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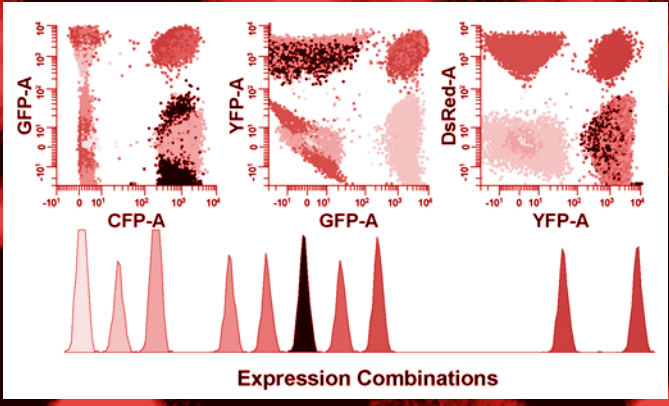

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Concurrent Flow Cytometric Analysis of DNA and RNA

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Summary

Concurrent analysis of DNA and RNA by flow cytometry provides information on the DNA content and the transcriptional status of cells. This can be accomplished using metachromatic fluorochromes that bind to DNA by intercalation and to single-stranded RNA electrostatically. Because cell viability is a prerequisite for the analysis, freshly prepared cells must be used. Simultaneous DNA/RNA analysis can be used primarily in the classifications and biological assessment of hematoreticular malignancies including multiple myeloma.

Key Words

Flow cytometry, nucleic acids analysis, RNA content, RNA and DNA analysis.

1. Introduction

Simultaneous analysis of DNA and RNA can be accomplished by using the metachromatic acridine orange fluorochrome (AO), one of the most known of the acridine fluorescent family members ***(1–10)***. AO is a unique and versatile dye that can be used in the detection of a wide variety of cellular components. By combining RNA and DNA quantitation using AO, it is possible to discriminate between cell populations with the same DNA content and to assess their translational activity, cell proliferation, and differentiation status ***(11)***.

AO is a 3,6-(dimethylamino) acridine that binds to double-stranded nucleic acids irrespective of base composition or ligand type. Cells to be stained must be permeabilized with non-ionic detergent (e.g., Triton X-100) at low pH with serum proteins. This is an important step that allows for the dissociation of histones from DNA for better accessibility of AO ***(12)***. The dye intercalates the double-stranded conformation of nucleic acids and stacks electrostatically

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on the RNA in the presence of EDTA. On excitation, DNA fluoresces approximately 530 nm (green) and RNA fluoresces with maximum emission above 630 nm (red). AO can also bind to cellular dsRNA leading to spurious elevation of DNA content measurement.

Since cellular rRNA and tRNA have double strand conformation, these have to be selectively denatured in the processing using EDTA ***(13)***.

2. Materials

2.1. General Guidelines

1. Prepare stock solutions prior to specimen preparation, with the exception of the AO working solution. The latter should be prepared only at the time of sample staining.
2. All glassware to be used for reagent preparation must be RNase free.
3. Rigorous adherence to molarity and pH conditions must be followed.
4. Peripheral blood lymphocytes and bone marrow aspirates are harvested after Ficoll-Hypaque gradient centrifugation. Solid tumor and bone marrow biopsies should be carefully minced and agitated to release individual cells.
5. Fluids and fine-needle aspirates are either washed directly in buffer or separated by gradient centrifugation depending on the extent of red blood cell contamination.
6. Specimens should be washed in phosphate-buffered saline (PBS) with $MgCl_2$ and cell concentrations adjusted to 1×10^6 cells/mL. If surface membrane markers are to be performed, the PBS with $MgCl_2$ step (*see* **Subheading 3.1., step 1**) should be eliminated.

