Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACSVerse™ System

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Application Note

Contents

Summary

Apoptosis, or programmed cell death, is a normal physiologic process for removal of unwanted cells. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein, has high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposed PS using flow cytometry. The BD Pharmingen™ Annexin V FITC apoptosis detection kit provides a set of reagents for the detection of apoptosis using flow cytometry. The BD FACSVerse™ system includes the cytometer, BD FACSuite™ software for acquisition and analysis, and BD FACSuite research assays for use with specific reagent kits. Based on the Annexin V FITC apoptosis detection kit, the Annexin V FITC assay in BD FACSuite software provides acquisition, analysis, and reporting functions for generating reliable and consistent data using the BD FACSVerse system. This application note describes proof-of-principle experiments for the detection of camptothecin-induced apoptosis in Jurkat cells and stimulated peripheral blood mononuclear cells (PBMCs) using the Annexin V FITC apoptosis detection kit on the BD FACSVerse system.

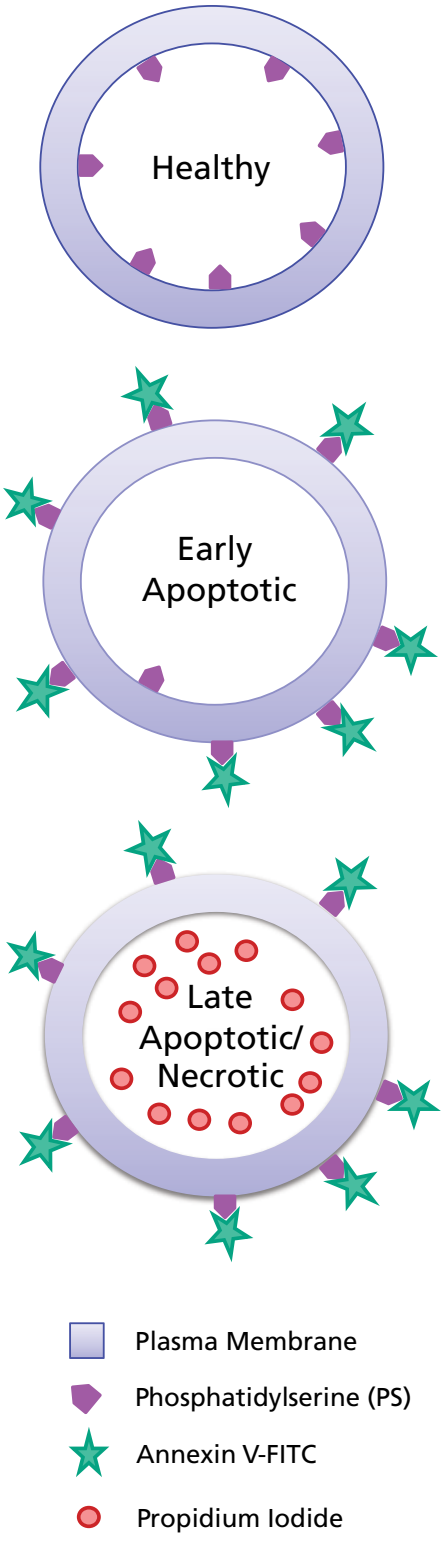


Figure 1. Diagram showing healthy and apoptotic cells with markers for detection of apoptosis.

Introduction

Apoptosis is a normal genetically programmed process that occurs during embryonic development, as well as in maintenance of tissue homeostasis, under pathological conditions, and in aging. The term apoptosis, from the Greek word for "falling off" of leaves from a tree, is used to describe a phenomenon in which a cell actively participates in its own destructive processes.[1] The process is characterized by specific morphologic features, including loss of plasma membrane asymmetry and attachment, plasma membrane blebbing, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane asymmetry is one of the earliest features of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS, and binds to exposed apoptotic cell surface PS.[2–4] Annexin V can be conjugated to fluorochromes while retaining its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis. This process is summarized in Figure 1.

