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# An ELISA for detection of apoptosis

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## ABSTRACT

**We describe a simple and convenient enzyme-linked immunosorbent assay (ELISA) for the detection of apoptosis in tissue culture. An early event in apoptosis is DNA fragmentation followed by release of nucleosomes into the cytoplasm. Our sandwich assay uses a pair of monoclonal antibodies specific for two nucleosomal epitopes to capture and detect cytoplasmic nucleosomes onto the ELISA plate. Our assay is about 500 times more sensitive than the detection of apoptotic DNA ladder by agarose electrophoresis and is especially suited for the testing of large numbers of samples.**

Apoptosis is an active process of cell death and is morphologically characterized by nuclear condensation and blebbing of the plasma membrane. Activation of endonucleases during the process of apoptosis leads to the fragmentation of chromatin into oligonucleosomal fragments of multiples of ~180 bp (1). Chromatin condensation and membrane blebbing, hallmarks of apoptosis, were initially assessed by electron microscopy, a labor intensive method. Another approach that has been widely used is visualization by agarose gels of the characteristic DNA laddering of oligonucleosomal fragments (2). This procedure is not readily amenable to the study of numerous samples. Recently, analysis of cell cycle status of populations by flow cytometry has been adapted for detection of apoptotic cells. Since apoptotic cells are smaller in size than normal cells due partly to nuclear condensation, they can be quantitatively detected as hypodiploid cells in flow cytometric histograms where they appear as a peak to the left of the $G_0/G_1$ peak (3). However, it has been reported that with human lymphocytes the peak representing apoptotic cells is not always sharp and overlaps with the $G_0/G_1$ peak (4). Cell viability assays using vital dyes are easy to perform (5), but very often underestimate the number of apoptotic cells, since DNA fragmentation is an early event in apoptosis, whereas cell membrane disintegration occurs later. Another quantitative method that is widely used for measuring DNA fragmentation is release of $^3H$ thymidine by prelabeled cells that are subjected to apoptosis induction (6). A disadvantage of this approach is the requirement for active cellular growth.

An early event in apoptosis is DNA fragmentation and release of nucleosomes into the cytoplasm. We describe in this report a sensitive enzyme-linked immunosorbent assay (ELISA) for apoptosis based upon the detection of cytoplasmic nucleosomes. The nucleosome is the basic unit of chromatin and results from the ordered association of histones and DNA (7). The double-antibody sandwich ELISA that we describe is based upon the specific recognition of nucleosomes by a pair of monoclonal antibodies (mAb). In our assay, nucleosomes in cytoplasmic lysates are captured onto ELISA plates coated with a first mAb reacting with an exposed epitope of histone H2B. The bound nucleosomes are then detected by a second mAb specific for the complex formed by the H2A–H2B dimer and DNA.

We used serum starvation as a means to induce apoptosis in the human T cell line Jurkat in tissue culture. We first verified the induction of apoptosis using a DNA fragmentation assay as a reference method (8). The intensity of the DNA ladder increased with the duration of serum starvation, indicating a corresponding increase in apoptosis in the Jurkat cell line (not shown). In subsequent experiments, cytoplasmic lysates from day one cell culture served as the apoptosis negative control, whereas lysates from day four culture were used as the apoptotic sample. Two murine mAbs generated in our laboratory were used in our ELISA (both mAbs can be requested from the corresponding author). The capture mAb, LG11-2, an IgG2aκ obtained from an autoimmune MRL/*lpr* mouse, is specific for the N-terminus of histone H2B and does not react with other histone molecules (not shown). The detection mAb, PL2-3, is an IgG2aκ specific for the nucleosome subparticle composed of histones H2A, H2B and DNA (9).

In the double-antibody sandwich ELISA described in this report, cytoplasmic nucleosomes are captured onto plates coated with LG11-2 mAb and then detected with biotinylated PL2-3 mAb and a streptavidin–alkaline phosphatase conjugate. We first determined the concentration of detection antibody required for an optimal signal to noise ratio. Serial dilutions of biotinylated PL2-3 antibody from 10 to 0.08 μg/ml were tested, while a constant amount of $10^4$ cell equivalents of cytoplasmic lysate per well was used in the assay. In pilot ELISA experiments, we had previously determined that this amount of lysate yielded a consistently strong signal (not shown). Figure 1A displays the titration of PL2-3 antibody and indicates that the signal difference between control and apoptosing samples increases proportionally to antibody concentration until a virtual plateau at the highest PL2-3 concentrations. The specificity of this assay is evidenced