2.2. Stock Solutions

1. 1 mg/mL of AO: (**Caution:** AO is a mutagen. Chemical, physical, and toxicological properties are not fully established for this material. Handle with care). Measure 50 mg of AO powder (Polysciences, Warrington, PA; *see* **Note 1**) into a clean glass beaker. Add 50 mL of distilled H_2O, cover with aluminum foil and stir until dissolved, protected from light. Filter solution through a no. 1 Whatman filter paper. Store solution protected from light at 4°C, wrap in aluminum foil. Stable for 1 yr (*see* **Note 2**).
2. 0.2 *M* Citric acid: Add 19.21 g of citric acid (Sigma, St. Louis, MO) to 500 mL of distilled H_2O. Stir until dissolved and store at 4°C. Stable for 2 mo.
3. 10 m*M* Ethylenediaminetetraacetic acid (EDTA): Add 2.17 g of EDTA (Gibco™ Invitrogen Corporation, Carlsbad, CA) to 500 mL of distilled H_2O, stir until dissolved and store at 4°C. Stable for 2 mo.
4. PBS with 2 m*M* $MgCl_2$: Dilute 10X PBS without calcium and magnesium salts (Irvine Scientific, Santa Ana, CA) to a 1X solution. Add 0.408 mL of 4.9 *M* $MgCl_2$ for each liter of PBS. Adjust the pH to 7.2–7.4. Bulk preparations may be stored at room temperature up to 3 mo. Store working aliquots at 4°C.
5. 1 *M* Sodium chloride (NaCl): Add 29.0 g of NaCl (Fisher, Houston, TX) to 500 mL of distilled H_2O and stir until dissolved. Store at 4°C. Stable for 3 mo.

6. 0.4 *M* Sodium phosphate dibasic (Na_2HPO_4): Add 28.39 g of Na_2HPO_4 (Fisher) to 500 mL of distilled H_2O and stir until dissolved. Store at room temperature, 22°C. Stable for 2 mo.
7. Triton 10X: Add 10 mL of Triton X-100 (CMS, Houston, TX) to 90 mL of distilled water. Store at 4°C. Stable for 3 mo.

2.3. Working Solutions

Working solutions are prepared from the stock solution described above.

1. Solution A: Add 1 mL of Triton X-100, 8 mL of 1 *N* HCI, 15 mL of 1 *M* NaCl to 76 mL of distilled H_2O. Adjust to pH 1.2 with 1 *N* HC1. Store at 4°C. Stable for 2 wk.
2. Solution B: Add 50 mL of 10 m*M* EDTA, 75 mL of 1 *M* NaC1, 157.5 mL of 0.4 *M* Na_2HPO 92.5 mL of 0.2 *M* citric acid to 120 mL of distilled water. Adjust to pH 6.0 with 1 *N* NaOH or 1 *N* HC1. Store at 4°C. Stable for 1 mo.
3. AO working solution: Add 0.1 mL of AO stock solution to 9.9 mL of solution B. Prepare daily and keep on ice. EDTA enhances AO interaction with RNA to form condensed samples.

3. Method

3.1. Staining

The following steps should be performed at 0–4°C in disposable glass tubes.

1. Adjust cell suspension at a concentration of 1.0×10^6 cells/mL in PBS with 2 m*M* $MgC1_2$. Cells can either be fixed in ethanol or permeabilized with Triton X-100.
2. Place solution A and AO working solution on ice.
3. Aliquot 0.2 mL of freshly prepared cell suspension into a 12 × 75 mm glass disposable test tube.
4. Add 0.4 mL of solution A (*see* **Note 3**). Incubate for 45 s at 0–4°C.
5. Add 1.2 mL of AO working solution and analyze immediately.
6. Repeat **steps 3–5** for each specimen.
7. Wash the cytometer with bleach and rinse thoroughly with distilled H_2O (*see* **Note 4**).

3.2. Analysis

3.2.1. Instrumentation and Acquisition

Excitation of AO is achieved between 455 and 490 nm. Because the emission spectra of AO DNA binding overlaps with the red fluorescence from RNA, filter combinations should be chosen to minimize this occurrence. We recommend a 550-longpass (LP) dichroic with a 525-bandpass (BP) for DNA fluorescence and a 630-LP for RNA fluorescence. Further compensation may be needed to reduce overlapping emissions (*see* **Note 5**).

Instrument performance should be monitored to ensure consistent resolution and mean peak channels for calibration particles. Protocols should be designed to acquire light scatter signals, dual-parameter DNA/RNA histograms, along with single-parameter fluorescence distributions of each fluorescent signal. Doublet discrimination should be performed if required. An example of acquisition protocol is depicted in **Fig. 1**.