PS translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) for identification of early and late apoptotic cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because they will also stain with both Annexin V and PI. However, when apoptosis is measured over time, cells can often be tracked from Annexin V and PI negative (viable, or no measurable apoptosis), to Annexin V positive and PI negative (early apoptosis with intact membranes), and finally to Annexin V and PI positive (end stage apoptosis and death). The presence of cells with these three phenotypes within a mixed cell population, or the "movement" of a synchronized cell population through these three stages, suggests apoptosis. In contrast, a single observation indicating that cells are both Annexin V and PI positive, in and of itself, reveals less information about the process by which the cells underwent their demise.

BD FACSVerse System and BD FACSuite Software

The BD FACSVerse system is a high-performance flow cytometer that incorporates easy-to-use, task-based workflows. The system streamlines every stage of operation from automated setup through data analysis and reporting. The system includes unique features such as the BD™ Flow Sensor option for volumetric counting, automated procedures for setting up the instrument and assays, and configurable user interfaces that provide maximum usability for researchers. These functions are integrated to provide simplified routine applications while simultaneously providing powerful acquisition and analysis tools for more complex applications. In addition, the BD FACS™ Universal Loader option (the Loader) provides the capability to use either tubes or plates for samples, with or without barcoding for sample identification and tracking.

The Annexin V FITC assay in BD FACSuite software is a specific module based on the BD Pharmingen Annexin V FITC apoptosis detection kit which contains all the acquisition, analysis, and reporting functions for generating data to measure the viability and Annexin V status of cells within a given population.

Pre-defined assays such as the Annexin V FITC assay can also be used as a starting point for creating custom experiments and assays to suit the needs of researchers. These user-defined assays can then be run in a worklist, or deployed to other BD FACSVerse cytometers within the laboratory or to an external site.

Objective

The objective of this application note is to demonstrate the ease of use of the Annexin V FITC apoptosis detection kit and the Annexin V FITC assay in conjunction with the BD FACSVerse system for the detection of camptothecin-induced apoptosis in Jurkat cells and stimulated peripheral blood mononuclear cells (PBMCs).

Methods

Kits

Product Description	Vendor	Catalog Number
BD Annexin V: FITC Apoptosis Detection Kit I (Contains Annexin V-FITC, Propidium Iodide Staining Solution, Annexin V Binding Buffer)	BD Biosciences	556547

Reagents and Materials

Product Description	Vendor	Catalog Number
BD Falcon™ Round-Bottom Tubes, 12 x 75 mm	BD Biosciences	352052
BD Falcon Conical Tubes,15 mL	BD Biosciences	352096
BD Falcon Cell Culture Flask, 25 cm^2	BD Biosciences	353108
BD Vacutainer® Tubes with Heparin	BD Medical	367874
BD IMag™ Cell Separation Magnet	BD Biosciences	552311
BD FACSuite CS&T Research Beads	BD Biosciences	650621 (50 tests) 650622 (150 tests)
BD FACSuite FC Beads - 4c Research Kit	BD Biosciences	650625

BD FACSVerse Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Fluorochrome
488	D	560 LP	586/42	Propidium iodide
	E	507 LP	527/32	FITC

Software

Product Description	Catalog Number
BD FACSuite Research Assay Software	651363

Cell Lines

Cell Line	Source	Designation	Culture Medium
Jurkat, Clone E6-1	ATCC	TIB-152	RPMI 1640 medium + 10% FBS

Specimens

Blood specimens were collected from normal donors who consented to participate in an Institutional Review Board–approved protocol.

Methods

Sample Preparation and Camptothecin Treatment

Jurkat

1. Jurkat cells were maintained in the culture medium containing RPMI 1640 (ATCC, No. 30-2001) supplemented with 10% fetal bovine serum (FBS) (ATCC, No. 30-2020).
2. Jurkat cells, in log phase of growth, were harvested by centrifugation at 300*g* for 5 min at room temperature (RT).
3. The supernatant was discarded and the cells were resuspended at a concentration of 1 x 10^6 cells/mL in culture medium and used for camptothecin treatment.
4. A 10-mM stock solution of camptothecin (Sigma-Aldrich, No. C-9911) was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, No. D-2650) and diluted into the culture medium at a final concentration of 5 µM for treated samples. An untreated sample with equivalent DMSO concentration was used as the control. To determine the dose-dependent effect of camptothecin, the 10-mM stock solution was diluted to final concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 µM.
5. Cells were incubated at 37°C with 5% CO_2 in air for 4 hours.

