

# Measuring Proliferation in Leukemia Cell Lines via Carboxyfluorescein Succinimidyl Ester (CFSE) upon Treatment with Cytostatic Concentrations of Cytosine Arabinoside (Ara-C)

## APPLICATION NOTE



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### IN THIS PAPER YOU WILL LEARN

A method to monitor and measure cell proliferation

Method to measure the activity of a cytostatic compound

## Principal of the Technique

### Background

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent cell staining dye<sup>1</sup>, which is cell permeable and covalently couples, via its succinimidyl group, to intracellular molecules. CFSE is retained within cells for a relatively long period of time and once incorporated does not transfer to other cells. CFSE has been utilized as a marker for cell counting and tracing purposes. Due to the fact that CFSE fluorescence decreases with each cell division, as the CFSE is divided between daughter cells, it is generally applied to cell proliferation studies. Given the stability of CFSE in cells it can be used to track 7-8 cell divisions typically. One drawback of using CFSE is that it exhibits very high cytotoxicity; thus careful optimization of the assay under specific circumstances is necessary.

### Research Applications: Introduction

Chemotherapeutic treatment of acute myeloid leukemia is based on the standard combination of daunorubicin and cytosine arabinoside (Ara-C). Ara-C is also utilized in the treatment of non-Hodgkin lymphoma. Ara-C works by combining a cytosine base with an arabinose sugar, and thus disrupts DNA synthesis.

Standard chemotherapy drugs are used at cytotoxic levels, however they are cleared from the body typically within 24 hours. At lower concentrations these drugs are likely to have a cytostatic effect, which would contribute to the effectiveness of the therapeutic regime.

Here we aim to utilize the CFSE proliferation assay to determine the cytostatic effect of sub-toxic concentrations of Ara-C on a number of leukemic cell lines.

## Protocol

### Standard Procedure

HL-60 and OCI/AML-3 cell lines were maintained at a culture density of  $1 \times 10^5$  -  $1 \times 10^6$  cell/mL in 10 mL of alpha-MEM medium supplemented with 10 % fetal calf serum (FCS, v/v), 100 units of penicillin per ml and 100 µg of streptomycin per mL at 37°C and 5 % CO<sub>2</sub>.

The U937 cell line was maintained at a culture density of  $1 \times 10^5$  -  $1 \times 10^6$  cell/mL in 10 mL of RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS, v/v), 100 units of penicillin per mL and 100 µg of streptomycin per mL at 37°C and 5 % CO<sub>2</sub>.

1. Cell lines were cultured to a density of  $5 \times 10^5$  cells/mL and 2 mL of these cells were collected in 15 mL centrifuge tubes
2. Cells were centrifuged at 400x g for 5 minutes
3. Supernatant medium was removed
4. Cells were washed with 1 mL of PBS
5. Cells were resuspended in 1 mL of PBS containing 0.5 µM CFSE dye (see Note 1)
6. Cells were incubated for 5 minutes at 37°C
7. Cells were centrifuged at 400x g for 5 minutes
8. PBS containing CFSE was removed
9. Cells were washed with 1 mL of PBS
10. Cells were resuspended in culture medium at the concentration of  $1 \times 10^5$  cells/mL and a sample was analysed for CFSE fluorescence. This will represent the CFSE uptake of the cells initially.
11. Cells were treated with 20 nM of Ara-C
12. 500 µL of cells are taken for flow at 48 hours for measurement of proliferation *via* CFSE fluorescence decrease.
13. CFSE fluorescence was read on the FITC channel
14. Cells were analysed for fluorescence on a CytoFLEX flow cytometer (Beckman Coulter)
15. Cells were gated on FSC vs. SSC to identify the correct, viable cell population.
16. Gated cells were further gated on FSC-area vs. -height to discriminate singlet cells from doublet cells
17. Analysis of CFSE fluorescence was done using overlay histograms of singlet cells
18. CFSE fluorescence is collected in the standard FITC channel of the CytoFLEX

