# Apoptosis detection by annexin V and active caspase-3 with the Agilent 2100 bioanalyzer

Application

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**Abstract**

This Application Note describes how the Agilent 2100 bioanalyzer can be used to analyze apoptotic cell samples. Performance of two assays for apoptosis detection is demonstrated and the data are compared to those obtained with a conventional flow cytometer. Histogram quality of the low number of fluorescent cells counted with the microfluidic sytem is in good agreement with data obtained with the flow cytometer. Detailed protocols and reagent recommendations for an annexin V assay and a caspase-3 assay are given. High reproducibility of chip results, low cell consumption and ease-of-use are advantages the compact Agilent 2100 bioanalyzer system offers for apoptosis cell assays.

Agilent Technologies

## Introduction

The Agilent 2100 bioanalyzer was introduced by Agilent Technologies as the first commercially available lab-on-a-chip analysis system for the life science laboratory using LabChip® products, developed by Caliper Technologies Corp. Chip-based approaches for a variety of separation-based techniques have been introduced, addressing DNA, RNA, and protein separations. The Agilent 2100 bioanalyzer is capable of two-color fluorescence detection and runs disposable microfluidic glass chips. The applications presented here are based on the controlled movement of cells by pressure-driven flow inside the interconnected networks of microfluidic channels. Cells are hydrodynamically focused in these channels before passing the fluorescence detector in single file. Each chip accommodates up to six samples and data acquisition of all samples is fully automated while data analysis allows for user-specific settings. Specific advantages of the instrument are the low number of cells required for analysis and the ease-of-use. Here, the detection of apoptotic cell death is investigated.

### Apoptosis

Apoptosis, or programmed cell death, is a genetically controlled response of cells to commit suicide. The purpose of this process is to kill unwanted host cells. It is used in three situations: for development and homeostasis, as a defense mechanism and in aging. Apoptosis is characterized by a distinct set of morphological events involving plasma membrane blebbing, loss of cell volume, nuclear condensation, fragmentation of DNA at nucleosomal intervals and ultimate fragmentation of the cell into membrane-enclosed "apoptotic bodies"[1].

During the early phase of apoptosis, changes occur at the plasma membrane. Phosphatidyl serine (PS) that is actively confined to the inner leaflet of the lipid bilayer in healthy cells translocates to the outer layer, where it is exposed at the external surface of the cell[2]. Annexin V belongs to the family of calcium and phospholipid binding proteins with high affinity for PS[3]. It can be used as a sensitive probe for PS exposure on the cell membrane. Translocation of PS to the external cell surface is not unique to apoptosis, as it also appears during necrosis. These mechanisms of cell death differ in their initial stages when the cell membrane remains intact during apoptosis but loses integrity, leaking cellular contents, during necrosis[4]. Therefore, measuring annexin V binding to the cell surface as indicator for apoptosis has to be performed in conjunction with a calcein retention test that verifies the integrity of the cell membrane. As electrically neutral or nearly neutral molecules, calcein acetyloxymethyl (AM) esters freely diffuse into most cells. Once inside the cell, these non-fluorescent substrates are converted by nonspecific intracellular esterases into fluorescent products that are retained by cells with intact plasma membranes. In contrast, both the unhydrolyzed substrates and their products rapidly leak from dead or damaged cells with compromised membranes, even when the cells retain some residual esterase activity[5].

Apoptosis involves active participation of endogenous cellular enzymes in the mediation of death well before membranes lose their integrity. A family of cysteine proteases (caspases) appears to represent the effector arm of the apoptotic program[6]. Caspase activation can be achieved by different pathways, by the cell surface death receptor pathway and by the mitochondria-initiated pathway. In the cell surface death receptor pathway, activation of caspase-8 following its recruitment to the death-inducing signaling complex (DISC) is the critical event that transmits the death signal. This event is regulated at several different levels by various viral and mammalian proteins. Activated caspase-8 can activate downstream caspases by direct cleavage or indirectly by inducing cytochrome c release from the mitochondria. In the mitochondrial-initiated pathway, caspase activation is triggered by the formation of a multimeric Apaf-1/cytochrome c complex that is fully functional in recruiting and activating procaspase-9. Activated caspase-9 then cleaves and activates downstream caspases such as caspase-3, -6, and -7[7]. Caspase-3 is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis

and/or cleavage by another protease[8]. Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which is derived from the 32 kDa proenzyme[9]. A convenient and broadly accepted method to study the activity of caspase-3 during apoptosis involves intracellular antigen staining with anti-active caspase-3 antibodies. Following fixation, permeabilization and staining, the activity of caspase-3 can be measured by flow cytometry or fluorescence microscopy techniques.