3.2.2. RNA Content Standards

RNA and DNA cellular contents can be quantitatively measured by comparison to the same contents in biological standard (lymphocyte, cell line, and so on). Freshly harvested lymphocytes is a popular and stable control for such purpose. RNase treated and untreated standard should be run and measured concurrently. RNA and DNA contents of the test cells must be measured under conditions similar to those used for the control. The RNA index should be expressed by dividing the test level by the level of the control. Lymphocytes can also be used for DNA content calibrations to determine the DNA index.

3.2.3. Standards

Normal peripheral blood lymphocytes (NPBLs) are used as a biological control to assess the quality of AO staining and the stability of the diploid G_0/G_1 mean channels **(Fig. 2)**. Lymphocytes harvested after Ficoll-Hypaque gradient separation and cell count adjusted to 1×10^6 cells/mL can be used to establish an acceptable range of RNA mean channel values (*see* **Note 6**). The lymphocyte cell suspension should be simultaneously stained and concurrently run with each set of specimens to be analyzed.

NPBLs can also be used to determine DNA ploidy, especially in tumors with near-diploid peaks (*see* **Notes 7** and **8**). Equal parts (i.e., 100 µL each) of NPBL and tumor cell suspension are mixed together and stained with acridine orange **(Fig. 3)**.

3.2.4. Histogram Analysis

DNA indices and cell cycle data can either be generated using commercial software programs or manually. For manual analysis, cursors may be placed around G_0/G_1 peaks and $S+G_2/M$ areas. DNA indices are calculated by dividing the mean channel value (x-axis) for the G_0/G_1 aneuploidy peak by the mean channel value for the intrinsic diploid G_0/G_1 peak. By definition, diploid DNA

Fig. 1. *(see facing page)* A typical acquisition protocol displaying a doublet discrimination histogram (peak vs integral fluorescence) **(A)**, a two-parameter DNA/RNA display **(B)**, and one-parameter DNA and RNA histograms (**C**,**D**, respectively).

A
R1
FL1-Peak -->
DNA -->
B
RNA -->
DNA -->
C
Number
DNA -->
D
Number
RNA -->

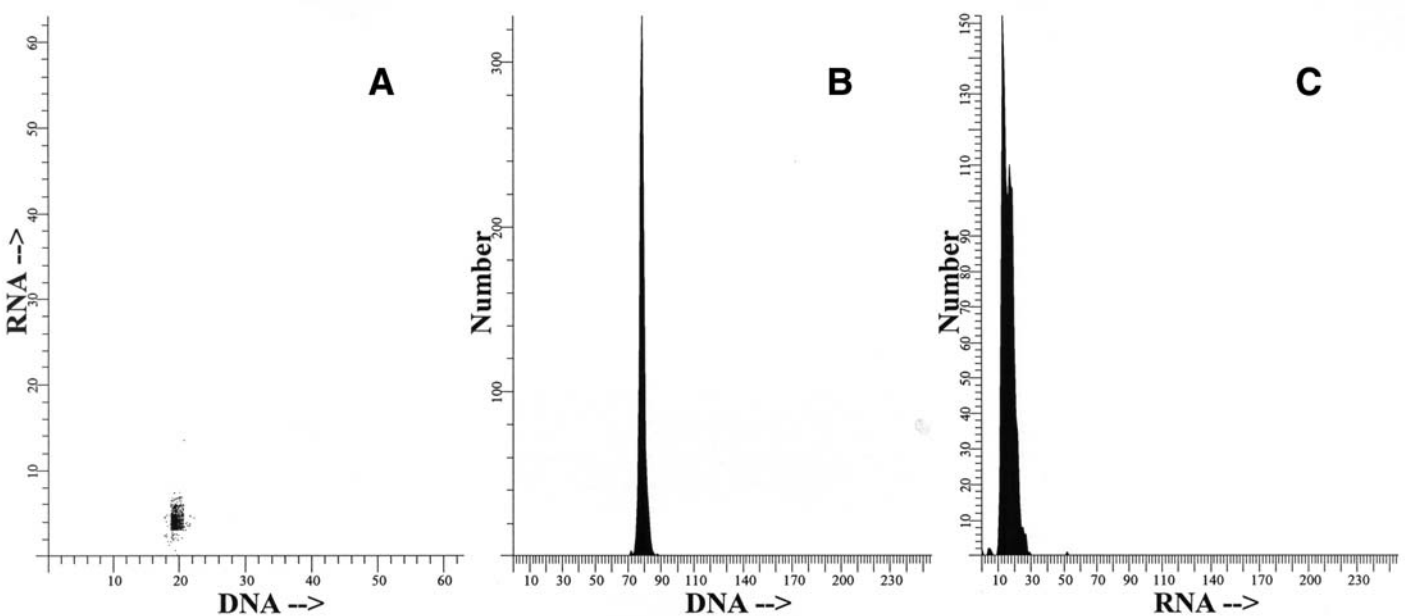

Fig. 2. Two-parameter DNA/RNA **(A)** and one-parameter DNA and RNA histograms (**B**,**C**, respectively) of NPBLs stained with AO.

