A fast protocol for apoptosis detection by annexin V with the Agilent 2100 bioanalyzer

Application

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Abstract

This Application Note describes a simplified and fast protocol for the detection of apoptotic cells with the Agilent 2100 bioanalyzer and the cell fluorescence LabChip®. The specific advantage of the simplified protocol is the amount of time saved and its ease-of-use.

The note describes how the Agilent 2100 bioanalyzer can be used to analyze apoptotic cell samples. Annexin V staining of live cells is demonstrated and the data are compared to that obtained with a conventional flow cytometer. Histogram quality of the microfluidic system is comparable to that of a conventional flow cytometer. Detailed protocols and reagent recommendations for staining of cells are provided. The high reproducibility of the chip results, low cell and reagent consumption, the speed and the ease of use are advantages the Agilent 2100 bioanalyzer offers for monitoring apoptotic cells by annexinV staining.

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Introduction

The 2100 bioanalyzer was introduced by Agilent Technolgies as the first commerially 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[1, 2, 3]. The Agilent 2100 bioanalyzer system is capable of two-color fluorescence detection and runs disposable microfluidic glass chips. Most recently a set of applications has been presented based on the controlled movement of cells by pressure-driven flow inside the interconnected network of microfluidic channels. Cells are hydrodynamically focused in these channels before passing the fluorescence detector in single file. Each chip accommodates six samples and data acquisition of all samples is fully automated while analysis allows for user-specific settings. These applications include:

- "Apoptosis detection by annexin V and active Caspase 3 with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4319EN)
- "Monitoring transfection efficiency by green fluorescent protein (GFP) detection with the Agilent 2100 bioanalyzer" (Agilent publication 5988-4320EN)
- "Detecting cell surface and intracellular proteins with the Agilent 2100 bioanalyzer by antibody staining" (Agilent publication number 5988-4322EN)

In this Application Note a fast and reliable protocol for staining of apoptotic cells with annexin V and the live cell dye calcein is described. Specific advantages of the fast protocol are the speed and the ease-of-use of the cell staining.

Apoptosis

Apoptosis, or programmed cell death, is a genetically controlled response for cells to commit suicide. The purpose of this process is to kill unwanted host cells. It is put to use 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"[4]. 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 is translocated to the outer layer, by which PS becomes exposed to the external surface of the cell[5]. Annexin V is a member of the family of calcium and phospholipid binding proteins with high affinity for PS[6] and 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, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment when necrosis occurs the cell membrane looses its integrity and becomes leaky[7]. Therefore, the measurement of annexin V binding to the cell surface as indicator for apoptosis has to be performed in conjunction with a test to verify the integrity of the cell membrane, typically done by the inclusion of acetyloxymethyl (AM) or Acetate ester derivatives of fluorescent compounds. As electrically neutral or near-neutral molecules, the calcein AM esterase substrate freely diffuses 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[8].

Here, we describe the measurement of percentages of apoptotic cells within cell populations with the Agilent 2100 bioanalyzer together with a fast and reliable protocol for the staining of apoptotic cells. The apoptotic process was induced in Jurkat cells either by treatment with camptothecin or by incubation with anti Fas-antibody. Annexin V-stained cells within the live cell population were detected.

Experimental

The Agilent 2100 bioanalyzer and cell assay extension were obtained from Agilent Technologies Deutschland GmbH (Waldbronn, Germany). Detection of stained 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. 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.

Reagents

- Camptothecin (prepare a 10 mM solution in DMSO).
- Anti-Fas Antibody
- 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 H_20 to yield a concentration of 1 µg/µl)
- Calcein-AM (dilute the original stock (5 mM) with DMSO to yield a 500 µM solution)

For ordering information refer to page 7.

Fast protocol for annexin V Assay

- Treat Jurkat cells at a concentration of 5 x 10^5/ml with 1 µM camptothecin or 0.5 µg/ml anti Fas antibody to induce apoptosis.
- Prepare the following solutions prior to use. Required for each staining reaction (scale up volumes to number of samples to be stained):
  A. Mix 5 µl of Medium Binding Reagent with 0.25 µl of annexin V-biotin.
  B. Prepare 250 µl 1x Binding buffer (Solution B) per sample with dest. H_20 and 5x Binding Buffer
  C. Mix 250 µl of 1x Binding Buffer with 0.25 µl of calcein and 0.25 µl of Fluorolink Cy5. (for optimal results it may be necessary to optimize the Fluorolink Cy5 concentration by testing several dilutions of the reagent).