Here, we describe the measurement of percentages of apoptotic cells within cell populations using the Agilent 2100 bioanalyzer. The apoptotic process was induced in Jurkat cells, a cell line derived from an acute human T-cell leukemia, either by treatment with camptothecin or by incubation with anti Fas-antibodies. Different readouts were chosen – either annexin-presenting cells within the live cell population were detected or the percentage of cells with active caspase-3 was measured in the total cell population.

## Experimental

The Agilent 2100 bioanalyzer and Cell Assay Extension were obtained from Agilent Technologies Deutschland GmbH (Waldbronn, Germany). Detection of apoptotic cells was performed on the Agilent 2100 bioanalyzer in combination with the Cell Fluorescence LabChip® Kit and the Cell Fluorescence software. The kit includes 25 chips and reagents required to perform the analysis of pre-stained cells. Stained cell samples were resuspended in an isobuoyant cell buffer at 2 x $10^6$ cells/ml and loaded onto the chips as described in the reagent kit guide. Data acquisition was performed using an intuitive software package with no requirement to manually set instrument specific parameters.

## Results

### a) Induction of apoptosis with camptothecin; annexin V staining

In a first application example, Jurkat cells were induced with camptothecin and harvested after 0, 4 and 16 hours of treatment. After washing, the cells were incubated with annexin V-biotin. Cells were washed again and stained

