-D (0.5 µg/mL; Sigma USA), Etoposide (100 µM, Dabur, India) were directly added to the culture media of cells. Cells were harvested at 8, 24 and 48 h time-points and for analysis of DNA fragmentation.

Live cell morphology analysis

Cells undergoing apoptosis were observed by Nomarski-DIC time-lapse imaging using the Axiovert 200

Zeiss inverted microscope (Carl Zeiss, Germany) and Axiovision software (version 4.0).

Detection of DNA fragmentation (ladder assay)

DNA isolation was done using following methods:

- (1) *The conventional “phenol–chloroform” DNA isolation method:* 3–4 million Sf9 cells were taken as starting material for DNA isolation. Cells were dislodged in the culture medium, and centrifuged at 5,000g to obtain the pellet containing both intact and apoptotic cells. Cells were further washed with PBS (phosphate buffer saline, pH 7.4) and again centrifuged at 5,000g before overnight lysis of the cell pellet with 150 μ L of lysis buffer (10 mM EDTA, 0.5% SDS, 10 mM Trizma Base and 0.5 μ g/mL Proteinase K; pH 7.5). Proteinase-K was deactivated by heating at 75 °C for 15 min followed by addition of RNase-A (0.5 μ g/mL) at 37 °C for 2 h. DNA was isolated by phenol–chloroform–isoamyl alcohol method and DNA was dissolved in 40 μ L TE buffer (pH 7.4). The total 40 μ L DNA solution was loaded on 2% agarose gel (Hermann and Frischauf 1987; Zhuang et al. 2008).
- (2) *The new DMSO (dimethyl sulphoxide)–SDS (sodium dodecyl sulphate)–TE (Tris-EDTA) method:* 1.8–2 million Sf9 cells were taken as starting material for DNA isolation. Cells were dislodged and washed with PBS in the same manner as used in conventional method. Instead of overnight lysis used in the conventional method, 100 μ L of DMSO was added directly to the cell pellet and mixed well followed immediately by vortexing. Equal volume (100 μ L) of TE buffer (pH 7.4) with 2% SDS was added, followed by mixing and vortexing. The resulting solution was centrifuged at 12,000g at 4 °C and 40 μ L of the supernatant (equal volume as used for the conventional assay) was loaded on agarose gel. The isolated DNA was analyzed using UV-spectroscopy by recording the absorbance at 260, 280 and 230 nm respectively. Further, the A260/A280 and A260/A230 was calculated to analyze the purity of the isolated DNA and the isolated DNA was found to be of good quality. Stability of the isolated DNA was also analyzed by running the same sample

after few days. When samples were stored at 4 °C, DNA degradation was not observed up to 3 days of storage.

Results and discussion

The DNA fragmentation assay involves two basic steps, viz., isolation of genomic DNA and separation of DNA fragments using agarose gel electrophoresis. The untreated normal cells yielded a very high molecular weight DNA in the range of 5–7 kbp but a ladder could be seen in apoptotic cells using the phenol–chloroform (PC) method of DNA isolation. Although this method yields reasonably good quality of DNA, it involves multiple time-consuming steps and is cumbersome to use for a large number of samples. Besides, it is prone to loss of low molecular weight fragments during centrifugation and phase-separation steps, and DNA may also get degraded due to mishandling during these multiple steps. Therefore, good level of skill is required to isolate DNA using the PC method.

In the new protocol detailed herein, we modified the DNA isolation steps in such a manner as to retain good quality DNA while considerably reducing the overall time of processing and minimizing chances of losing smaller DNA fragments. For this method we selected DMSO as the component of lysis buffer because of being a polar-aprotic solvent (Wang et al. 2008; Di and Kerns 2006). Lipids and proteins have limited solubility in DMSO, which removes proteins through its denaturing and precipitating action and also inhibits the activity of nucleases (Arakawa et al. 2007), which helps reduce false DNA fragmentation during sample processing. In addition, DMSO is known to inhibit the formation of folded DNA structure (Kang et al. 2005), which results in increased band intensity on the agarose gel following staining with fluorochromes such as ethidium bromide.