index is equal to 1.00 ***(14)***. Cell cycle percentages may be calculated using the actual number of cells included within the cursor boundaries (area). RNA indices are calculated by dividing the mean G_0/G_1 RNA fluorescence of the test sample by the mean G_0/G_1 RNA fluorescence for NPBL.

3.2.5. Principles of RNA Content Analysis

RNA measurement is largely a rough estimation of the ribosomal RNA content which comprises more than 80% of the cellular RNA content. This in turn is an indirect reflection of the translational potential of cells analyzed.

RNA analysis can be used in:

1. The identification of different cell populations (i.e., lymphocytes, host cells and tumor cells) in a given sample.
2. Assessment of cell cycle status. Progression of the cell cycle is associated with increased RNA content which occurs during the G_0/G_1. Cellular RNA can, therefore, be used in the DNA histogram to determine the number of cycling cells.

3.3. Applications of Concurrent DNA/RNA Analysis

Various applications of this technique have been investigated including the assessment of cellular differentiation of cellular phenotypes, determination of cell cycle phases, and biological assessment of hematologic malignancies and solid tumors. Reproducible measurements of DNA/RNA contents depend on the quality of specimen preparation and staining conditions (*see* **Note 9**).

3.3.1. Lymphoreticular Malignancies

Cells from leukemias and the leukemia phase of lymphoma are typically uniform, do not require processing, and are ideal for such analysis. Analysis of bone marrow specimens, however, varies depending on the extent of neoplastic involvement and residual normal marrow elements. RNA values and the histogram profile of normal bone marrow aspirates should be used to guide interpretation of data generated from these specimen types. RNA/DNA values can be used in the diagnosis and biological assessment of multiple myeloma, acute and chronic leukemias, and chronic myeloid leukemias ***(15–31)***. Examples of AO staining in different hematologic malignancies are displayed in **Figs. 4** and **5**.

3.3.2. Solid Tumors

AO analysis of solid neoplasms are more complicated because of tumor cell heterogeneity, cell membrane disruption, and contamination by host normal cells. Evaluation of Wright–Giemsa stained cytospin preparations is of vital importance in specimen qualification and interpreting these results (*see* **Note 10**). If sufficient intact tumor cells are present, histogram interpretation can be reliably analyzed. In general, at least 70% of the cells in a given specimen should contain

