Measuring multiple apoptosis parameters with the Agilent 2100 bioanalyzer

A simplified and fast protocol for the analysis of DNA fragmentation during apoptosis

Application

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Abstract

This Application Note describes a simplified and fast protocol for apoptosis DNA fragmentation analysis with the Agilent 2100 bioanalyzer using the DNA LabChip® kit. The combination of Annexin V, active-caspase-3 and DNA laddering analysis on the same platform together with the Cell Fluorescence LabChip kit permits quick confirmation and quantitation of apoptosis in cell populations. A fast and simple protocol was applied for the extraction of fragmented, chromosomal DNA. The increased sensitivity and sizing accuracy of the DNA LabChip kit compared to conventional agarose gel electrophoresis also means that a significant lower amount of apoptotic cells is required for the analysis.

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Introduction

The Agilent 2100 bioanalyzer was introduced by Agilent Technologies as the first commerially available lab-on-a-chip analysis system for the life science laboratory using LabChip® products, developed in collaboration with Caliper Technologies Corp. Chip-based approaches for a variety of separation-based techniques have been introduced, addressing DNA, RNA and protein separations.[1, 2, 3]. Recently the Agilent 2100 bioanalyzer has been extended to enable dual color simple flow cytometric assays[4]. Apoptosis assays have been demonstrated for quantitative measurements of whole cells[5]. The combination of cytometric and gel-like separations on the same instrument platform makes it an ideal tool for multi-parametric apoptosis quantitation and confirmation.

Apoptosis

Apoptosis, or programmed cell death, is the outcome of a metabolic cascade that results in cells dying in a controlled manner. It is used by nature during development[6], homeostasis, aging and in defense mechanisms. Apoptosis is a key factor in cancer and also suspected to be a characteristic mechanism for degenerative disorders such as Alzheimer[7] or Parkinson's diseases. The process is universal and has been described in eukaryotic cells as well as in individual bacteria[8], protozoa[9] and amoeba[10]. It is a target for research scientists in a wide variety of fields. Apoptosis is characterized by a distinct set of morphological events involving plasma membrane blebbing and asymmetry loss, reduction of cell volume, loss of mitochondrial membrane potential, nuclear condensation, fragmentation of DNA at nucleosomal intervals and other cytoplasmatic changes. Ultimately fragmentation of the cell into membrane-enclosed “apoptotic bodies” occurs. Differential expression patterns or alternate mechanisms in the apoptosis pathways among tissues and cell lines makes it necessary to have two or even three parallel experiments to confirm, quantitate and compare kinetic events on apoptosis.

The appearance of DNA laddering is unambiguously connected to apoptosis. Targeting of phosphatidyl serine (PS) in the outer leaflet of cell membrane with fluorescently labeled annexin V and antibody labeling of active-caspase-3 in cells has been previously described in conjunction with the Agilent 2100 bioanalyzer as a quantitative measurement for apoptotic cell samples[5].

This Application Note describes the combined measurement of annexin V and the extracted DNA of apoptotic cells on one platform – the Agilent 2100 bioanalyzer. A fast protocol for DNA preparation and recommendations for data evaluation are discussed.

Experimental

Apoptosis was induced in Jurkat cells by incubation in a 5 µM solution of the topoisomerase inhibitor Camptothecin (Sigma, # C9911) in medium. Cells were harvested and counted at the indicated time intervals. Cell lysis and DNA purification was performed as described in the fast protocol below. Eluted DNA was directly loaded into the chip wells, prepared according to the LabChip Reagent Kit Guide.

Fast protocol for DNA fragment purification (based on original protocol in reference 11)

- Pellet 1-2 × 10^6 cells by centrifugation (2 min × 400 g). Remove medium.
- Add 100 µL 4 °C lysis solution (0. 2% Triton X, 10 mM Tris, 10 mM EDTA) and gently resuspend.
- Incubate 5 min at 4 °C.
- Centrifuge for 5 min at 13000 g.
- Transfer supernatant to new vial and discard the pellet.
- Purify DNA using a low volume elution method. Here a PCR purification kit* was used (QIAGEN MinElute PCR Purification Kit. Cat.28004).
- Run in Agilent 2100 bioanalyzer with the DNA 12000 LabChip Kit, selecting the DNA 12000 Laddering assay from the biosizing software.