1. Count and harvest cells. Adjust cell density to 1 x 10^6 cells/ml in original culture medium.
2. Transfer 200 µl of cell suspension to a microcentrifuge tube (0.5 ml). Add 4 µl of Solution A. Mix and incubate for 10 min at RT.
3. Centrifuge cells at 400 x g for 2 min. Remove medium carefully by aspiration and resuspend the cells in 200 µl of Solution C. Mix by gentle vortexing. Incubate for 10 min at RT in the dark.
4. Centrifuge cells at 400 x g for 2 min. Remove medium carefully by aspiration and carefully resuspend the cells in 100 µl of CB (final cell concentration 2 x 10^6/ml).
5. Load chip with 10 µl of cell sample and insert into instrument.
6. From the menu bar of the cell fluorescence software choose Assay/Cells/"Apoptosis – fast protocol" and start the run.

Results

Induction of Apoptosis with anti-Fas antibody

Apoptosis was induced in Jurkat cells by treatment with 0.5 µg/ml anti-Fas antibody and cells were harvested after 0, 2.5 and 5 hours of treatment and incubated for 10 min at RT with annexin V-biotin. Cells were centrifuged, resuspended and stained with Cy5®-streptavidin and calcein according to the fast protocol. After resuspending the cells in cell buffer the samples were loaded onto the chip according to the reagent kit guide and analyzed with the Agilent 2100 bioanalyzer. The predefined "Apoptosis – fast protocol" assay was chosen from the cell fluorescence software for chip measurement. For comparison, cells stained with the fast protocol, as well as cells stained with the conventional protocol[9] were analyzed on a flow cytometer. Representative histograms and dot plots of an untreated control and a sample treated for 2.5 h are shown in figure 1. The sub-population of all living cells was defined by setting a marker in the blue color for all calcein stained cells (figure 1, C, E). These sub-populations are further analyzed in the red color by setting a marker for all cells displaying a positive red annexin V staining (figure 1, D, F). These double positive apoptotic cells can also be seen in a dot plot view as represented in figure 1, A, B. The histogram quality obtained with the 2100 bioanalyzer and the fast protocol are of comparable quality as data generated with the same samples on a conventional flow cytometer.

Figure 2A shows the annexin-histogram of live untreated cells whereas figure 2B shows the annexin-histogram of live Fas-antibody treated cells, both obtained with a flow cytometer.

We tested the accuracy and reproducibility of the fast staining protocol in comparison to the conventional protocol (as described in another Application Note[9]). Apoptotic cell samples were stained with the fast protocol and run on the 2100 bioanalyzer (3 chips loaded with one untreated control sample and 5 treated apoptotic samples each). The same samples and samples stained with the conventional protocol were measured on a standard flow cytometer in parallel. Results are expressed as percentage of apoptotic cells within the live cell population (figure 3).
The data obtained demonstrate the good reproducibility of the measurement over several chips and with different protocols. No significant difference was observed between the data obtained with a conventional flow cytometer and the data obtained with the 2100 bioanalyzer.

Time course of apoptosis induced by treatment with anti-Fas antibody

Apoptosis was induced in Jurkat cells by treatment with 0.5 µg/ml anti-Fas antibody and harvested after 0, 1, 2, 3, 4 and 5 hours of treatment. Cells were incubated with annexin-biotin and stained with Cy5-streptavidin and calcein according to the fast protocol. After resuspending the cells in cell buffer the samples were loaded onto the chip according to the