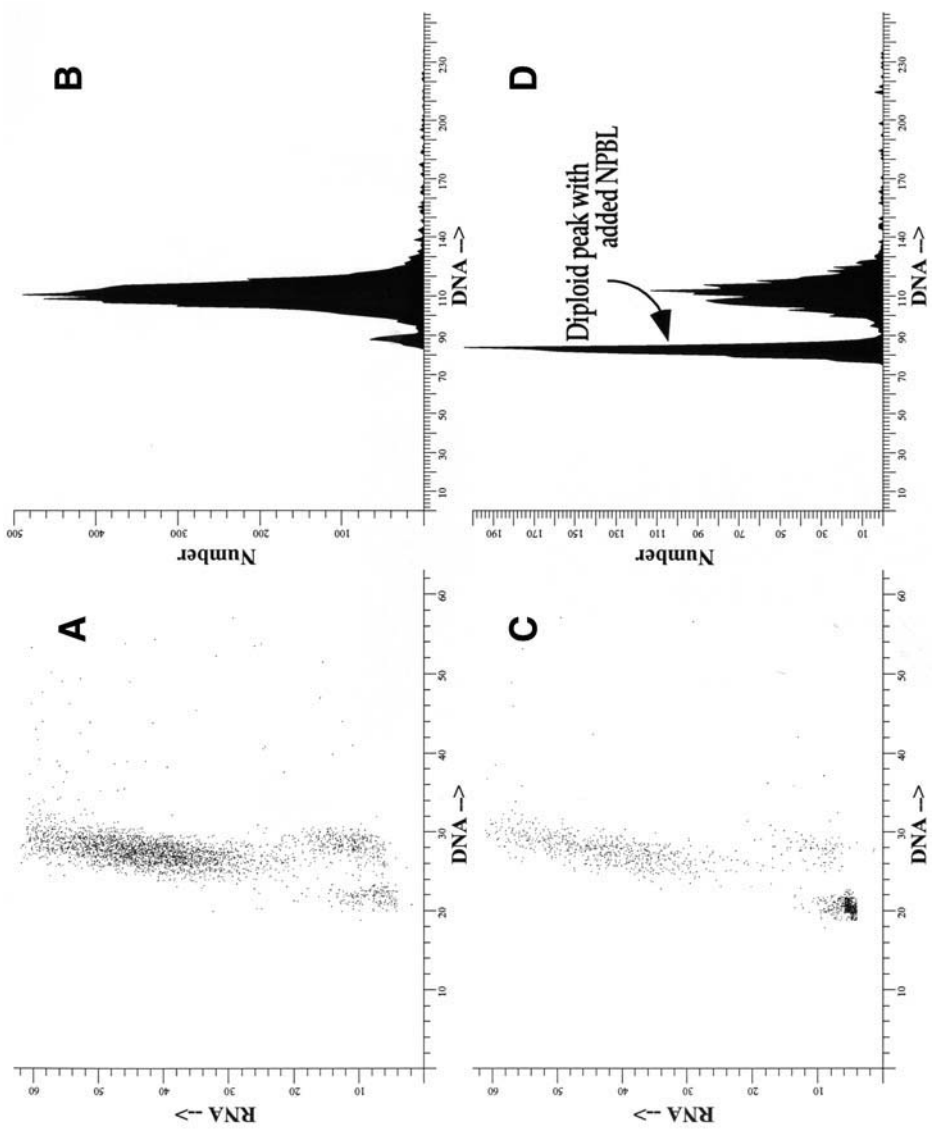

Fig. 3.

neoplastic elements and, of these, 60% should be intact. RNA indices should be reported for the diploid stemline in DNA diploid histograms and the aneuploid stemline for DNA aneuploid histograms.

4. Notes

1. The purity of AO is critical to the successful application of this technique. We recommend AO from Polysciences (Warrington, PA).
2. AO concentration may be adjusted to achieve optimum stainability for different instruments in a given setting ***(1)***. We recommend trials of various concentrations to achieve the optimal working solution conditions.
3. Maintain intact cells during permeabilization by suspending cells in the presence of serum or albumin. Solution A is used to permeabilize cells and remove histones and acid-soluble proteins. Incubate cell suspensions in this solution at 0–4°C **(Subheading 3.1., step 4)**. Vortex-mix gently, as vigorous agitation of cell suspension in solution A may disrupt cell membranes.
4. Cleaning flow cytometers is essential after using AO. This is further stressed if other applications are to follow. Regular cleansing with bleach followed by water should alleviate any AO carryover. If problems persist after cleaning, tubing can be changed between protocols.
5. Paired samples treated with and without RNAse and DNAse should be stained with AO ***(1)***. The purpose is twofold; to assess the specificity of staining and determine the amount of spectral overlap. Acquire DNase-treated preparations to ensure AO specificity for RNA (red fluorescence). Likewise, RNase-treated preparations should be acquired to collect DNA (green) fluorescence. Acquire consecutive, independent samples and lymphocyte preparations with and without RNase. These data are important in determining the extent of spectral overlap to adjust compensation accordingly.
6. A series of lymphocyte preparations should be used to determine an acceptable range of RNA values only after spectral overlap has been accounted for

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Fig. 3. *(see opposite page)* Two parameter **(A)** DNA/RNA and one-parameter **(B)** DNA histograms of a tumor specimen before mixing with NPBLs. (**C**,**D**) AO analysis after mixing equal portions of the tumor cell suspension with NPBLs. Note the increase in cell number for the diploid G0/G1 peak in **(D)**.