*As an alternative to the PCR purification kit, the following protocol has been successfully tested:

- Add 2 µL 5M NaCl to the lysate and vortex 5 seconds.
- Add 200 µL ice-cold absolute ethanol and vortex 5 seconds.
- Incubate on ice for 10 minutes
- Centrifuge at 13000 g for 5 minutes. Discard supernatant.
- Dry residual ethanol in speed-vac or desiccator.
- Resuspend precipitate in 30 µL of pre-warmed (65 °C) distilled water or 10 mM Tris/10 mM EDTA.
- Run in Agilent 2100 bioanalyzer with the DNA 12000 LabChip kit, selecting the DNA 12000 laddering assay.

UV measurements

UV spectroscopy readings were performed to determine total DNA concentration. The Agilent 8453 UV-visible spectrophotometer was used in conjunction with a 0.2-cm light path ultra-micro cell (10 µl volume). The whole spectrum (190–1100 nm) was acquired and evaluation was automatically done by the Warburg-Christian method[12] with internal wavelength correction at 320/10 nm.

Annexin V apoptosis measurements

A fast annexin protocol as described in reference 13 was used.

Results and Discussion

Besides DNA laddering, cytometric assays like annexin V or caspase analysis are commonly used for apoptosis detection. Figure 1A shows the analysis of annexin V in Jurkat cells on the Agilent 2100 bioanalyzer after 6 hours treatment with 5 µM campthotecin. After switching the instrument to electrophoresis mode, extracted DNA from treated Jurkat cells was analyzed using the DNA 12000 LabChip kit. The characteristic DNA laddering further confirmed apoptosis in the sample (figure 1B).

Sizing information, provided by the 2100 bioanalyzer software, shows nucleosomal fragmentation of DNA by evenly distributed peaks in the electropherogram (figure 2). Apoptosis is confirmed in samples by fragmentation of the chromosomal DNA in 180–200bp intervals.[14]

Typical DNA extraction with the described fast protocol leads to 10 µL of 2-30ng DNA/µL (figure 3). The DNA 12000 concentration range specification is 0.5–50 ng/µL, so no further treatment is required. Band concentration was calculated to be approximately 1 ng/µL (1 µL loaded on the chip).

