Benchmarks

Detergent and Enzyme Treatment of Apoptotic Cells for the Observation of DNA Fragmentation

BioTechniques 24:558-560 (April 1998)

The uniform degradation of genomic DNA into oligomers of approximately 180–200 bp, or multiples of that, characterizes internucleosomal cleavage of DNA. Such fragmentation is a biochemical hallmark of apoptosis (6). Agarose gel electrophoresis is the most common way to demonstrate the ladder pattern of apoptotic DNA. Traditional purification protocols for the isolation of DNA from apoptotic cells require RNase and proteinase digestion of samples. DNA is then extracted using potentially hazardous solvents such as phenol and chloroform, followed by precipitation. Additional time is needed to dissolve the precipitated DNA into buffer before the sample can be analyzed by agarose gel electrophoresis for the presence of internucleosomal DNA fragmentation. This procedure can take two days. The quantity and quality of DNA recovered from apoptotic cells varies depending on the manipulation of the sample and the protocol used (5). Shearing of DNA during extraction results in the appearance of smears on agarose electrophoretic gels that can obscure the internucleosomal-sized DNA fragments seen in apoptosis. These ambiguous results could easily be misinterpreted as necrosis based on the DNA degradation patterns observed (1,2). In addition, many figures in journals depicting gel electrophoresis of apoptotic DNA lack the complete spectrum of internucleosomal-sized fragments because of the absence of either large or small oligomers, therefore resulting in loss of data (3,4).

We demonstrate that solvent extraction and subsequent precipitation are not required for analysis of DNA fragmentation. Only gentle detergent and enzyme treatment of apoptotic cells at 37°C are required to observe DNA fragmentation by agarose gel electrophoresis. Our method provides a rapid test for detection of the smaller apoptotic oligomers produced in a ma-

jority of apoptotic cells. It could also be used to analyze large DNA fragments by pulse gel electrophoresis in which genomic DNA is not completely fragmented.

This new procedure uses high-percentage agarose gels to increase resolution for smaller oligomers. This allows us to quickly determine whether a cell undergoes apoptotic internucleosomal DNA fragmentation. With 45 min of DNA preparation and 1 h of electrophoresis, we are able to discern that DNA fragmentation has occurred in various cell lines. Thus, preparation and electrophoresis time is greatly reduced, and the yield and integrity of the DNA sample are not compromised by extraction and precipitation procedures. In addition, reduced cost of the reagents and limited exposure to hazardous solvents make this protocol a much more attractive alternative to conventional DNA extraction methods.

For demonstration of this method, we induced apoptosis in L929 and WEHI-164 cells with tumor necrosis factor (TNF) and BW5147 cells with dexamethasone. Treatment of L929 and WEHI-164 cells with 1000 U of murine recombinant TNF (Genentech, San Francisco, CA, USA) per milliliter for 18 h induces apoptosis (1). BW5147, a T-cell lymphoma cell line, also undergoes glucocorticoid-induced apoptosis within several hours of treatment with 10^{-7} M dexamethasone. Treated and untreated cells were collected in 1.5-mL microcentrifuge tubes and washed once with 1 mL of 0.02% EDTA in Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} . The cells were pelleted by centrifugation for 5 min at $700\times g$; then 40 μL of the TE lysis buffer (10 mM Tris-HCl, 1 mM sodium-EDTA, pH 7.5) containing 0.25% Nonidet® P-40 (NP40) or Triton® X-100 were added along with 5 μL of an RNase A solution (20 mg/mL) (Sigma Chemical; St. Louis, MO, USA). The mixture was suspended by gentle vortex mixing and incubated for 20 min at 37°C. Then, 5 μL of a proteinase K solution (20 mg/mL) (Sigma Chemical) were added to the sample and incubated an additional 20 min or until the solution cleared. A 6 $\times$ loading buffer (5 μL of 0.025% bromophenol blue, 0.025% xylene cyanol FF, 30% gly-

cerol) was added to 25 μL of the sample. The sample was then analyzed by electrophoresis on a 1.8% agarose minigel in TE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 1–4 h at 36 V. Ethidium bromide-stained DNA was visualized by transillumination with UV light (300 nm) and photographed (5).

