

DRAQ5-Based DNA Content Analysis of Hematolymphoid Cell Subpopulations Discriminated by Surface Antigens and Light Scatter Properties

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Background: Analysis of cell cycle kinetics offers important information regarding the behavior of normal and neoplastic cells. Most often, cell cycle determinations by flow cytometry (FCM) have been performed using whole-sample analysis with intercalating dyes like propidium iodide (PI). The cell cycle phase assessment in individual cell subsets in heterogeneous samples is best performed using combined antigen/scatter and DNA analysis. DRAQ5, a novel DNA binding dye that excites at 488 nm and emits in the far red spectra, rapidly penetrates intact live cells while preserving their light scatter properties and expression of surface antigens. We evaluated the ability of this dye to measure cell cycle phases in a variety of clinical hematolymphoid samples.

Methods: We first compared whole sample DRAQ5 and PI cell cycle analyses in 26 clinical hematolymphoid samples. Next, we analyzed cell subpopulations in 39 samples of nonpathologic bone marrow by performing simultaneous CD45/CD34 and DRAQ5 staining. We assessed cell cycle characteristics specific to each population identified by CD45/CD34/side light scatter: lymphocytes, monocytes, immature and mature granulocytes, nucleated erythroid cells, and early precursors.

Results: Whole sample DNA cell cycle analyses by DRAQ5 and PI showed no significant differences in S-phase. DRAQ5, however, produced slightly larger coefficients of variation. DRAQ5-based DNA content analysis was easily performed on the distinct marrow cell subpopulations, since light scatter and antigen expression were completely preserved. Significant differences in S-phase were noted between subpopulations of cells exhibiting different degrees of maturation.

Conclusions: Because of its simplicity of use, excitability with 488 nm lasers, and the ability to stain viable cells, DRAQ5 should prove most useful in the kinetic evaluation of normal and neoplastic hematolymphoid cell subsets identified by light scatter and antigenic expression. © 2004 Wiley-Liss, Inc.

Key terms: DRAQ5; DNA content; cell cycle; S-phase; flow cytometry; proliferation; tumor kinetics; bone marrow; combined antigen and DNA content analysis

Analysis of cellular kinetics offers important information regarding the biologic behavior of both normal and neoplastic cell populations. Measurement of cell cycle kinetics of neoplastic cells in tumor samples, however, is hampered by the difficulty of specifically measuring malignant cells, since these cells are usually admixed with a variable, and sometimes significant, number of normal cells. Flow cytometry (FCM) has been extensively used in the analysis of cell cycle kinetics. In particular, it has been very popular to measure cell cycle fractions in neoplasias, including those of hematologic and lymphoid origin (1,2). Intercalating dyes such as propidium iodide (PI), which are excitable with argon ion lasers commonly used in most commercial flow cytometers, have been frequently utilized in the rapid determination of cell cycle phases in

tumors, generally producing DNA analysis histograms of consistent quality. However, the vast majority of these analyses were performed on whole samples, without taking into account the effect of normal cells on the mea-

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surements. Normal elements that are usually quiescent may affect kinetic results by producing a "dilution effect." For instance, a rapidly proliferating tumor with a relatively high S-fraction will appear less proliferative if a significant number of nonmalignant, slowly dividing cells are present. Conversely, proliferating normal cells (e.g., marrow progenitors) may cause the opposite effect.

In the past, flow cytometry-based kinetic analysis of selective cell subsets has been accomplished using simultaneous DNA and surface antigen labeling. This approach was previously reported with the use of UV-excitabile Hoechst 33342 on viable cells (3), and PI with FITC-labeled antibody detection of surface immunoglobulin in B-cell lymphomas (4,5). Simultaneous DNA and surface antigen labeling was also performed on unfixed bone marrow cells using lineage-specific primary monoclonal antibodies with a secondary FITC-conjugated antibody, followed by staining with hypotonic PI (6). PI-based procedures are relatively simple and can be performed using standard clinical instruments. However, the emission spectrum of PI makes it difficult to use more than one fluorochrome for simultaneous antigen labeling. Multicolor analysis is possible with alternative DNA dyes. For example, three-color analysis with the DNA binding dye 7-amino-actinomycin D (7-AAD), in conjunction with FITC- and PE-conjugated antibodies, has been performed with the efficient use of a single 488 nm laser (7). Additionally, 3-color (8) and 4-color analysis using TO-PRO-3 iodide has been reported, with minimal emission into the green, orange, and deep red fluorescence detectors (8).