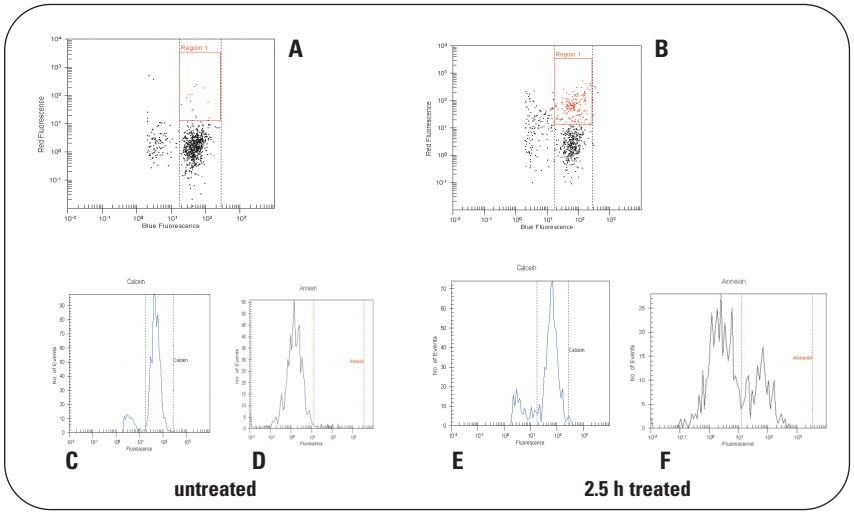

Figure 1
Typical results of the "Apoptosis – fast protocol" on the 2100 bioanalyzer. Jurkat cells were treated with 0.5 µg/ml anti Fas-antibody and harvested after 2.5 hours of treatment. Cells were stained according to the fast protocol and loaded onto the chip according to the reagent kit guide and analyzed with the 2100 bioanalyzer. The sub-population of all living cells was defined by setting a marker in the blue color for all calcein stained cells (Fig.1 C+E). These sub-populations are further analyzed in the red color by setting a marker for all cells displaying a significant red staining by annexin-V (Fig1 D+F). These apoptotic cells are also visible in a dot plot view as represented in the region in Fig.1 A+B.

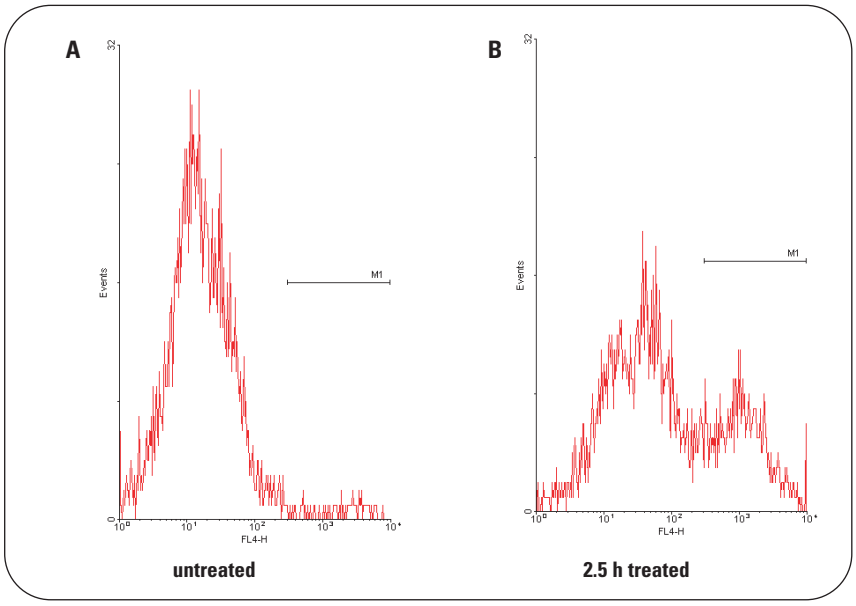

Figure 2
Typical results of the "Apoptosis – fast protocol" on a conventional flow cytometer. Jurkat cells were treated with 0.5 µg/ml anti Fas-antibody and harvested after 2.5 hours of treatment. Cells were stained according to the fast protocol and analyzed with a conventional flow cytometer. Only the annexin V histograms of the live treated (B) or the untreated (A) cells are shown.

reagent kit guide and analyzed with the 2100 bioanalyzer. The predefined "Apoptosis – fast protocol" assay was chosen from the cell fluorescence software for chip measurement. The sub-population of all living cells was defined by setting a marker in the blue color for all calcein stained cells (figure 4A). These sub-populations are further analyzed in the red color by setting a marker for all cells displaying a positive annexin V staining. Figure 4B shows the time course of the induction of apoptosis. Apoptosis is detectable in a significant amount of cells after 2 h. Following a treatment of 4 h, 95% of the cells are apoptotic.