PBMC Stimulation

1. Whole blood was collected from normal donors into BD Vacutainer tubes (heparin).
2. PBMCs were isolated using Ficoll-Paque™ PLUS (GE Healthcare, No. 17-1440-02) as outlined in the product technical data sheet (TDS).[5]
3. Isolated PBMCs were suspended in culture medium at a concentration of 1 x 10^6 cells/mL and stimulated with Dynabeads® Human T Activator CD3/CD28 (Invitrogen, No. 111-31D) for 3 days in a T25 flask following the manufacturer's instructions.
4. Cells were harvested, beads removed using the BD IMag Cell Separation Magnet following the manufacturer's instructions, and the cell concentration was determined using the Trypan blue exclusion method.
5. The cell suspension was centrifuged (500*g*, 10 min, RT) and resuspended in fresh culture medium at a concentration of 1 x 10^6 cells/mL and cultured for an additional 16–18 hours in two T25 flasks.
6. A 10-mM stock solution of camptothecin was diluted into the culture medium at final concentrations of 0, 0.03, 0.06, 0.125, 0.3, 0.6, 1.25, 2.5, and 5.0 µM.
7. Cells were incubated at 37°C with 5% CO_2 in air for 16 hours.

Staining the Cells

Treated and untreated cells were stained as described in the Annexin V FITC Apoptosis Detection Kit TDS.[6]

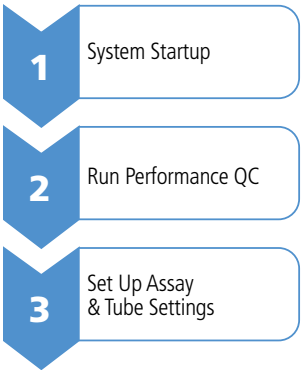


Figure 2. Workflow for instrument setup.

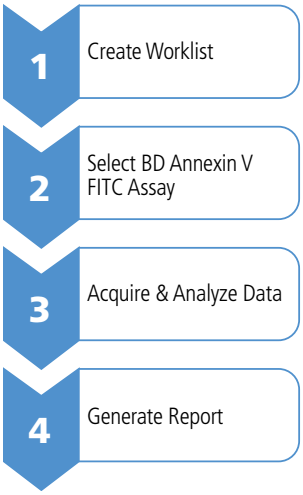


Figure 3. BD Annexin V FITC assay workflow.

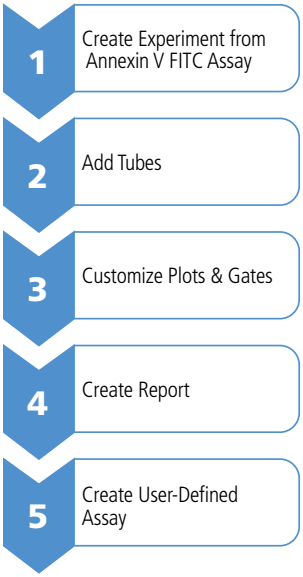


Figure 4. Workflow for creating a user-defined assay from a BD-defined assay.

Instrument Setup

The basic workflow for BD FACSVerse instrument setup is shown in Figure 2. Performance quality control (QC) was performed using BD FACSuite CS&T research beads as outlined in the *BD FACSVerse System User's Guide*.[7] The Annexin V FITC assay setup was then performed following the instructions in *BD FACSuite Software Research Assays Guide*.[8] The reference settings for compensation were automatically applied.

Annexin V Apoptosis Detection Assay

Data was acquired using a BD FACSVerse system and BD FACSuite software using the Annexin V FITC assay. As shown in Figure 3, a worklist was created from the assay and the samples were acquired automatically using the Loader with acquisition criteria of 10,000 events for each tube. The data was analyzed and a report was automatically generated.