Despite the successful use of DNA dyes with multicolor surface antigen labeling, the aforementioned analyses require the use of UV-emitting lasers that are not commonly available on clinical flow cytometry instruments, or cell fixation and/or permeabilization, as intact cells are impermeable to most of these compounds (7). Gentle cell fixation and permeabilization methods have been reported to improve DNA content analysis (9). However, any type of fixation and permeabilization is likely to alter the light scatter properties, and affect the quality of surface antigen labeling, hampering resolution of cell subpopulations.

DRAQ5, a novel synthetic anthraquinone excitable at 488, 568, 633, and 647 nm (optimal excitation), and fluorescing from 670 nm into the far-red visible light spectrum, specifically binds to DNA by rapidly penetrating intact cell membranes. With this dye, no fixation or permeabilization is necessary for DNA staining, and the physical and antibody labeling properties of the cells are preserved. Thus, DRAQ5 allows easy and rapid cell cycle measurements of subpopulations that are clearly defined by light scatter and immunophenotype. This dye should be particularly useful in measuring cell cycle phases in heterogeneous populations such as those of bone marrow, peripheral blood, or lymphoid tissues. Normal bone marrow contains cell populations of diverse origin that traverse multiple differentiation stages, each with different growth kinetics. Even in pathological states, marked cell heterogeneity may be present. In this study of heterogeneous clinical samples, we show the feasibility of per-

forming DRAQ5-based cell cycle phase analysis on specific cell subpopulations identified by intact scatter properties and antigen labeling.

MATERIALS AND METHODS

Sample Collection and Initial Preparation

For comparison of DNA cell cycle analyses between DRAQ5 and PI, we collected a total of 26 fresh discarded samples used for routine diagnostic analysis in our clinical hematopathology service. They included 20 bone marrows, four peripheral bloods, an orbital mass, and a submental lymph node. The diagnoses included peripheral T-cell, large B-cell, Burkitt, follicular and marginal zone lymphomas, myelodysplastic syndrome, polycythemia vera, and hairy cell leukemia, as well as nondiagnostic cases that showed no evidence of hematopoietic malignancy. For evaluation of DRAQ5 in specific bone marrow subpopulations, an additional 39 cases of nonpathologic bone marrow aspirates were obtained from discarded staging samples in lymphoma patients. Cases were defined as nonpathologic based on routine microscopic and flow cytometric immunophenotype evaluation.

Bone marrow and peripheral blood samples were received in EDTA. Lymphoid tissues were received fresh and cell suspensions were prepared by mincing the tissue with scalpels in RPMI media, and filtering through a wire mesh screen (#80 mesh). Erythrocytes in the peripheral blood and bone marrow cell suspensions were lysed by incubating with lysing solution (0.15 M NH_4Cl [8.29 gm NH_4Cl], 1.0 gm KHCO_3 , 37 mg EDTA, and 1 L distilled H_2O) for 10 min at room temperature at a ratio of 1:9 (volume of sample:volume of lysing solution), with a final volume of 50 mL. For erythrocyte lysing of lymph node/tissue cell suspensions, cells were pelleted by centrifugation (500 g for 5 min at room temperature), media was aspirated from the pellet, and lysing solution (45 mL) was added directly, with subsequent incubation at room temperature for 10 min. After incubation, cells were pelleted by centrifugation (500 g for 5 min at room temperature), the media was aspirated, and the cells washed twice in a PBS solution containing 0.1% NaN_3 . After the final wash step, cells were resuspended in RPMI medium with 10% FCS containing a mixture of antibiotics.