Working with few cells

Since the fast protocol might prove especially useful when working with limited amounts of cells and reagents we developed steps to facilitate working with few cells. The fast protocol was optimized for use with 200,000 cells per sample. In order to test the performance of the fast protocol with less cells, Jurkat cells were treated with 1 µM camptothecin for 16 h. Between 200,000 and 50,000 cells were used per sample, stained according to the fast protocol and resuspended in CB to yield $2x10^6$ cells/ml (see procedure B below). The chips were loaded on the instrument and run after selecting the assay "Apoptosis – fast protocol". The data obtained show that 50,000 cells are sufficient to obtain accurate results and good histogram quality (figure 6). It is important to note, that when working with few cells the pellets after the centrifugation steps are quite small.

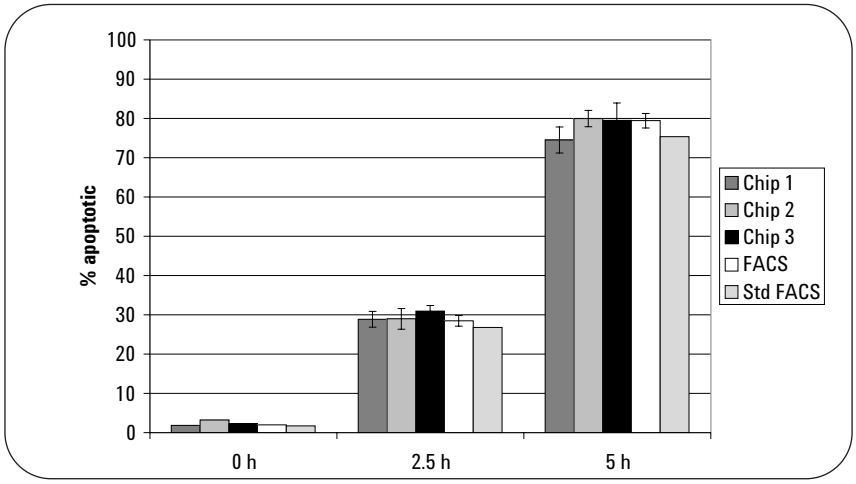

Figure 3

Performance of the "Apoptosis – fast protocol" on the bioanalyzer in comparison to the conventional protocol and flow cytometer. Jurkat cells were treated with 0.5 µg/ml anti Fas-antibody and harvested after 0, 2.5 or 5 hours of treatment. Cells were stained according to the fast protocol (Chip 1-3 or FACS) or according to the conventional protocol and analyzed on a standard flow cytometer (Std FACS). The results obtained with all three methods proofed to be practically identical.