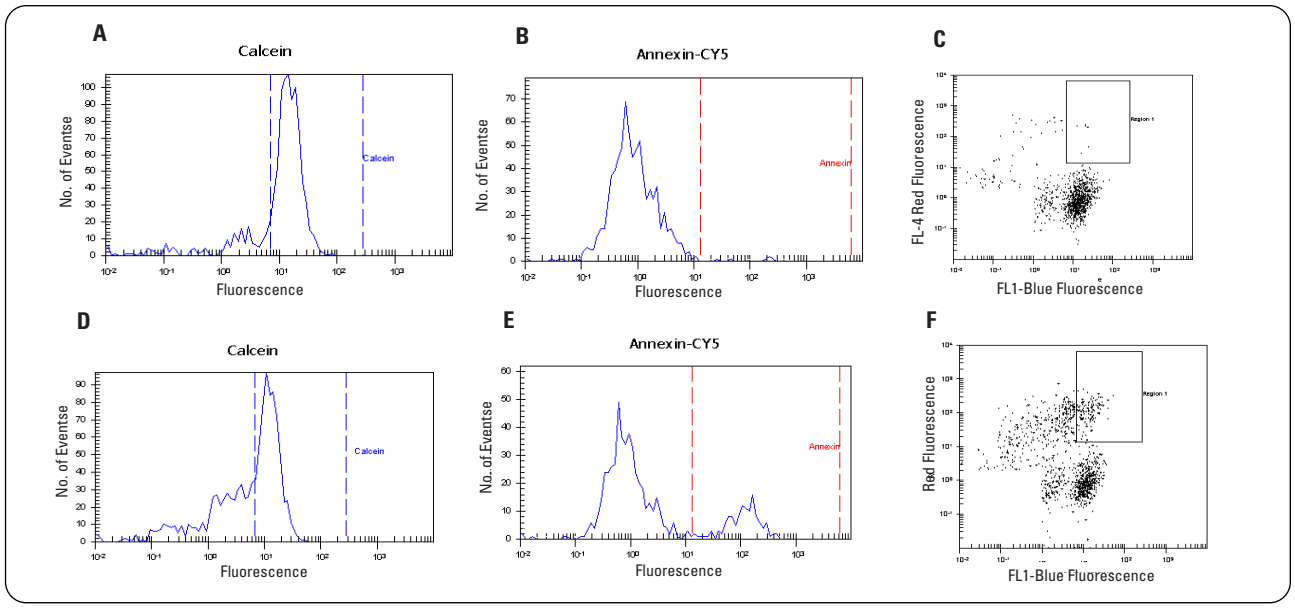


**Figure 1**
**Induction of apoptosis in Jurkat cells with camptothecin; annexin V staining. Jurkat cells were treated with camptothecin for 16 h, subsequently stained with calcein and annexin V-Cy5 and analyzed on the Agilent 2100 bioanalyzer (500-1000 cells counted per sample).**
**A) Calcein B) Annexin-Cy5 histogram of untreated sample C) Dot plot of untreated sample D) Calcein E) Annexin-Cy5 histogram of 16h treated sample F) Dot plot of 16h treated sample**

with Cy5®-streptavidin and calcein according to protocol. After washing and resuspending the cells in cell buffer the samples were loaded onto the chip and inserted into the Agilent 2100 bioanalyzer. A predefined assay was chosen from the system software for chip measurement. Figure 1 shows representative histograms and dot plots of an untreated control sample and a sample treated for 16 hours. The sub-populations of all living cells were defined by setting a marker for all calcein stained cells (figure 1 A+D). These sub-populations are further analyzed in the red color by setting a marker for all cells showing a significant red staining by annexin-V (figure 1 B+E). In this way, all cells that show strong annxin-V binding and a strong calcein signal, indicating an intact cell membrane, are detected. These apoptotic cells can also be seen in a dot plot view as shown in the rectangular areas in figure 1 C and 1 F. The data quality is comparable to the data quality generated with the same samples on a conventional flow cytometer (figure 2), which usually counts many more cell events for analysis. Final results are expressed as percentage apoptotic cells of all live cells. Figure 3 shows the results which demonstrate a good reproducibility of the measurement over several chips. The figure also shows data from a conventional flow cytometer which nicely compares with the data obtained with the Agilent 2100 bioanalyzer.

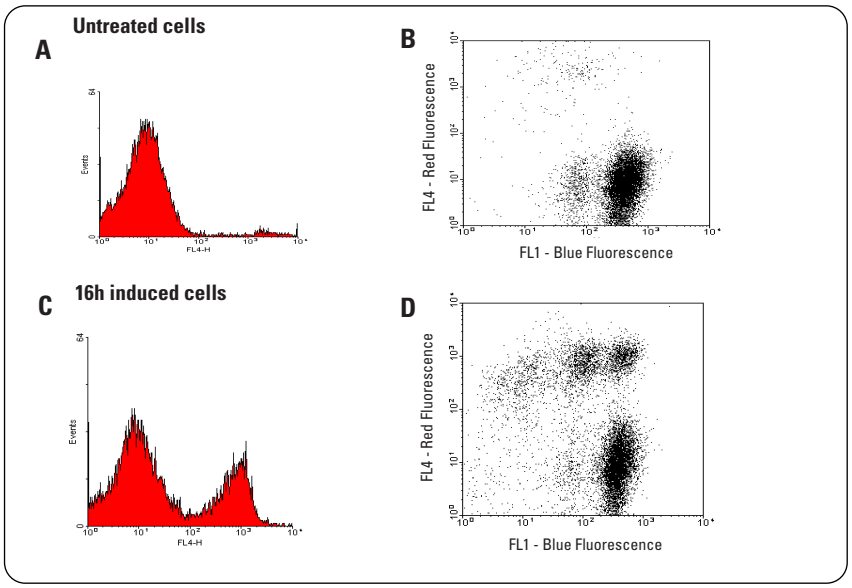


**Figure 2**
**Induction of apoptosis in Jurkat cells with camptothecin; annexin V staining.**
**Jurkat cells were treated with camptothecin, subsequently stained with calcein and annexin V-Cy5 and analyzed on a conventional flow cytometer (10,000 cells counted per sample).**
**A) Red color/FL-4 (annexin-Cy5) histogram of untreated sample. B) Dot plot of untreated sample. C) Red color/ FL-4 (annexin-Cy5) histogram of 16h treated sample. F) Dot plot of 16h treated sample.**