Cell Surface Staining

Surface staining of the cells was performed in albumin (Sigma Chemical Company, Saint Louis, MO)-precoated wells in Falcon 96-well U-bottom assay plates (BD Labware, Franklin Lakes, NJ). FITC-conjugated CD45 antibody (CD45-FITC) and PE conjugated CD34 antibody (CD34-PE) (Becton Dickinson [BD], San Jose, CA) were used for staining. The antibody was prepared at a five-fold dilution by adding 200 μL of CD45-FITC and 200 μL of CD34-PE to 600 μL of a 1:1 solution of human type AB serum (Bio-whitaker, Walkersville, MD) and Hanks balanced salt solution (Mediatech Cellgro, Herndon, VA). Next, 20 μL of the resulting diluted antibody mixture was added to each microtiter well. For surface staining, approximately 3×10^5 cells were added to the coated wells containing the

Table 1
Comparison Between DRAQ5 and PI Derived Cell Cycle Analysis Parameters in a Variety of Hematolymphoid Samples

	Coefficient of variation of G0/G1 peak		S-phase (%)		Cell doublets (%)	
	PI	DRAQ5	PI	DRAQ5	PI	DRAQ5
Mean	2.9	4.3	8.9	7.8	1.7	1.2
SD	0.55	0.61	5.56	5.13	0.8	0.7
<i>P</i> value (paired <i>t</i> -test)	<0.0005		0.4		<0.003	

diluted fluorochrome-conjugated antibody and incubated for 15 min on ice in the dark. Subsequently, 50 μ L of PBS were added to each well, the tray was centrifuged at 500 g for 5 min and the supernatant was discarded. Next the cells were washed in 100 μ L of PBS, centrifuged at 500 g for 5 min, and the supernatant discarded. This wash step was then repeated for a total of two wash steps. After the last centrifugation and supernatant discard, cells were transferred to microtubes in a final volume of 250 μ L of PBS or To-Pro-3 iodide (Molecular Probes, Eugene, OR). The latter was used for viability gating as needed (10,11). The microtubes were inserted into corresponding 12 \times 75 mm tubes in the loader rack of a FACScalibur flow cytometer (Becton, Dickinson [BD], San Jose, CA) for data acquisition. Daily calibration of the instrument was performed using standardized CaliBRITE Beads (BD) with FACSComp Software (BD), and compensation was performed using appropriately stained normal peripheral blood samples.

DNA Staining

For DRAQ5 staining, 3×10^5 cells in 250 μ L of PBS, previously stained for surface antigens were incubated with 2 μ L of DRAQ5 (Biosstatus Ltd., Leicestershire, UK) for 5 min at room temperature and protected from bright light. For PI staining, reagents from CycleTEST Plus (BD), were used according to manufacturer's recommendations. In summary, cells were exposed to a trypsin solution for 10 min at room temperature, followed by a solution containing trypsin and RNase A, and subsequently incubated in PI for 10 min at 4°C at a final concentration of 125 μ g/mL.

Data Acquisition

Cells were acquired on a FACScalibur four-color flow cytometer (BD) equipped with both a 488-nm argon laser and a 635-nm diode laser. The data were acquired using CellQuest software (BD). Acquisition of the whole sample was performed without gating to exclude cell doublets and debris. The total number of cells collected in each case ranged from 40×10^3 to 50×10^3 .

Data Analysis

For DRAQ5 and PI comparisons, DNA cell cycle analysis was performed on unselected populations. DRAQ5-based DNA content analysis of discrete marrow subpopulations was performed by identifying and selecting those subpopulations by CD45 expression and side scatter proper-

ties. The following cell types were identified: lymphocytes, monocytes, mature granulocytes, immature granulocytes, nucleated erythroid cells, and early precursors (blast region) (12,13). The latter population was defined by CD34 expression, along with intensity of CD45 expression and side scatter. Each subpopulation's data were saved as separate Flow Cytometry Standard files using CellQuest software (BD), and analyzed for cell cycle phases using Modfit LT 3.1 (Verity Software House, Topsham, MA).