Since the DNA fragmentation is known to proceed before the onset of morphological changes during apoptosis, we studied morphological changes and DNA fragmentation at an early time-point (8 h) when very few apoptotic bodies were visible (Fig. 1). No DNA fragmentation could be detected using the PC method (lane 3, 4, 5 in Fig. 2), whereas the DMSO–SDS–TE method clearly shows the presence of

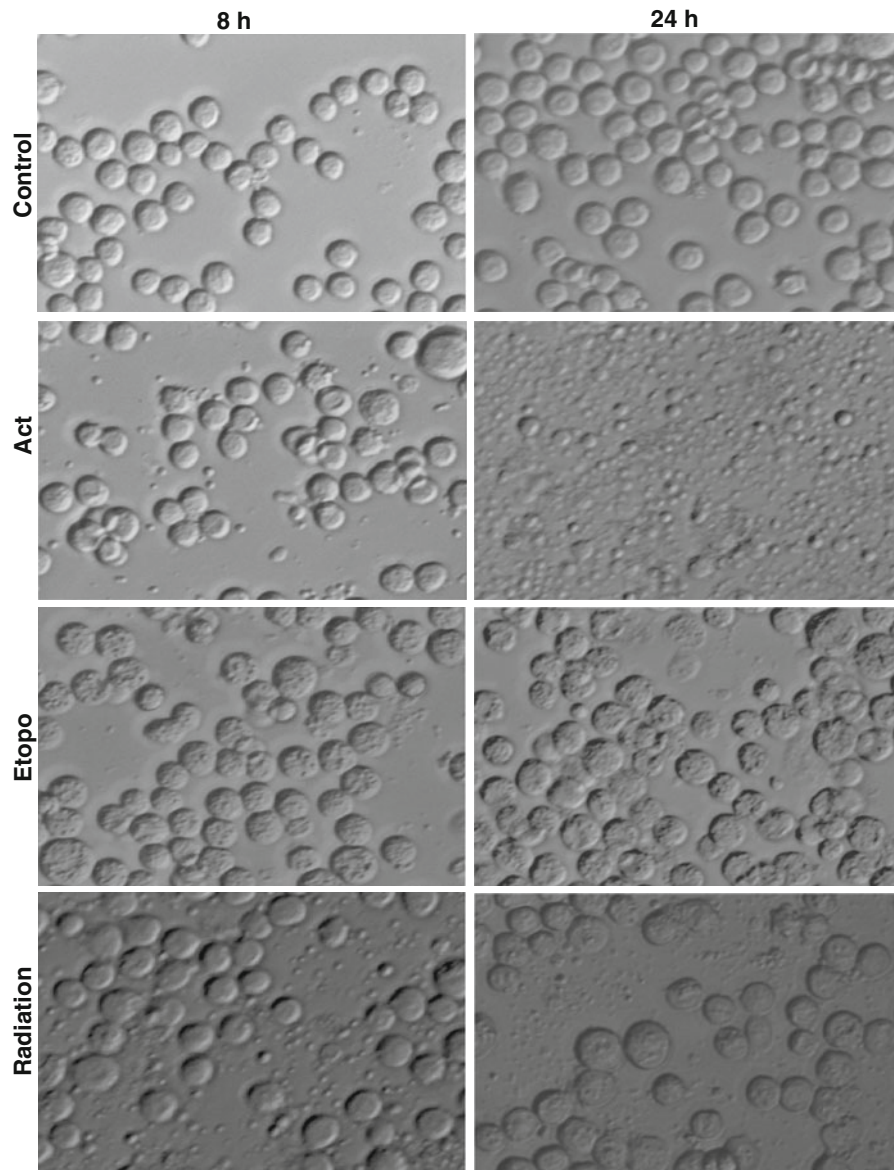


Fig. 1 Morphological analysis of stress-induced apoptotic body formation in Sf9 cells. Cells were treated with known apoptogenic agents; Actinomycin D (0.5 $\mu\text{g}/\text{mL}$), Etoposide (100 μM) and γ -radiation (2,000 Gy). Images were acquired

with an Axiovert 200 Zeiss inverted microscope (Carl Zeiss, Germany) using Nomarski-DIC mode and Axiovision (version 4.0) software

fragmented DNA (lane 3, 4, 5 in Fig. 3). This ability of the new DMSO-SDS-TE method to detect DNA fragmentation at early time point was accompanied by a better yield of low molecular weight fragments at later time-points, while high molecular weight band was predominant in the PC method especially after etoposide treatment or irradiation (lane-7, 8 in Figs. 2b, 3b). Therefore, our results show that the DMSO-SDS-TE method can detect the low molecular

weight DNA fragments with much better sensitivity compared to the conventional PC method.

Although DNA ladder is seemingly a simple assay, yet the extensive use of this technique prompted us to enhance its sensitivity and reliability through this super-short and highly simplified procedure. The obvious multiple advantages of the new DMSO-SDS-TE method are: (1) negligible loss of DNA fragments that increases the yield of DNA fragments