The report generated from the apoptosis assay includes the following plots and gates for untreated and treated samples (Figure 5):

1. FSC-A vs SSC-A with a gate for cells
2. Annexin V FITC-A vs Propidium Iodide-A (PI-A) with gates for following populations:
   a. Annexin V^-/PI^-
   b. Annexin V^+/PI^-
   c. Annexin V^+/PI^+
   d. Annexin V^-/PI^+

In addition, a summary of assay results with statistics for untreated and treated samples was automatically calculated in the report. The summary includes:

- Absolute number of events acquired
- % Cells
- % Annexin V^-/PI^-
- % Annexin V^+/PI^-
- % Annexin V^+PI^+
- % Annexin V^-PI^+

User-Defined Assay

The Annexin V FITC assay was used as a starting point for creating a user-defined assay to accommodate additional tubes required for performing a camptothecin dose response experiment. Figure 4 outlines the workflow for creating a user-defined assay from a BD-defined assay. This user-defined assay was used to acquire camptothecin dose-dependent data from Jurkat cells and PBMCs in a worklist using the Loader. For details about creating a user-defined assay, see the *BD FACSVerse System User's Guide*.[7]

Results and Discussion

Camptothecin, a topoisomerase I inhibitor that has been shown to induce apoptosis and cell death in a variety of cell types, was used to demonstrate the Annexin V FITC assay in BD FACSuite software. Two commonly used model systems were used: Jurkat, a cell line derived from an acute human T-cell leukemia, and human PBMCs stimulated with the polyclonal T-cell activators CD3 and CD28. In these two models, apoptosis was induced and detected using Annexin V FITC apoptosis detection kit reagents and the Annexin V FITC assay in BD FACSuite software on a BD FACSVerse system.

Annexin V Apoptosis Detection Assay Using Untreated and Camptothecin-Treated Jurkat Cells

Untreated and camptothecin-treated (5 µM) Jurkat cells were stained using the Annexin V FITC apoptosis detection kit. Data from the untreated and treated samples was acquired in a worklist using the Annexin V FITC assay. Figure 5 shows the assay lab research report that was automatically generated after acquisition and analysis. FSC-A vs SSC-A plots were used for gating cells and to identify any changes in the scatter properties of the cells. Annexin V FITC-A vs Propidium Iodide-A plots from the gated cells show the populations corresponding to viable and non-apoptotic (Annexin V^-PI^-), early (Annexin V^+PI^-), and late (Annexin V^+PI^+) apoptotic cells.

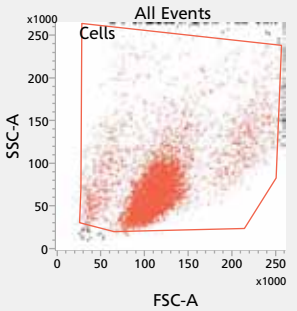
In the untreated (control) samples, the majority of cells (93.3%) were viable and non-apoptotic (Annexin V^-PI^-). In contrast, when cells were treated with 5 µM of camptothecin for 4 hours, 69.6 % of Annexin V^-PI^- cells were observed. There was an increase in early apoptotic cell populations (Annexin V^+PI^-) from untreated to treated cells (2.2% to 23.0% Annexin V^+PI^-, respectively). A slight increase in the Annexin V^+PI^+ population was also observed which indicates late apoptotic or dead cells. The increase in apoptotic cells was also reflected by changes in the light scatter properties for the untreated and treated tubes (FSC-A vs SSC-A plots). During apoptosis, cell shrinkage occurs, which is associated with a decrease in forward scatter. Further, the formation of apoptotic vesicles in the cells during apoptosis leads to an increased side scatter profile.