DNA content analysis included determination of the mean channel fluorescence and the coefficient of variation (CV) of the G1 peaks, number of cell doublets, and S-phase fractions. All data were generated using the Auto-analysis function of the Modfit LT 3.1 program, with active aggregates and debris modeling, according to software manufacturer's recommendations. Paired *t*-test was used for statistical calculations.

For the purposes of data display and creation of figures, dot plots were generated using WinMDI 2.8 (Joe Trotter, Scripps Institute).

RESULTS

Comparison Between DRAQ5 and PI

Results of DNA content analysis, using DRAQ5 and PI in parallel samples, are shown in Table 1. DRAQ5 staining produced slightly but significantly wider CVs of the diploid G0/G1 peak than PI, with CVs that ranged from 3.1% to 5.6% (mean 4.3%), compared to the CycleTEST PI CVs that ranged from 2.2% to 4.2% (mean 2.9%). Analysis of 25 samples showed diploid DNA content (single G0/G1 peak), while aneuploidy was detected in a single case (a submental lymph node, Fig. 1B). Although the CV in this case was slightly wider in the DRAQ5 (5.42%) than in the PI (2.16%) stained sample, the modeled DNA index was identical in both (1.7). No significant differences in the resulting total S-phase fraction was noted in the 25 samples, but a significantly lower number of cell doublets were noted in the DRAQ5 stained specimens (Table 1).

Analysis of Bone Marrow Subpopulations by DRAQ5

Marrow subpopulations were delineated in 39 cases of nonpathologic bone marrow specimens by CD45/SSC. Using this approach, lymphocytes, monocytes, mature granulocytes, immature granulocytes, early precursors, and nucleated erythroid cells were clearly demonstrated as discrete clusters (Fig. 2). All bone marrow samples and subpopulations within samples