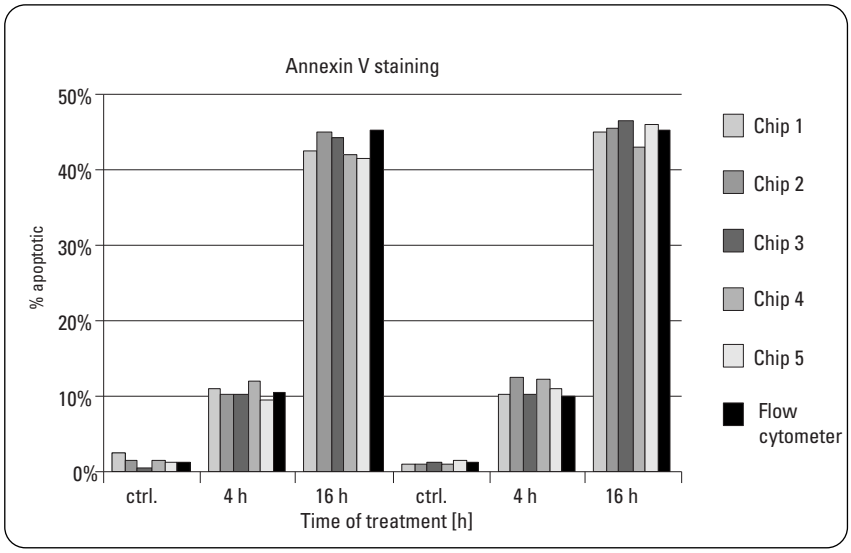


**Figure 3**
**Induction of apoptosis in Jurkat cells with camptothecin; annexin V staining. Jurkat cells were treated with camptothecin, stained with calcein and annexin V-Cy5 and analyzed on the 2100 bioanalyzer. Data of five chips was compared with measurements from a conventional flow cytometer.**

### b) Induction of apoptosis with Fas antibody; caspase-3 staining

In a second application example Jurkat cells were treated for two and four hours with anti Fas antibodies to induce apoptosis. In this application a different detection method for apoptosis, intracellular staining of active caspase-3 is used. After washing half of the cells were stained with SYTO16 (an intercalating nucleic acid stain), washed again and treated with a permeabilization/fixation solution. They were subsequently stained with anti active caspase-3 antibodies and a Cy5- conjugated secondary antibody (figure 4 A). The other half of the treated cells were stained with calcein/annexin V-Cy5 as described above (figure 4 B). Both sample groups were measured on the 2100 bioanalyzer and on a conventional flow cytometer. Only 20,000 cells of each prepared cell sample were loaded onto the chip (500-1000 cells analyzed per sample), whereas 100,000 cells were required for measurement on the flow cytometer (10,000 cells analyzed per sample). As expected, the percentages of live apoptotic cells are similar for both assays. The Agilent 2100 bioanalyzer displayed good reproducibility and yielded data comparable to the flow cytometer system.