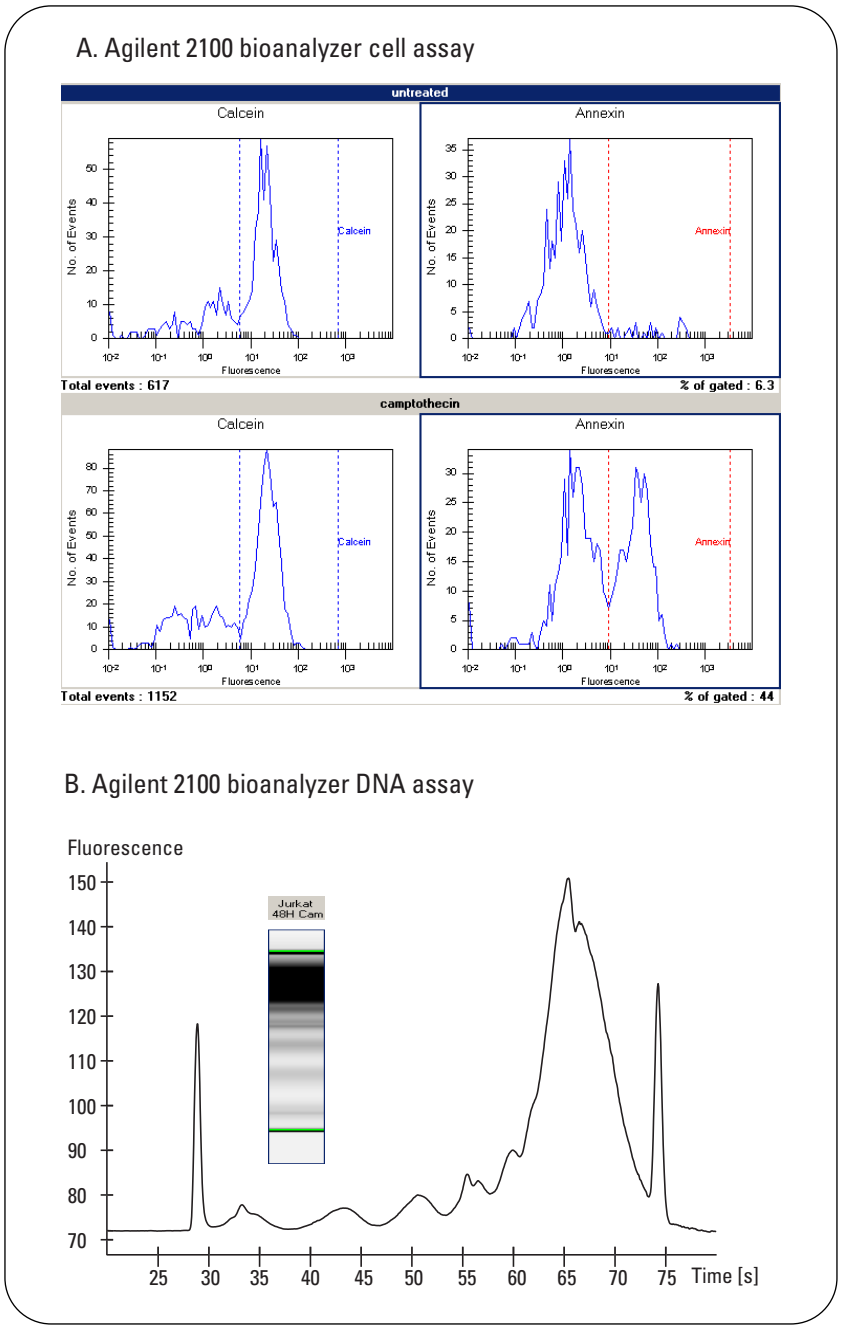


Figure 1
A. Histogram view of the two-color flow cytometric analysis showing annexin V-biotin, streptavidin-Cy5® staining of phosphatidyl serine in untreated (upper panel) and apoptosis induced Jurkat cells (lower panel). Left panels show reference staining with calcein for life cells, as annexin also stains cells with a damaged membrane, e.g. necrotic and dead cells.
B. Apoptosis confirmation by DNA laddering assay, electropherogram and gel like image are shown.

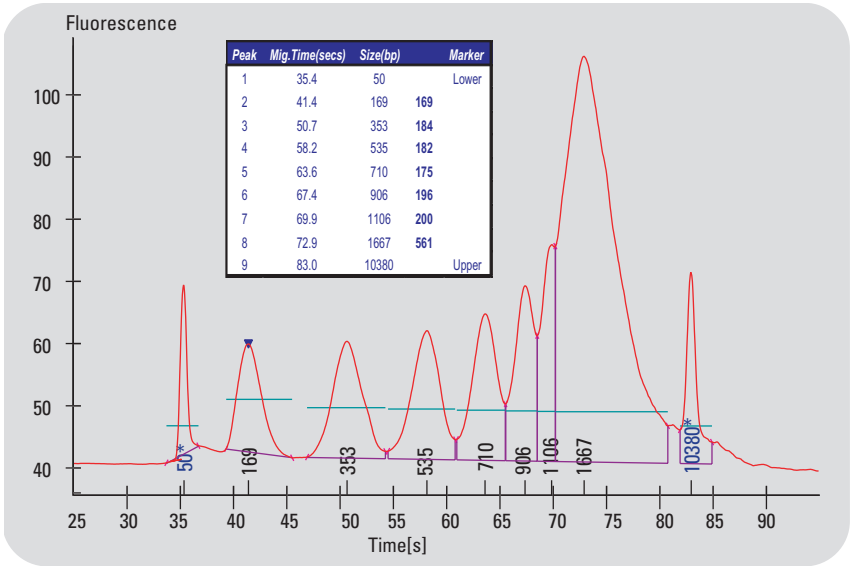
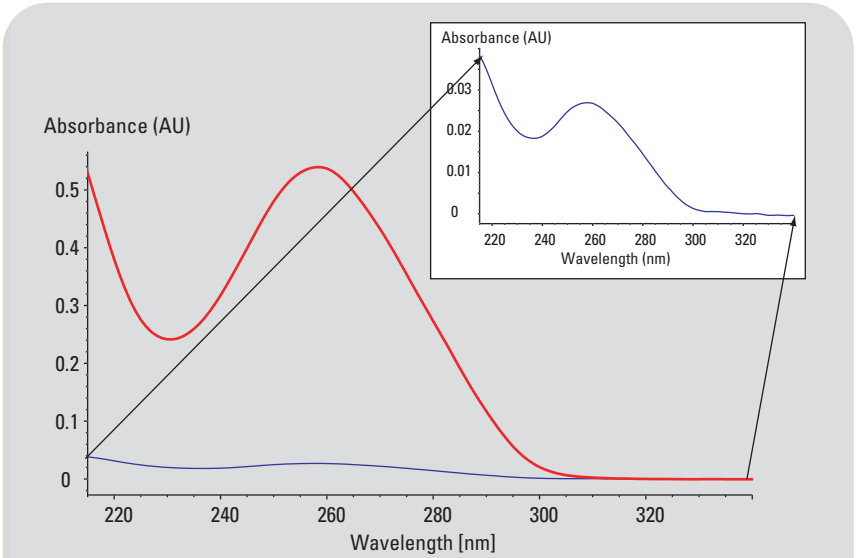


Figure 2
Electrophrogram of U937 cells lyophilized positive control (Roche diagnostics). Automated size calibration allows a quick verification of DNA fragmentation sizes. DNA peaks at 180–200bp intervals were well according to the expected values for nucleosomal fragmentation.