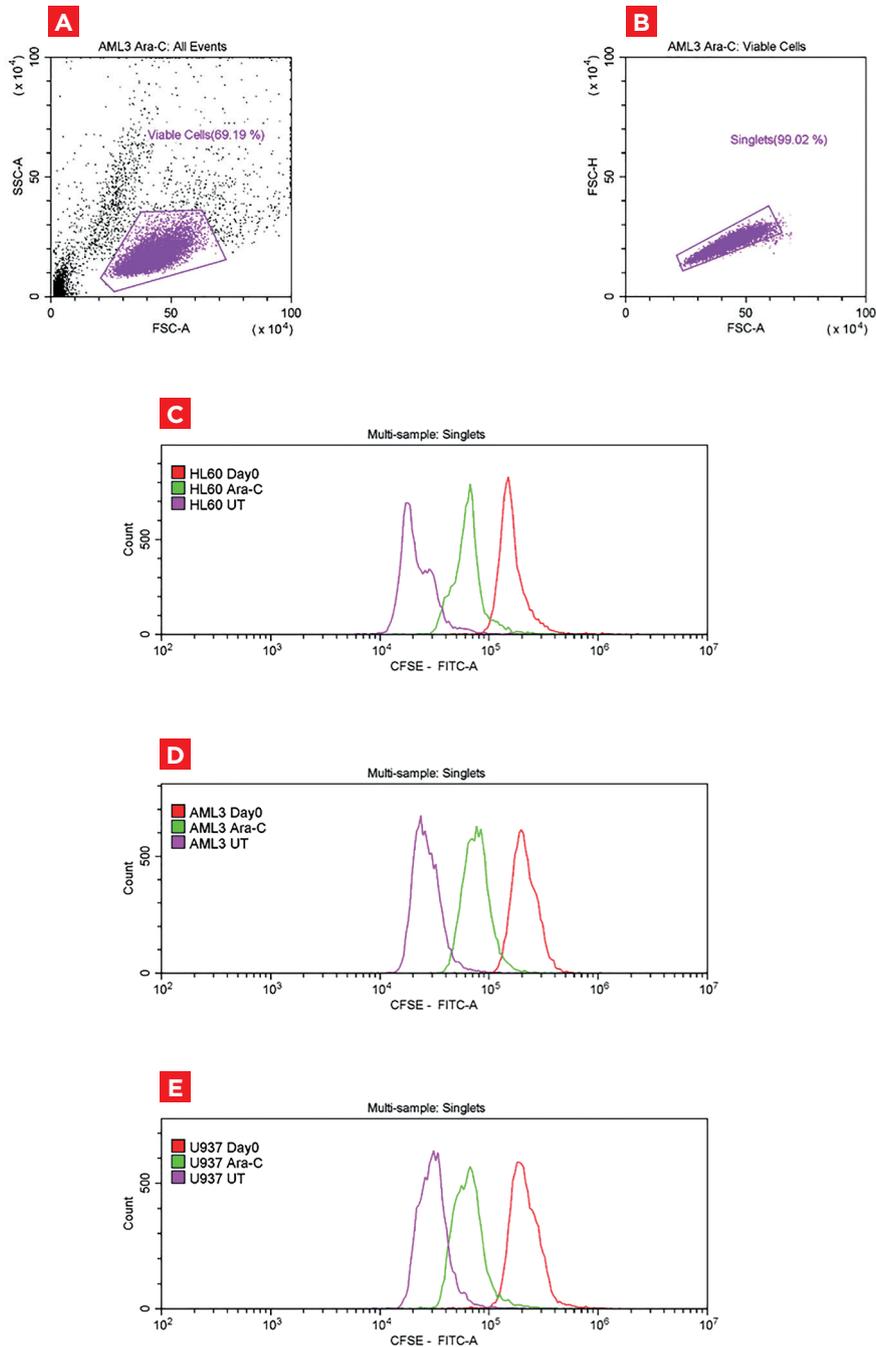
### Materials & Methods

**List material required but not supplied** Beckman Coulter CytoFLEX , OCI/AML-3 cells, HL-60 cells, U937 cells, Centrifuge, microcentrifuge, Gilson pipette (P10, P20, P200, P1000), 15 mL Centrifuge tubes, 1.5 mL microcentrifuge tubes.

**Reagents** alpha-MEM medium (supplemented with 10 % fetal calf serum (FCS, v/v), 100 units of penicillin per ml and 100 µg of streptomycin per mL). RPMI 1640 medium (supplemented with 10 % fetal calf serum (FCS, v/v), 100 units of penicillin per mL and 100 µg of streptomycin per mL). CellTrace CFSE Cell Proliferation kit (Life Technologies). Cytosine Arabinoside (Ara-C).

### Sample prep

Sample Type (include cell line information if available)	Species	Age of specimen (if available-or time since prep)	Prep Method
OCI/AML3	Human		
HL-60	Human		
U937	Human		



**Figure 1:** Measuring Proliferation in Leukemia Cell Lines via Carboxyfluorescein succinimidyl ester (CFSE) upon treatment with cytostatic concentrations of Cytosine Arabinoside (Ara-C). (A) Gating strategy for viable cell population on a FSC vs. SSC dot-plot, showing OCI/AML-3 cells treated with Ara-C. All samples are gated in a similar manner. (B) Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area vs. -height. All samples are gated in a similar manner. CFSE fluorescence was analysed on the FITC-A channel in cells immediately post-staining (red histograms), after 48 hours in standard culture (pink histograms) or after 48 hours in Ara-C treatment (green histograms) in (C) HL-60, (D) OCI/AML-3 or (E) U937 cells.

## Results

HL-60, OCI/AML-3 and U937 cells were grown for 48 hours in the presence or absence of a 20 nM Ara-C. These cells exhibited effectively no loss in viability, as seen by FSC vs. SSC profiles. Thus, this level of Ara-C is determined as sub-toxic (this concurs with previous studies, by our group and others). We next set out to determine if this concentration of Ara-C inhibited cell proliferation. This was done by measuring the reduction of CFSE fluorescence. The greater the reduction in CFSE fluorescence, the more proliferation will have occurred in the culture. CFSE fluorescence was measured in cells immediately subsequent to staining and 48 hours later, with- and without Ara-C treatment.

From the data we see that in all cultures examined, Ara-C treatment resulted in a reduced proliferation than in untreated cultures. Interestingly, all cells proliferated to some extent. So, while Ara-C treatment did affect the rate of proliferation it did not prevent cell growth entirely. The level of CFSE fluorescence decreased from levels after the initially staining even with Ara-C treatment, but not to the same extent as the untreated cells. This could be due to the cells continuing to cycle and divide initially in the presence of Ara-C before the cells arresting eventually.

Thus CFSE staining is a useful tool to rapidly measure the effect of drug treatment on the proliferative capacity of cells in culture.

## References

1. Parish CR. 1999. Immunol Cell Biol. 77(6):499-508. Fluorescent dyes for lymphocyte migration and proliferation studies.

## Notes

In our optimization of this assay we determined that concentrations greater than 0.5  $\mu$ M of CFSE and incubation times of greater than 5 minutes resulted in high levels of apoptosis. The staining parameters we used here resulted in the greatest cellular staining with minimal (< 5%) reduction in cell viability.

## Reagent Details

Reagent	Supplier	Order Details
CellTrace CFSE Cell Proliferation kit	Life Technologies	C34554
Cytosine Arabinoside (Ara-C)	Sigma Aldrich	C1768

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