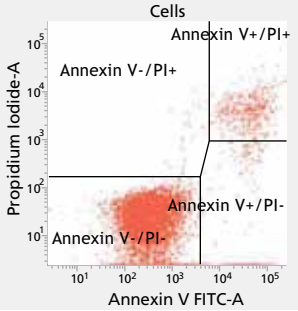
BD Annexin V FITC v1.0: Lab Report

Cytometer Name: BD FACSVerse
Cytometer Serial #: 123456789
Software Name & Version: FACSuite Version 1.0.0.1477
Operator Name: BDAdministrator
Report Date/Time: 11-Jul-2011 23:39:18

Tube Name: Untreated

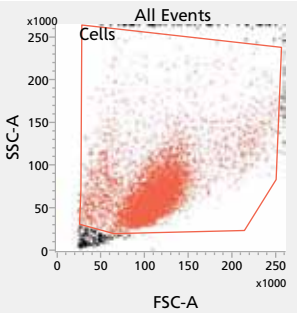
Sample ID	Jurkat	Acquisition Date	11-Jul-2011
Time (hrs)	4 h	Acquisition Time	23:39:12
Conc.	0 µM		

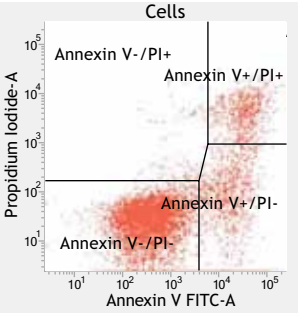




Tube Name: Treated

Sample ID	Jurkat	Acquisition Date	11-Jul-2011
Time (hrs	4h	Acquisition Tim	23:39:44
Conc.	5 µM		





Cytometer Name: BD FACSVerse
Software Name & Version: FACSuite Version 1.0.0.1477
Operator Name: BDAdministrator
Cytometer Serial #: 123456789
Report Date/Time: 11-Jul-2011 23:39:18

Results Summary

Label	Untreated	Treated
Events Acquired	10000	10000
% Cells	97.5	96.0
% Annexin V-/PI-	93.3	69.6
% Annexin V+/PI-	2.2	23.0
% Annexin V+/PI+	4.3	6.5
% Annexin V-/PI+	0.2	0.9

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Figure 5. BD FITC Annexin V Apoptosis assay lab report showing detection of apoptosis in untreated and camptothecin-treated (4 hour) Jurkat cells.

Dose-Dependent Effect of Camptothecin on Jurkat Cells and Stimulated PBMCs

Jurkat cells and stimulated PBMCs were treated with varying concentrations of camptothecin to demonstrate the dose-dependent effect on apoptosis. Figure 6 shows proof-of-principle data from the Jurkat dose response experiment as Annexin V FITC-A vs PI-A contour plots with quadrant gates showing four populations. In the untreated control sample, the majority (93.44%) of cells were viable and non-apoptotic, and with increasing doses of camptothecin, there was a decrease in the Annexin V^-PI^- population and an increase in cells undergoing early apoptosis (Annexin V^+PI^-). Data from the four populations was further plotted against the concentration of camptothecin (Figure 6, Panel B) which also shows a dose-dependent increase in the Annexin V^+PI^- population and a decrease in the Annexin $^-PI^-$ population. A slight increase in the Annexin V^+PI^+ population indicating dead or necrotic cells was observed at higher doses of camptothecin.