	Necrotic control J 20H 10 % ETOH	Sample 4 J 48H 5 µm CAM	Unit
260	2.7E-02	0.54	AU
280	1.5E-02	0.27	AU
320	1.1E-04	0.00	AU
Warburg-Christian	2.3	24.0	ng/µL
Protein	4.7	17.0	ng/µL

Figure 3
Accurate UV quantitation of DNA was achieved with 5 µL of sample (diluted to 10 µL with water). The high sensitivity of the Agilent 8453 spectrophotometer in combination with an ultra micro-cell provides high quality spectra even at very low absorbance levels (spectrum magnification). The UV spectra with higher absorbance (red) corresponds to the apoptic sample 4 (figure 4). The spectra with lower absorbance (blue) corresponds to the necrotic control sample.

Even at low concentrations, apoptotic samples are clearly distinguishable from the negative control and necrotic samples (figure 4, samples 1 to 7). The positive samples in figure 4 turned out to be 35 % apoptotic as confirmed by the annexin V assay. A clear discrimination of samples that contain a lower number of apoptotic cells is achieved by overlaying negative controls and samples in the electropherogram view (figure 5).

If alternative DNA purification methods are used, special attention must be paid as the injection of large amounts of genomic DNA (like whole cell lysates) may lead to clogging of the chip. The recommended extraction protocol eliminates such interference of genomic DNA. In addition, fragmented DNA is concentrated, which permits the apoptosis measurement of low cell numbers.

Conclusion

A combination of complementary assays is usually required to confirm that cells are apoptotic. The 2100 bioanalyzer offers the flexibility to quantitatively measure flow cytometric parameters and subsequently perform a DNA fragmentation assay. It eliminates the need for outsourcing samples and helps keep consolidated electronic track of all results. In addition to faster results and ease-of-use, the high sensitivity and optimized protocols allow minimum cell consumption and saves sample preparation time.

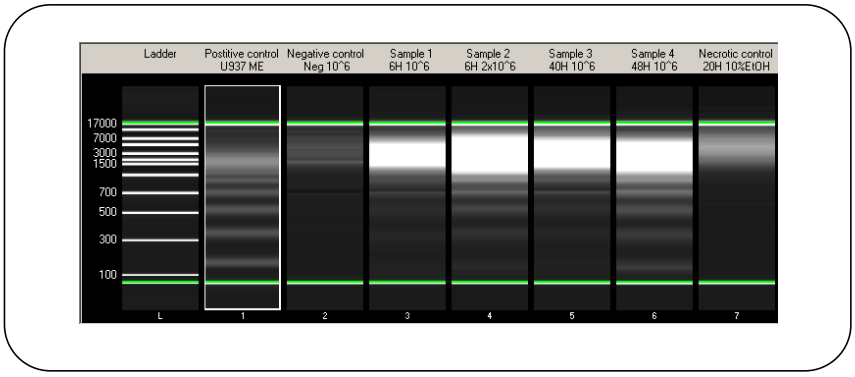


Figure 4
Gel-like image showing positive and negative controls together with 4 apoptotic samples. Negative and necrotic samples display an overall smaller DNA signal and no trace of laddering.