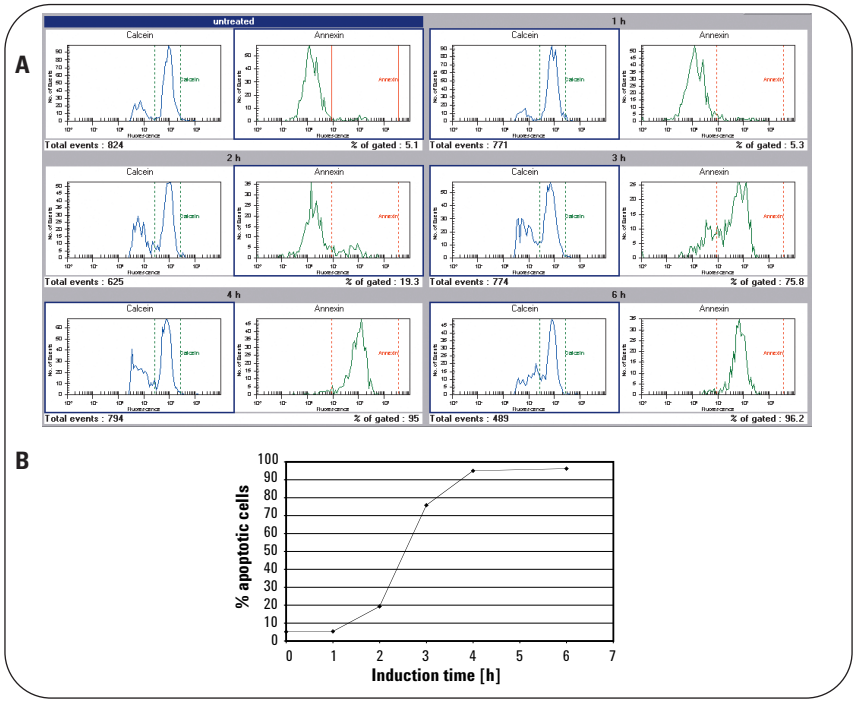

Figure 4

Time course of apoptosis induced by treatment with anti-Fas antibody.
Apoptosis was induced in Jurkat cells by treatment with 0.5 µg/ml anti-Fas antibody and harvested after 0, 1, 2, 3, 4 and 6 hours of treatment. Cells were stained with annexin V-biotin, Cy5-streptavidin and calcein according to the fast protocol. The sub-population of all living cells was defined by setting a marker in the blue color for all calcein stained cells. These sub-populations are further analyzed in the red color by setting a marker for all cells displaying a significant red staining by annexin-V. A) 2100 bioanalyzer histogram view of the analysis. B) Time course of the induction of apoptosis by anti-Fas antibody in Jurkat cells. Apoptosis is detectable in a significant amount of cells after 2 h. Following a treatment of 4 h, approximately 95% of the cells are apoptotic.

Steps for handling few cells

Any of these three procedures may be used after the calcein and Fluorolink Cy5 incubation (step 3) of the fast protocol according to user preferences.

Procedure A

1. Remove medium completely.
2. Carefully resuspend cells at 3 x 10^6 cells/ml in at least 20 µl CB. Strong vortexing or vigorous pipetting may damage cells.

Procedure B

1. Remove an aliquot of medium to yield 6 x 10^6 cells/ml.
2. Add remaining volume size of 2 x CB to yield 3 x 10^6cells /ml.
3. Carefully mix the cells and buffer well. Strong vortexing or vigorous pipetting may damage cells.

Procedure C

1. Remove medium by decanting.
2. Carefully resuspend cells in remaining liquid and measure the volume with a micropipette.
3. Add an equal volume of 2 x CB.
4. Adjust cell density to 3 x 10^6 cells/ml by adding CB.
5. Carefully mix the cells and buffer well. However, strong vortexing or vigorous pipetting may damage cells.

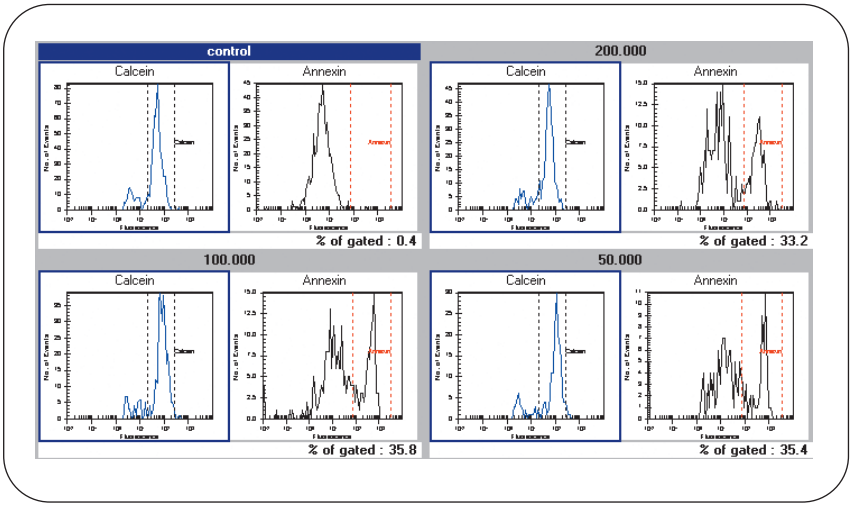

Figure 5

Assay performance when working with few cells. Jurkat cells were treated with 1 µM camptothecin for 16h. Between 200,000 and 50,000 cells were used per sample and stained according to the fast protocol. The % of gated value represents the percentage of annexin V positive stained cells within the live cell population. The data obtained show that 50,000 cells are sufficient to obtain accurate results.