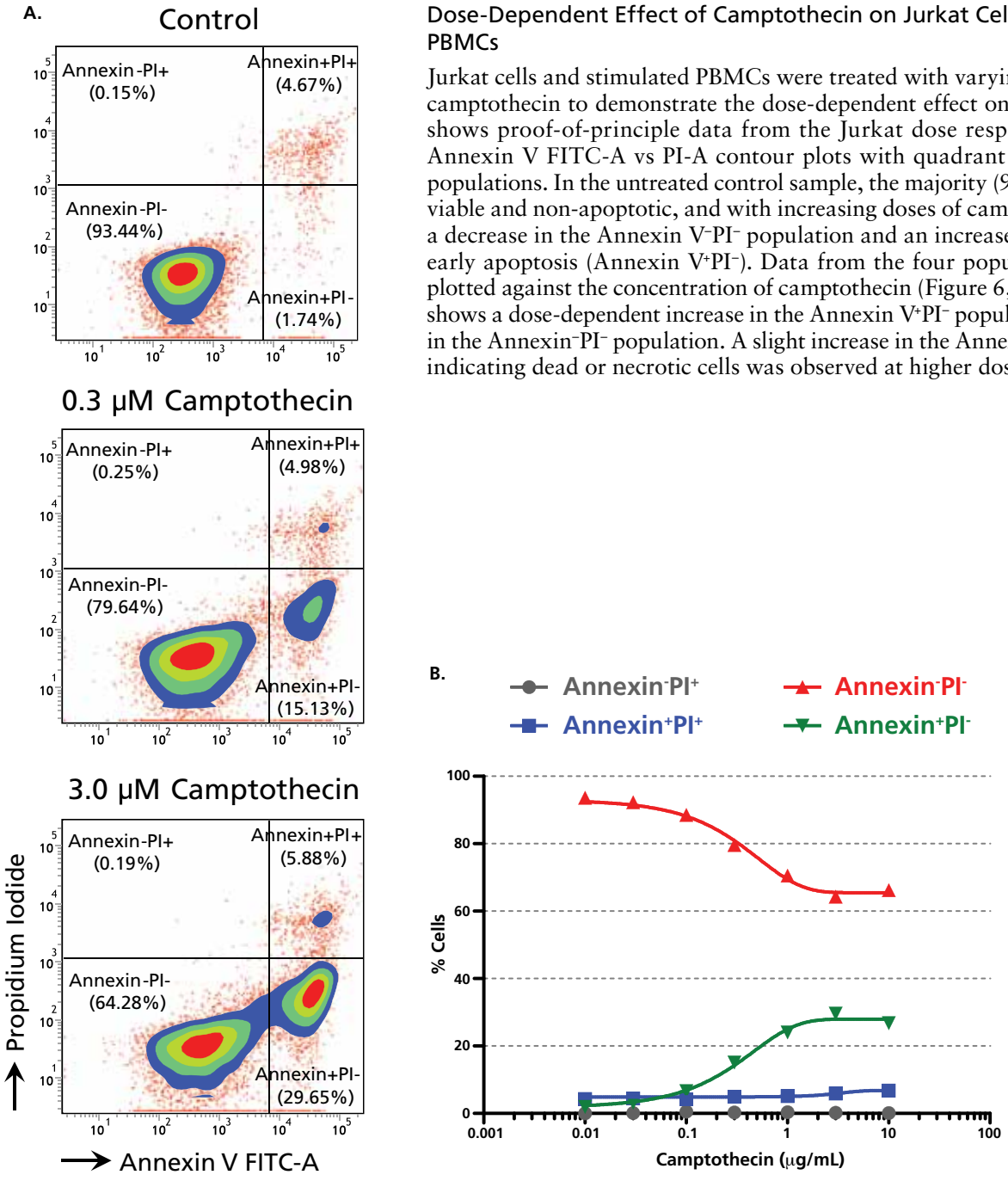

Figure 6. Dose-dependent effect of camptothecin on apoptosis in Jurkat cells.
Jurkat cells were treated with various concentrations of camptothecin for 4 hours followed by staining with Annexin V FITC and propidium iodide. **Panel A.** Representative Annexin V FITC-A vs Propidium Iodide-A contour plots from two concentrations of camptothecin. **Panel B.** Dose response curve of camptothecin showing the percentages of cells in four populations gated as shown in panel A.