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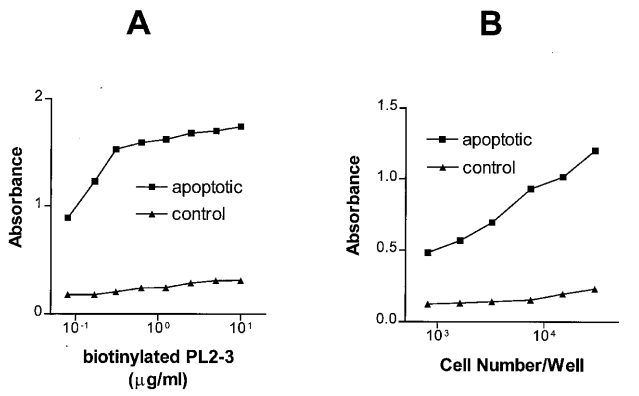


**Figure 1.** ELISA detection of apoptotic cytoplasmic lysates. Cells were washed, pelleted, counted and resuspended in ice cold lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris–HCl pH 7.5) at $25 \times 10^6$ cells/ml. After 30 min incubation on ice, the lysate was spun at 1500 *g* for 5 min to pellet cell debris and nuclei. Cytoplasmic apoptotic DNA in the supernatant was collected and stored at –20°C for the ELISA which was conducted as follows. Polyvinylchloride microtiter plates (Falcon ref. 3912) were coated with 50 µl/well of LG11-2 capture antibody (2 µg/ml) diluted in 0.05 M carbonate buffer pH 9.6. After overnight incubation at 4°C or 2 h at room temperature, plates were washed three times with 0.15 M PBS–0.05% Tween-20 (PT) and blocked with 50 µl/well PBS, 1% BSA, 0.05% Tween-20, 0.02% sodium azide (PBTN) for 1 h at room temperature. Following three washes in PT, 50 µl cytoplasmic lysate diluted in PBTN was added to each well and incubated at room temperature for 2 h. Plates were washed four times with PT and incubated with 50 µl/well of biotinylated PL2-3 detection antibody (2.5 µg/ml in PBTN in the standard assay) for 1.5 h at room temperature. After three washes with PT, 50 µl/well of alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Assoc., Inc.) diluted 1:2000 in PBTN was added. After 30 min at room temperature, the plates were washed twice in PT and then four times in substrate buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$). 150 µl/well *p*-nitrophenylphosphate (1 mg/ml substrate buffer) was added and permitted to develop in the dark at room temperature for 1 h. Optical densities were then measured at 405 nm using a $V_{max}$ kinetic reader (Molecular Devices). (**A**) Varying concentrations of biotinylated PL2-3 detection mAb were used in this assay while the amount of cytoplasmic lysate remained constant ($10^4$ cell equivalent per ELISA well). (**B**) Varying amounts of cytoplasmic lysate ($3 \times 10^4$ to $8 \times 10^2$ cell equivalents per ELISA well) were tested at a constant concentration (2.5 µg/ml) of biotinylated PL2-3 mAb.

in part by the saturability of the apoptotic DNA absorbance signal. From this experiment, we selected 2.5 µg/ml as the PL2-3 mAb concentration for the remainder of the assays.

In our experience, a minimum of $5 \times 10^5$ cell equivalents of apoptotic DNA can be clearly visualized on an ethidium bromide agarose gel. For maximum utility, we sought an ELISA that could detect as low a number of apoptotic cells as possible. We therefore tested cytoplasmic lysates that were serially diluted from $3 \times 10^4$ to $8 \times 10^2$ cell equivalents per ELISA well (Fig. 1B). Even at $8 \times 10^2$ cell equivalents the signal from the apoptotic cell lysate was five times higher than that of the control cell lysate. Over the same dilution range of cytoplasmic lysate, the signal of the control sample remained constant, confirming the inherent low background of the assay. These data indicate that our ELISA has ~500 times greater sensitivity in detecting apoptosis when compared to the benchmark DNA ladder fragmentation assay. Furthermore, our assay can be rendered semi-quantitative by expressing results relatively to an apoptotic positive control (10). This ELISA is easy to perform, extremely sensitive, and particularly suited to detect apoptosis in a large number of samples.

## ACKNOWLEDGEMENTS

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