Fig. 4. *(see page 380)* (**A–C**) Typical acute lymphocytic leukemia patterns. (*left:* DNA/RNA; *middle:* one-parameter DNA histogram; *right:* one-parameter RNA distribution). The specimen in **(A)** shows a high proliferative fraction and low RNA content. **(B)** shows an increasing RNA content with lower proliferative activity. **(C)** illustrates a classic DNA aneuploid ALL pattern.

Fig. 5. *(see page 381)* (**A**,**B**) Two multiple myeloma distributions with high RNA levels and different DNA indices. **(C)** Typical AML pattern, with high-trailing RNA and high proliferation (*left:* DNA/RNA; *middle:* one-parameter DNA histogram; *right:* one-parameter RNA distribution).

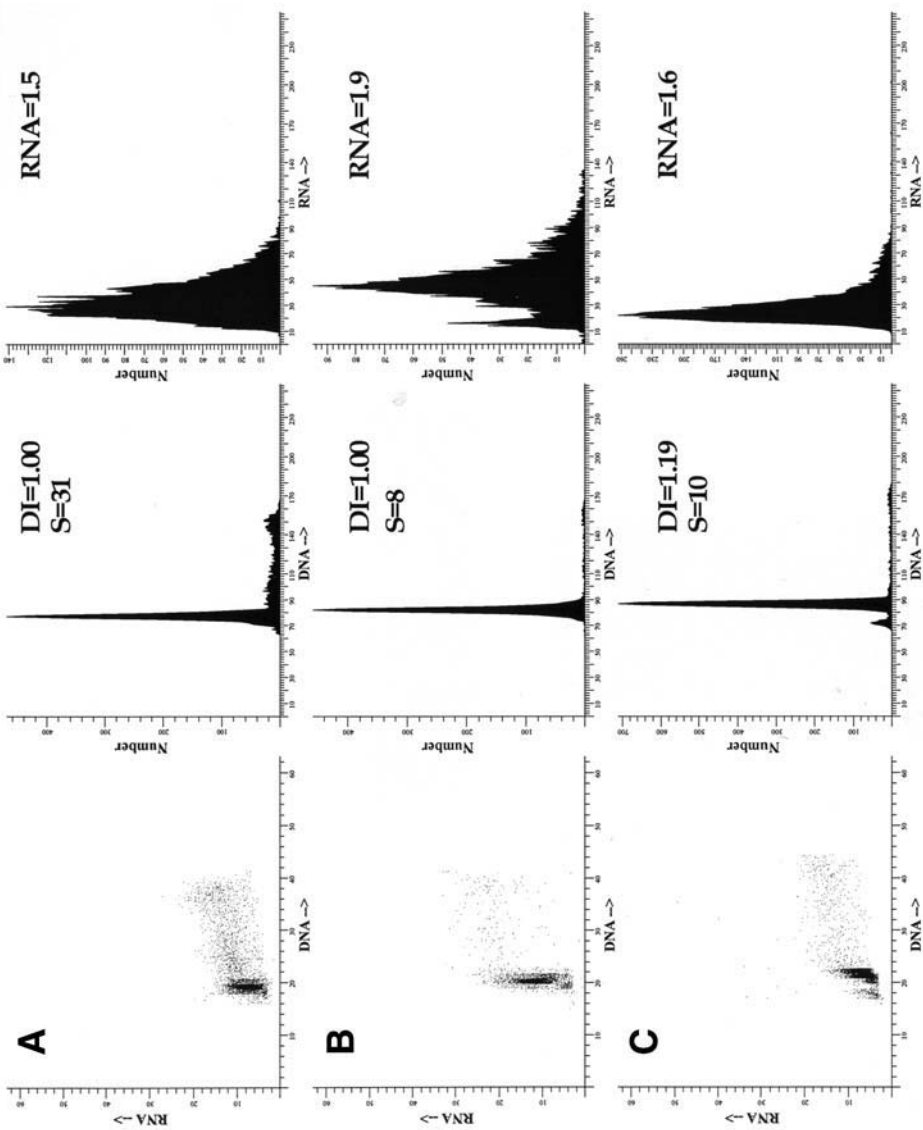

Fig. 4 (*see* legend on p. 379).