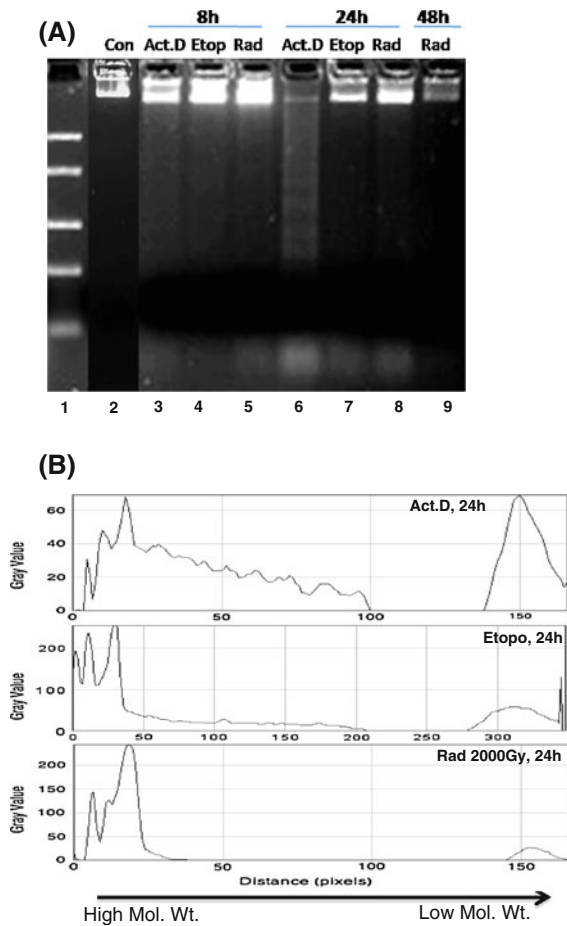


Fig. 2 DNA ladders of treated Sf9 cells obtained using the conventional phenol–chloroform method. **a** 3×10^6 cells were used and 40 μ L of final sample was loaded in all lanes. Electrophoresis was performed on 2% agarose gel. **b** Densitometric analysis of ladder lanes using Image-J software, with distinct peaks representing a single band on the gel, ranging from high to low molecular weight

and enhances sensitivity of detection, (2) early detection of DNA fragmentation due to increased sensitivity, (3) extremely short (less than 30 min) processing of cells as compared to the conventional (overnight) isolation method, (4) suitability for use in large number of samples, (5) cost-effectiveness (requiring fewer reagents and chemicals), and (6) higher stability of the isolated sample (up to 3 days when stored at 4 °C). Limitation of this method is that the DNA isolated using this protocol cannot be used for other purposes such as PCR, because of presence of high amount of DMSO. Therefore, experiments only aiming at detection or monitoring of DNA fragmentation

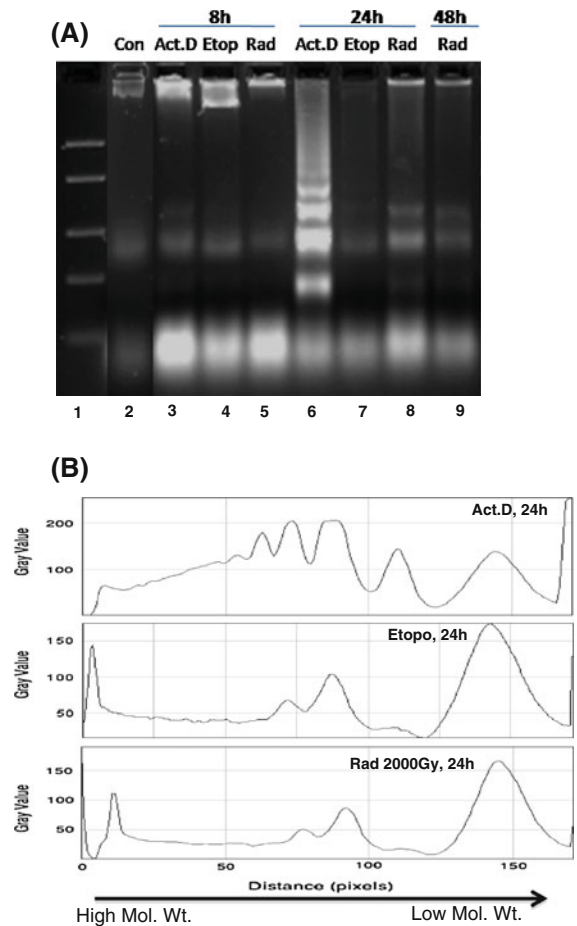


Fig. 3 DNA ladders of treated Sf9 cells obtained using the new DMSO-SDS-TE method. **a** Equal volume (40 μ L) of lysate was loaded in all lanes. Electrophoresis was performed on 2% agarose gel. Relative abundance of small DNA fragments is evident in the new assay compared to the conventional method. **b** Densitometric analysis of ladder lanes using Image-J software, with distinct peaks representing a single band on the gel, ranging from high to low molecular weight

should be performed using this technique, for which the new technique definitely offers an edge over the existing phenol–chloroform method. We believe that this short and sensitive technique will be very useful for numerous labs that routinely study cell death or carry out routine experimental/clinical screening of drugs and chemotherapeutics.

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