For fragmentation analysis, the sample preparation time using conventional organic solvent extraction methods required 2 days, compared to 45 min for the technique described above. The induction of internucleosomal DNA fragmentation in various cell lines used was highly discernible using this technique. Figure 1A shows electrophoresis of DNA isolated from L929 cells untreated and treated with TNF for various times. After 6 h of exposure to TNF, induction of apoptosis in L929 cells is clearly evident by distinct oligosomal DNA fragments ranging from 2.4 kb through the final 200-bp fragment (Figure 1A, lane 4). As determined by trypan blue exclusion, 72% of the L929 cells were still viable after 6 h of TNF treatment. DNA samples extracted from the same number of

Figure 1. A comparative gel demonstrating differences between proteinase/detergent and phenol/chloroform DNA preparation techniques using TNF-induced apoptotic DNA from L929 cells. (A) DNA isolation by proteinase and detergent treatments from 5×10^5 L929 cells untreated (lane 1) or treated with 1000 U TNF/mL for 2, 4, 6 and 24 h (lanes 2–5) were electrophoresed on a 1.8% mini agarose gel for 4 h. L929 cells exposed to TNF for 2 and 4 h were 96% viable by trypan blue exclusion; however, by 6 and 24 h, viability reduced to 72% and 9%, respectively. (B) DNA from 5×10^5 L929 cells either untreated (lane 1) or treated with 1000 U TNF/mL for 24 h (lane 2) was isolated by conventional phenol/chloroform extraction methods and electrophoresed on a 1.8% mini agarose gel for 4 h.

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TNF-treated L929 cells by conventional solvent extraction methods result in degradation of the sample and loss of smaller-sized oligomers (Figure 1B). As a result, the typical oligosomal ladder associated with apoptotic DNA fragmentation is very difficult to discern because of smearing and the absence of the lower portion of the DNA fragmentation ladder. Furthermore, induction of apoptosis and the resultant DNA fragmentation by dexamethasone treatment of BW5147 cells, a T-cell lymphoma, could be detected within 24 h before any significant loss of cell integrity could be detected by trypan blue exclusion (Figure 2, lane 3). DNA fragmentation in TNF-treated WEHI-164 cells is also highly discernible after electrophoresis for 1 h on a 1.8% agarose gel (Figure 2, lane 5). Interestingly, this technique is able to detect WEHI-164 cells going through senescence in culture, presumably by apoptosis, because DNA fragmentation could be detected in untreated samples (Figure 2, lane 4). Because WEHI-164 grows well in culture, we presume only a small proportion of the cells spontaneously undergoes DNA fragmentation. This indicates that the procedure can detect DNA fragmentation in a generally healthy cell population. Our laboratory has also successfully used this technique to isolate and visualize apoptotic DNA from various cell lines treated with camptothecin, a topoisomerase II inhibitor and apoptosis inducer (data not shown).

Figure 2. Apoptotic DNA fragmentation induction in a B-cell lymphoma and fibrocarcinoma by glucocorticoid and cytokine treatment. *Hind*III-digested λ DNA (lane 1) and DNA from untreated 5×10^5 BW5147 and WEHI-164 cells, either untreated (lanes 2,4) or treated with 10^{-7} M dexamethasone or 1000 U TNF/mL, respectively, for 24 h (lanes 3,5), were electrophoresed on a 1.8% mini agarose gel for 1 h.

This technique results in sharp 200-bp multiples indicative of the activation of endonucleases in the apoptotic processes. Importantly, high-percentage agarose gels allow tight band formation and thus distinct visualization of laddering patterns by smaller apoptotic DNA fragments after as little as one hour of electrophoresis. This protocol simplifies the identification of internucleosomal DNA fragmentation in a variety of apoptotic cells, thus improving the ability to produce rapid and concise results.

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This work was supported by the American Heart Association and the Geist Foundation. Address correspondence to Darren J. Park, Department of Microbiology, University of Hawaii, 2538 The Mall, Snyder Hall 318, Honolulu, HI 96822, USA. Internet: darrenp@hawaii.edu

Received 23 June 1997; accepted 15 December 1997.

Darren J. Park and Paul Q. Patek
University of Hawaii
Honolulu, HI, USA