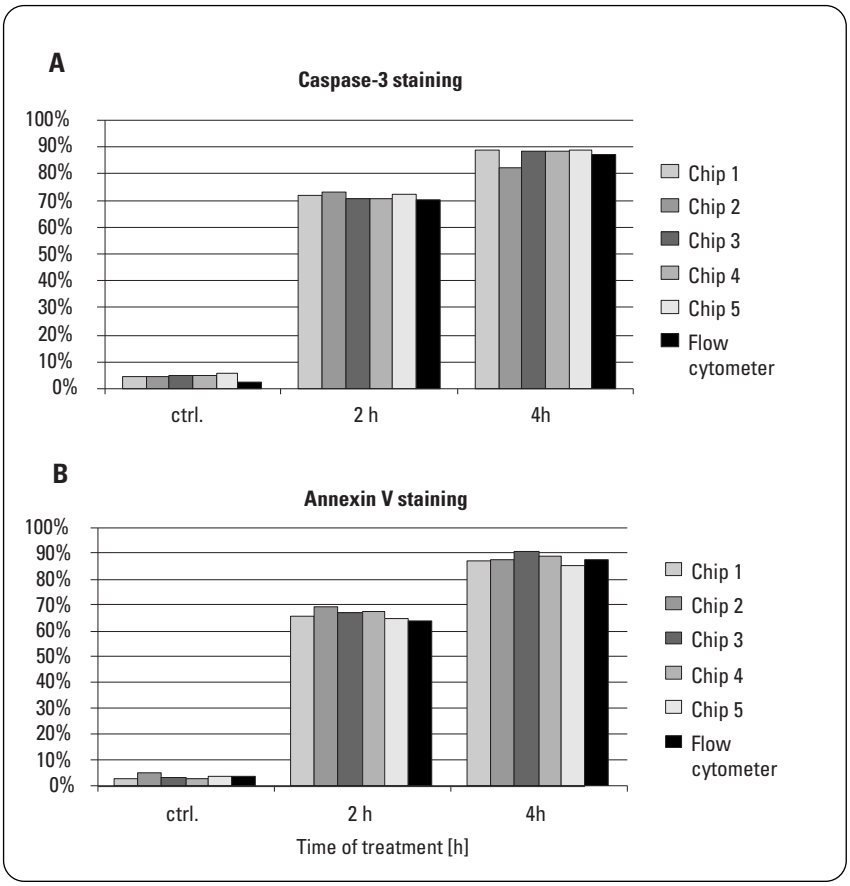


**Figure 4**
**Induction of apoptosis in Jurkat cells with anti-Fas antibody; caspase-3 (A) and annexin V (B) staining. Jurkat cells were treated with anti-Fas antibodies for different times. They were then stained with either calcein/annexin V-Cy5 or with SYTO16/caspase-3. Data of five chips was compared with measurements from a conventional flow cytometer.**

## Materials and methods

Ordering information for the reagents is listed in table 1.
Cells were cultured in RPMI medium containing 10 % fetal calf serum, penicillin/streptomycine, 1 mM Sodium Pyruvate and 2 mM L-glutamine.

### a) Induction of apoptosis and annexin V assay

#### Reagents

- Camptothecin: Prepare a 10 mM solution in DMSO.
- Annexin V-Biotin Apoptosis Detection Kit: Includes annexin V-biotin, 5x binding buffer and media binding reagent
- Fluorolink Cy5 labeled streptavidin: Reconstitute with 1 ml distilled water to yield a concentration of 1 mg/ml
- Paraformaldehyde 16 % solution EM Grade (optional)
- Calcein-AM: Dilute the original stock with DMSO to yield a 500 µM solution.

#### Protocol for annexin V assay

1. Treat Jurkat cells at a concentration of 5 x $10^5$/ml with 1 µM camptothecin or 0.5 µg/ml anti Fas-antibody for 2–24 h to induce apoptosis.
2. Harvest control and camptothecin-treated or Fas antibody-treated cells and adjust cell density to 1 x $10^6$ cells /ml in culture medium.
3. Add 10 µl media binding reagent per 500 µl of cell suspension (5 x $10^5$ cells).
4. Add 1.25 µl annexin V-biotin per 500 µl of cell suspension (5 x $10^5$ cells). Incubate for 15 minutes at room temperature (RT).
5. Centrifuge cells at 400x g for 4 minutes. Remove medium and resuspend cells at a cell density of 1 x $10^6$ cells /ml in 1x binding buffer. Use 5x binding buffer and dilute to 1x with distilled water.
6. Add 1 µl Cy5-streptavidin and 0.5 µl calcein (calcein stock = 500 µM, final = 0.5 µM) per 500 µl of cell suspension (5 x $10^5$ cells). Incubate in the dark for 10 minutes at RT.
7. Wash cells once with 500 µl of 1 x binding buffer.
8. *Optional step if cells are to be be stored overnight and measured later:*
   Remove medium and resuspend cells well at a cell density of 1 x $10^6$ cells /ml in 1x binding buffer. Make sure there are no cell clumps in the suspension. Add 70 µl paraformaldehyde 16 % while stirring. Incubate for 10 minutes at RT and store the cells at 4 °C.
9. Resuspend cells in cell buffer at 2 x $10^6$ cells/ml and load on chip according to the reagent kit guide.