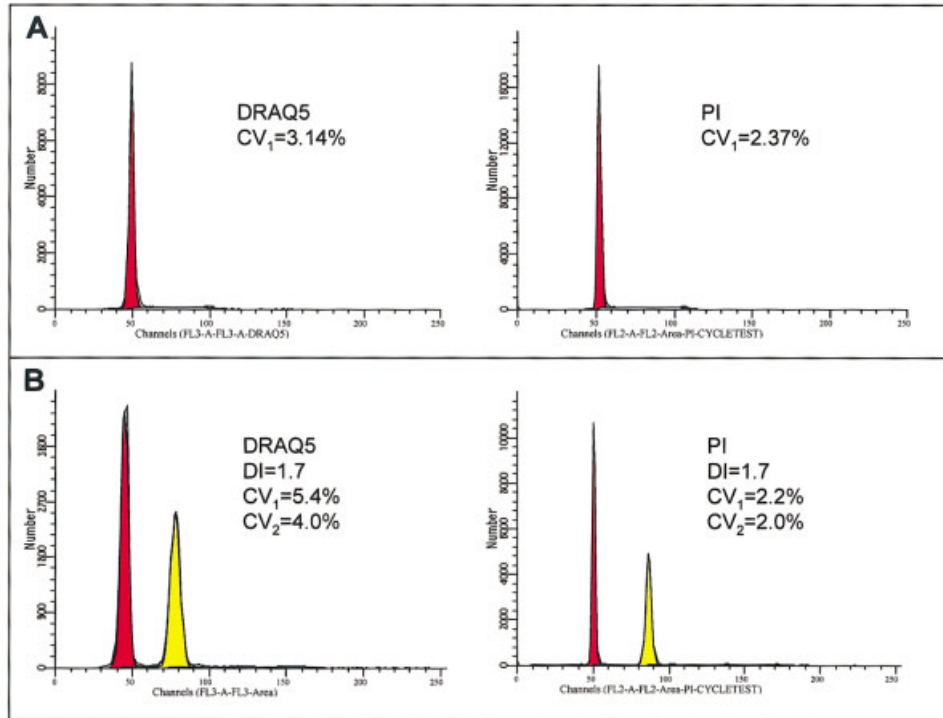


FIG. 1 Examples of two cases stained in duplicate with DRAQ5 and PI. The first case exhibits a single G0/G1 peak (A) and the second case exhibits two G0/G1 peaks (B), representing aneuploidy. CV₁ corresponds to the first G0/G1 peak located approximately at channel 50 and CV₂ corresponds to the second G0/G1 peak (aneuploid G0/G1 peak). DRAQ5 produces slightly larger CVs than PI in both cases (A,B). An identical DNA index (DI) of the second G0/G1 peak (B) was obtained with both DRAQ5 and PI staining.

exhibited a single G0/G1 DNA peak and their CVs ranged from 2.7% to 3.8%. The mean channel fluorescent intensity of the G0/G1 peaks showed some small but significant ($P < 0.0005$) variability when each subpopulation was compared to lymphocytes within the tissue sample as an internal standard. The percent difference ranged from 2.5% to 4.7% for all subpopulations (Table 2).

Figure 2 highlights a representative case illustrating the DRAQ5-derived S-phase determinations obtained in subpopulations of bone marrow cells identified by the CD45/SSC display. The S-phase of each subpopulation was compared to that of the lymphocytes in the same sample, which served as an internal standard. When all samples were analyzed in this manner, there was a significant difference in the mean S-phase values between each of these subpopulations (Table 2, Fig. 2). Mature subpopulations, such as monocytes and lymphocytes, exhibited low S-phase fractions overall ($3.8 \pm 3.3\%$ and $0.3 \pm 0.3\%$, respectively). Mature granulocytes demonstrated lower S-phase fractions ($1.1 \pm 0.76\%$) than granulocyte precursors ($8.6 \pm 3.9\%$). The erythroid population showed a very high S-phase of approximately $24 \pm 5.7\%$. Although the early precursor population appeared to exhibit a higher S-phase than mature populations, insufficient numbers of CD34-expressing cells in these nonpathological samples pre-

cluded a reliable S-phase determination by cell cycle modeling.

DISCUSSION

We report our validation and experience with DRAQ5, a novel, cell permeating, DNA intercalating dye. We demonstrate comparable cell cycle analysis between DRAQ5 staining and a well-established PI-based method in various hematology specimens. We also show, through analysis of nonpathologic bone marrow samples, that DRAQ5 is ideal for simultaneous DNA S-phase analysis in cell subpopulations identified by light scatter and surface antigen characteristics.

From standpoint of a clinical laboratory, the DRAQ5 reagent is quick and easy to use; only minutes of incubation with DRAQ5 are required for saturable DNA staining (14). Moreover, although DRAQ5 fluorescence is optimally excited at 647 nm, it is sufficiently energized at 488 nm, and is thus suitable for excitation with argon-ion lasers that are found in most clinical instruments. Because no fixation or permeabilization is needed, DRAQ5 is technically advantageous over other dyes that do require these extra steps, such as PI, Topro-3, and 7-AAD (4,7,8,15), which have previously been used in multiparameter DNA and surface antigen analysis. Hoechst 33342 is a DNA-binding fluorochrome that can be used without cell permeabilization since it enters viable cells by active uptake