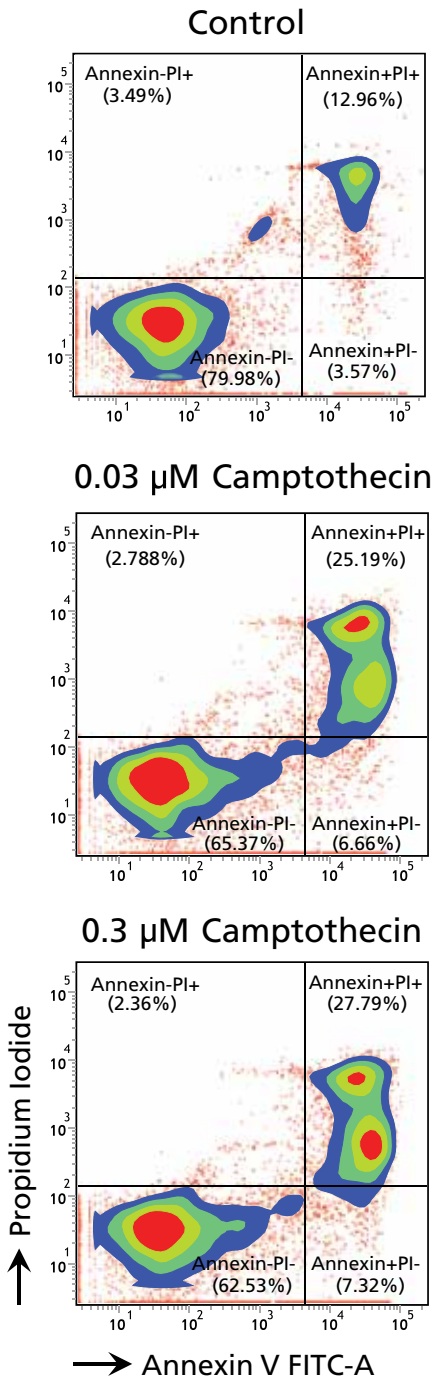

Figure 7. Dose-dependent effect of camptothecin on apoptosis in stimulated PBMCs.
PBMCs were stimulated with polyclonal T-cell activator (CD3/CD28) followed by treatment with various concentrations of camptothecin for 16 hours. Samples were then stained with Annexin V FITC and propidium iodide. Representative Annexin V FITC-A vs Propidium Iodide-A contour plots from two concentrations of camptothecin.

Figure 7 shows the effect of camptothecin on stimulated PBMCs. In the absence of camptothecin, the majority (79.98%) of untreated stimulated PBMCs were viable and non-apoptotic (Annexin V^-PI^-). However, with the increasing doses of camptothecin, there was an increase in the Annexin V^+PI^+ population accompanied by a lesser increase in the Annexin V^+PI^- population. Within the Annexin V^+PI^+ gate (late apoptotic cells), two populations were observed in camptothecin-treated stimulated PBMCs. This could be due to the heterogeneity in stimulated PBMCs since different subsets might undergo apoptosis in a non-synchronized fashion.

Conclusions

The BD Annexin V Apoptosis Detection Kit and BD Apoptosis Detection Assay in BD FACSuite software provide a quick and easy way to acquire and analyze data from apoptotic cells. Untreated and treated samples can be quickly acquired in an automated worklist mode and a laboratory research report with plots and statistics is automatically generated.

In addition, the pre-defined BD Apoptosis Detection Assay can be used as a starting point for creating a user-defined assay for use with multiple samples in high-throughput format such as screening for drug toxicity. In this application note, using the BD Annexin V Assay, we have presented proof-of-principle data to demonstrate camptothecin-induced apoptosis in Jurkat cells. In addition, dose-dependent effects of camptothecin on Jurkat cells and stimulated PBMCs have been shown using a user-defined assay.

References

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5. Ficoll-Paque Plus Instructions. 71-7167-00 AG.
6. FITC Annexin V Apoptosis Detection Kit I TDS. 556547, Rev 5.
7. *BD FACSVerse System User's Guide.* 23-11463-00, Rev. 01.
8. *BD FACSuite Software Research Assays Guide.* 23-11470-00, Rev. 01.

Tips and Tricks

- Annexin V binding is calcium-dependent, and defined calcium and salt concentrations are required for optimal staining. Always use the supplied Annexin V binding buffer for staining.
- We recommend using a positive control such as camptothecin to induce apoptosis in cells being tested.
- A DMSO-only control should be included for apoptosis-inducing compounds where DMSO is used as solvent.

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