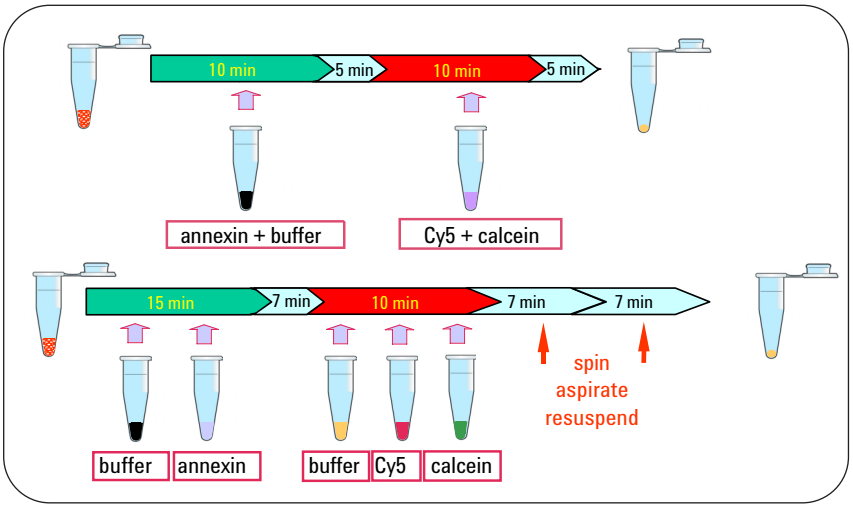

Figure 6

Workflow of the Apoptosis – fast protocol (upper panel) in comparison with the conventional protocol (lower panel). Working with the fast protocol will save approximately 16 min per staining procedure (1 chip).

Ordering information
Ordering details are listed in table 1.

Description	Order
Apoptosis inducing drugs	
Camptothecin	Sigma, # C9911
Anti-Fas antibody	Roche, # 1 922 432
Annexin V apoptosis assay	
Annexin V-Biotin Apoptosis Detection Kit	Oncogene Research Products, #PF036
Fluorolink Cy5 labeled streptavidin	Amersham, #PA 45001
Calcein-AM	Molecular Probes, #C-3099
Agilent 2100 bioanalyzer and cell fluorescence LabChip kit	
Agilent website	www.agilent.com/chem/labonachip

Table 1
Ordering Information

Conclusion

Apoptosis is a critical mechanism for all eucaryotic organisms to maintain tissue-homoeostasis. And with increasing importance, apoptosis assays such as the annexin V assays have become routine tools in many laboratories. Here we showed the use of the Agilent 2100 bioanalyzer in conjunction with a more convenient protocol as a versatile, fast and accurate tool to detect apoptotic cells. Protocols and a list of recommended reagents are provided. Excellent reproducibility of results from different chips and samples with the annexin V assay is demonstrated. Figure 6 compares the workflow and time investment for staining cells according to the conventional or the fast protocol. Compared to the conventional protocol, the "Apoptosis – fast protocol" shows several advantages:

- Faster and easier than conventional protocol (save 16 min per 6 samples)
- More versatile and less tedious when handling multiple samples
- Only 1/5 of the cells are required for apoptotic cell staining
- Elimination of washing and aspiration steps minimize loss of cells

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 does not require manual setting of instrument related parameters. Data analysis and reporting is straightforward with the intuitive software package.

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Related Application Notes

- "Apoptosis detection by annexin V and active caspase 3 with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4319EN)
- "Monitoring transfection efficiency by green fluorescent protein (GFP) detection with the Agilent 2100 bioanalyzer "(Agilent publication number 5988-4320EN)
- "Detecting cell surface and intracellular proteins with the Agilent 2100 bioanalyzer by antibody staining" (Agilent publication number 5988-4322EN)
- "Monitoring transfection efficiency in cells using an on-chip staining protocol. A rapid and accurate method to detect green fluorescent protein expression" (Agilent publication number 5988-7296EN)
- "Detecting cell surface proteins with the Agilent 2100 bioanalyzer by on-chip antibody staining. A rapid and accurate method to detect protein expression of B7-1 and B7-2 by on-chip antibody staining" (Agilent publication number 5988-7111EN)

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