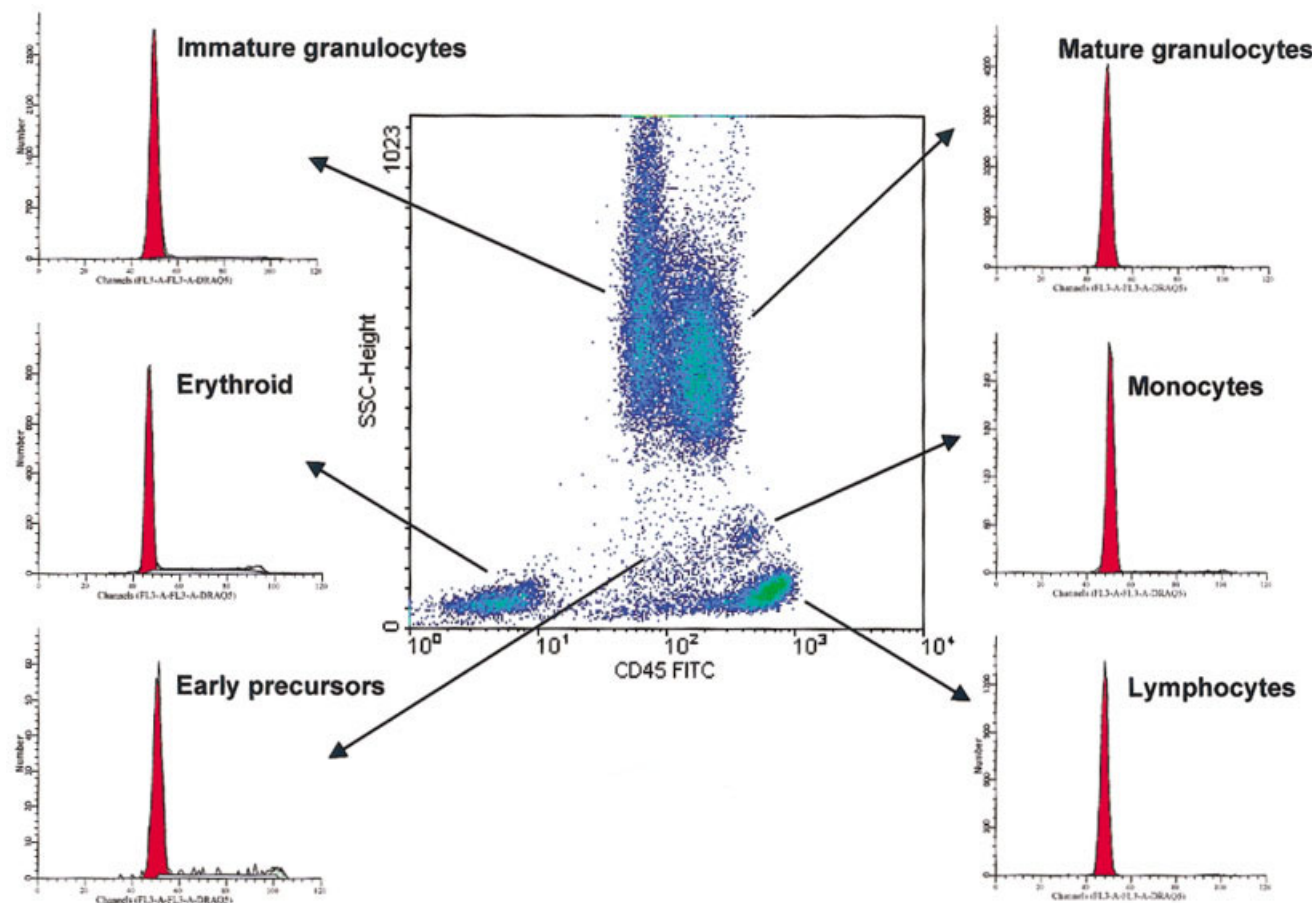


FIG. 2 A single representative case illustrating DRAQ5 derived S-phase fractions from a CD45/SSC display in a sample of nonpathologic marrow. *Early precursors were selected based on CD34/CD45 expression and SSC (not shown), but due to insufficient numbers of events, reliable determination of S-phase in this population could not be performed.

(3,16). However, this fluorescent dye requires UV excitation (16), and thus is not ideal for use in most commercial clinical instruments.