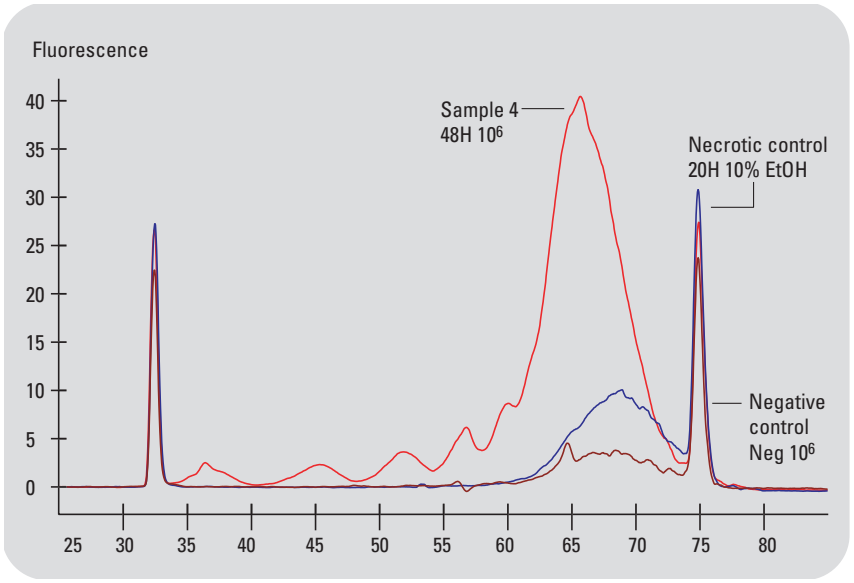


Figure 5
Electropherogram overlay of apoptotic sample, necrotic and negative controls (lanes 6, 7 and 2) Electropherogram view shows laddering as a succession of broad peaks between 150 and 1000bp. Peaks at 33s and 75s belong to the assay markers of each sample.

References

1.
Mueller, O., Hahnenberger K., Dittmann M., Yee H., Dubrow R., Nagle R., Ilsley D. "A microfluidic system for high-speed reproducible DNA sizing and quantitation." *Electrophoresis*, *21*, *128-34* **(2000)**.

2.
Ferrance, J., Snow, K., and Landers, J.P. "Evaluation of microchip electrophoresis as a molecular diagnostic method for Duchenne muscular dystrophy." *Clin Chem 48*, *380-3* **(2002)**.

3.
McCaman, M.T., Murakami, P., Pungor, E. Jr, Hahnenberger, K.M., and Hancock, W.S. "Analysis of recombinant adenoviruses using an integrated microfluidic chip-based system." *Anal Biochem 291*, *262-8* **(2001)**.

4.
Preckel, T., Luedke, G., Chan, S., Wang, B., Dubrow, R. and Buhlmann, C. "Detection of Cellular Parameters Using a Microfluidic Chip-Based System." *JALA 7(4)*, *85-89* **(2002).**

5.
Preckel, T. and Luedke, G. "Apoptosis detection by Annexin V and active Caspase 3 with the Agilent 2100 bioanalyzer" *Agilent Technologies Application Note* publication number 5988-4319EN **(2001)**.

6.
Oppenheim R.W. "Cell death during development of the nervous system. *Ann. Rev. Neurosci. 14*, *453-501* **(1991)**.

7.
Su, JH., Kesslak, JP., Head E, Cotman, CW. "Caspase-cleaved amyloid precursor protein and activated caspase-3 are co-localized in the granules of granulovacuolar degeneration in Alzheimer's disease and Down's syndrome brain." *Acta Neuropathol. (Berl) 104(1)*, *1-6* **(2002)**.

8.
Engelberg-Kulka,H ., Glaser G. "Addiction modules and programmed cell death and antideath in bacterial cultures." *Annu. Rev. Microbiol. 53*, *43-70* **(1999)**.

9.
Lu, E,. Wolfe, J. "Lysosomal enzymes in the macronucleus of Tetrahymena during its apoptosis-like degradation." *Cell Death Differ. 8(3)*, *289-297* **(2001)**.

10.
Arnoult D, Tatischeff I, Estaquier J, Girard M, Sureau F, Tissier JP, Grodet A, Dellinger M, Traincard F, Kahn A, Ameisen JC, Petit PX. "On the evolutionary conservation of the cell death pathway: mitochondrial release of an apoptosis-inducing factor during Dictyostelium discoideum cell death." *Mol Biol Cell 12(10)*, *3016-3030* **(2001)**.

11.
Kratzmeier, M., Albig, W.,Meergans, T. and Doenecke, D. "Changes in the protein pattern of H1 histones associated with apoptotic DNA fragmentation" *Biochem. J. 337*, *319-327***(1999)**.

12.
Warburg, O. and Christian, W. "Isolation and crystallization of enolase." *Biochem. Z. 310*, *384-421* **(1942)**.

13.
Preckel, T. Chan, S., and Luedke, G. "A fast protocol for apoptosis detection by annexin V with the Agilent 2100 bioanalyzer." *Agilent Technologies Application Note publication number 5988-7297EN* **(2002)**.

14.
Krohn, A.J. , Wahlbrink, T., and Prehn, J.H. "Mitochondrial depolarization is not required for neuronal apoptosis." *J. Neurosci. 19(17)*, *7394-7404* **(1999)**.

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