### b) Induction of apoptosis and caspase 3 assay

#### Reagents

- BD FACS Permeabilizing Solution 2 (500 tests)
- Camptothecin: Prepare a 10 mM solution in DMSO.
- Rabbit anti-active caspase-3 mAb
- Cy5-conjugated AffiniPure $F(ab')_2$ fragment goat anti-rabbit IgG (H+L)
- SYTO16
- Staining buffer (PBS, 2 % BSA, 0.05 % $NaN_3$)

#### Protocol for caspase 3 assay

1. Treat 5 x $10^5$/ml Jurkat cells with 1 µM camptothecin or 0.5 µg/ml anti Fas antibody for 2–24 hours to induce apoptosis.
2. Harvest control and camptothecin-treated or Fas antibody-treated cells and adjust cell density to 1 x $10^6$ cells /ml in staining buffer.
3. Add 1.5 µl SYTO16 per ml and incubate 10 minutes at 37 °C.
4. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
5. Resuspend 5 x $10^5$ cells in 500 µl 1x BD permeabilizing solution (dilute in distilled water) and incubate 10 minutes at RT.
6. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
7. Add anti caspase-3 antibody at 5 µg/ml in a total of 100 µl staining buffer to 1 x $10^6$ cells. Incubate 20 minutes on ice.
8. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
9. Add secondary antibody 5 µg/ml in 100 µl staining buffer. Incubate 30 minutes on ice in the dark.
10. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
11. Resuspend at 2 x $10^6$/ml in cell buffer and load on chip according to the reagent kit guide.

| Reagent | Supplier | Order No. |
|---|---|---|
| **Apoptosis inducing drugs** | | |
| Camptothecin: | Sigma-Aldrich<br>http://www.sigma-aldrich.com | C9911 |
| Anti-Fas antibody: | Roche Diagnostics<br>http://www.roche.com/diagnostics | 1 922 432 |
| **Annexin V apoptosis assay** | | |
| Annexin V-Biotin Apoptosis Detection Kit: | Oncogene Research Products<br>http://www.oncresprod.com | PF036 |
| Fluorolink Cy5 labeled streptavidin: | Amersham<br>http://www.amersham.com | PA 45001 |
| Paraformaldehyde:<br>16 % Solution EM Grade | Electron Microscopy Sciences<br>http://www.emsdiasum.com | 15710-S |
| Calcein-AM: | Molecular Probes<br>http://www.probes.com | C-3099 |
| **Caspase 3 apoptosis assay** | | |
| BD FACS Permeabilizing Solution 2<br>500 Tests | BD Biosciences<br>http://www.bd.com | 340973 |
| Rabbit anti-active caspase-3 mAb: | BD Biosciences<br>http://www.bd.com | 559565 |
| Cy5-conjugated Goat anti-rabbit IgG, AffiniPure F(ab')2 Goat-anti-rabbit | Jackson ImmunoResearch Labs<br>http://www.jacksonimmuno.com | 111-176-045 |
| SYTO16: | Molecular Probes<br>http://www.probes.com | S-7578 |

**Table 1**
**Ordering information for reagents used in the experiments described in this Application Note.**

## Conclusion

Apoptosis is a critical mechanism for organisms to maintain tissue-homoeostasis. In this Application Note we showed that the Agilent 2100 bioanalyzer is a versatile tool to detect apoptotic cells. Protocols and a list of recommended reagents for detection of two apoptotic markers are given. Excellent reproducibility of results from different chips for both the Annexin V and the caspase-3 assay is demonstrated.

Data from the 2100 bioanalyzer compares very well with that of a conventional flow cytometer in spite of a 5-fold lower cell consumption. Data acquisition is done automatically and data analysis is achieved by an intuitive software package which does not require manual setting of instrument related parameters.

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