Using DRAQ5, detection of aneuploidy specific to abnormal cell populations may provide diagnostic and/or prognostic information (17). In our cases, a single lymph

node exhibited aneuploidy, and the DNA index by DRAQ5 and PI staining was identical. The remaining cases demonstrated a single G0/G1 peak, however DRAQ5 produced a slightly, but significantly wider CV when compared to PI. In addition, when compared to lymphocytes, small, but significant, differences in G0/G1 mean fluores-

Table 2
DRAQ5 Derived Cell Cycle Analysis Parameters in Bone Marrow Cell Subpopulations

	Early precursors ^a		Erythroid		Immature granulocytes		Mature granulocytes		Lymphocytes		Monocytes	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Difference in G0/G1 mean channel fluorescence (%) ^b	4.3	1.8	2.5	1.1	4.7	1	3.3	3.4	b	b	3.1	3.1
CV(%) of G0/G1 peak	3.8	0.74	2.7	0.4	3.2	0.5	3.3	0.4	3.3	0.4	3.3	0.8
S-phase (%)	^a	^a	23.9	5.7	8.6	3.9	1.1	0.8	0.3	0.3	3.8	3.3

^aDefined by CD45/CD34/SSC expression. Insufficient number of events in these populations precluded a reliable S-phase determination.

^bThe mean G0/G1 channel fluorescence value of each subpopulation was compared to that of lymphocytes within the same sample, which served as the internal control for each sample. The percent difference between the two values is shown.

cence were observed among marrow subpopulations. These results likely represent minor differences in chromatin packing and/or DNA saturation by DRAQ5 staining within each distinct cell subpopulation. Thus, like To-pro-3 (15) or Hoechst 33342 (3) stains that result in comparable wider CVs, DRAQ5 staining should be acceptable for identifying aneuploidy in a sample with a large change in DNA content, but may not be adequate for detection of minor changes in DNA content or chromatin condensation.

Despite the slightly larger CVs, the results of DRAQ5 staining were within an acceptable range for satisfactory cell cycle modeling (18). The use of DRAQ5 is ideal for cell cycle analysis of discrete cell subpopulations within heterogeneous samples such as blood or bone marrow. In these samples, multiple cells representing different lineages or various cell differentiation stages coexist and require preserved scatter and antigen labeling for their identification. In our cases, S-phase analysis of bone marrow compartments was simple, given the clear delineation of cell subpopulations. Also, DRAQ5-stained specimens showed significantly fewer numbers of cell doublets when compared to PI staining, most likely due to the fact that DRAQ5 labeling requires virtually no cell manipulation that may cause an increase in numbers of cell doublets. Not surprisingly, DRAQ5-derived S-phase analysis of cell subpopulations in the marrow revealed that immature cells exhibited higher S-phase fractions than mature elements. Granulocyte precursors identified by CD45/SSC (12,13) had a higher S-phase than mature granulocytes, monocytes, or lymphocytes. A high S-phase fraction was noted in the nucleated erythroid cell population, a finding that reflects normal erythroid cell turnover *in vivo*, and is comparable to previously reported erythroid cell proliferation rates that were assessed by two-color analysis in normal bone marrow donors (19). The early precursor population, as defined by CD45 intensity and CD34 expression, most likely included both myeloid and lymphoid precursors; however, their numbers were too few for an adequate S-phase fraction calculation. Acquisition of a greater number of events (for example, $\geq 100,000$) would enable a more accurate S-phase assessment in this early precursor population.

Determination of proliferative fractions provides an objective estimation of cell growth and may contribute essential information in predicting tumor behavior. However, the analysis of proliferative fractions in clinical samples in the past has typically been compromised by the inability of most analytical methods to appropriately measure tumor-specific proliferation. By enabling the assessment of proliferative fractions within clearly definable subpopulations, DRAQ5 staining should be particularly useful in evaluating the kinetics of tumor cells in samples that contain significant numbers of normal cells admixed with neoplastic elements.

In summary, the speed and simplicity of performing DRAQ5-based DNA cell cycle analysis on cell subpopula-

tions with unique surface antigen and scatter characteristics lends itself to multiple applications with potentially significant diagnostic and therapeutic utility in preneoplastic and neoplastic disorders of the hemolymphoid system.

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