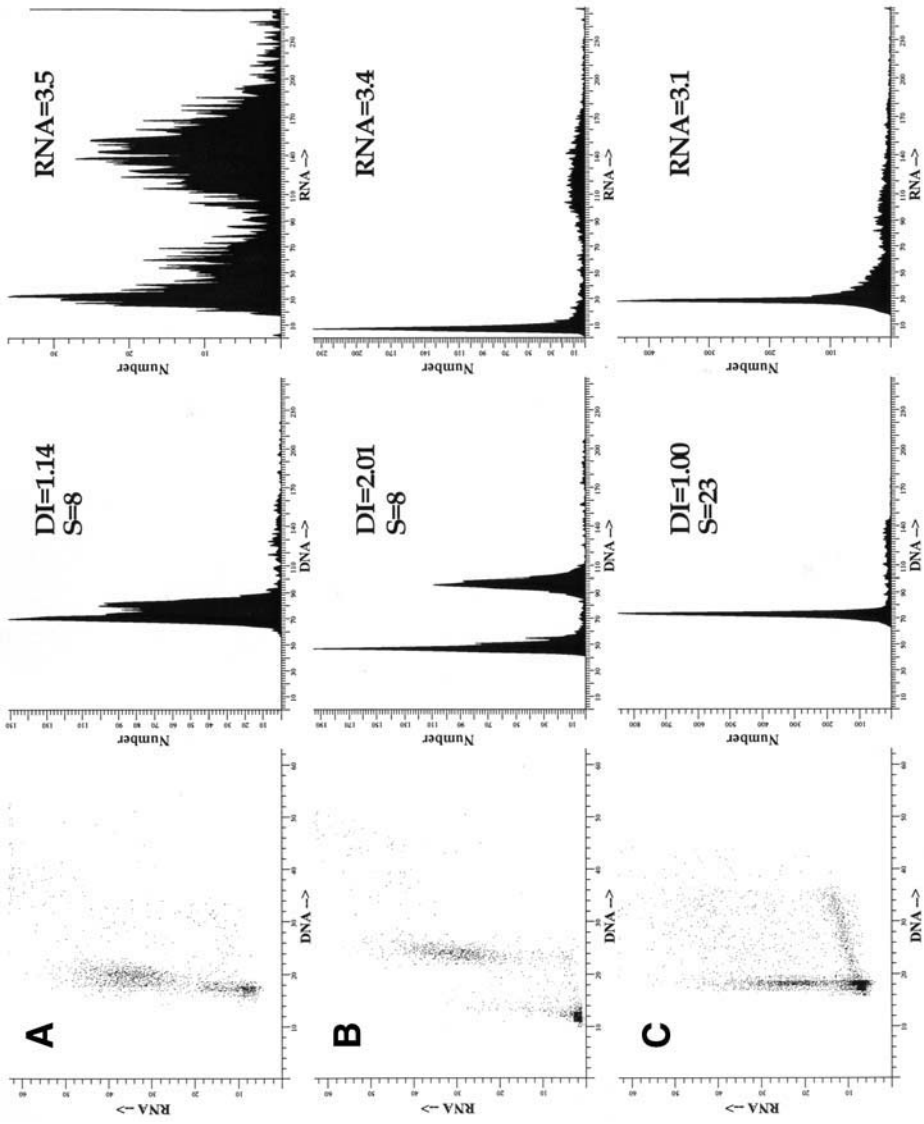

Fig. 5 (*see* legend on p. 379).

(Note 5). Acquire NPBL with each group of specimens and record the mean RNA fluorescence.

7. Cell concentration is critical, particularly for lymphocyte and tumor mixtures. Equal portions of each are necessary to classify near-diploid populations accurately as hypodiploid (DNA index <1.00) or hyperdiploid (DNA index >1.00).
8. Chromatin condensation differences in solid tumor cells and NPBLs may lead to incorrect histogram classification. Epithelial-derived tumor cells may allow more accessibility to the dye, therefore fluorescing brighter than NPBLs.
9. Debris or red blood cell contamination appear on the left of the diploid population on histograms and may interfere with the detection of a hypodiploid peak and/or cause inaccurate S-phase values, but the use of commercial programs with debris subtraction and aggregate modeling may overcome these problems.
10. Specimens with more than 30% non-neoplastic elements should be eliminated. If lymphocytes are predominant, gate on light scatter and reacquire DNA/RNA fluorescence. Should more than 40% bare nuclei be present, the specimen should be deemed inappropriate for RNA evaluation. Specimens should be used only for DNA ploidy and cell-cycle analysis.

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