DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis

In flow cytometry, analysis of DNA ploidy (DNA index or DI) and/or discrimination of cells in G_0/G_1 versus S versus G_2/M phases of the cell cycle is generally done by measuring cellular DNA content alone. Indeed, univariate DNA content analysis is an established clinical assay in oncology and is also widely used for research in cell and molecular biology (see *UNIT 7.1* for an overview of nucleic acid analysis). A large number of DNA fluorochromes can be used for this purpose, and the binding characteristics and spectral properties of most nucleic acid probes are described in *UNIT 4.2*. A great variety of techniques for measuring DNA utilizing these fluorochromes have been developed since the mid 1970s (Darzynkiewicz et al., 1994). The techniques differ primarily in the mode of cell permeabilization (detergent versus prefixation with alcohols) and composition of the stain solution. The most commonly used procedures are described in this unit.

Relatively simple and universally applicable methods for staining ethanol-fixed cells are presented in Basic Protocol 1 and Alternate Protocol 1. Because cells may be stored in fixative for extended periods and may be transported while in the fixative, this method allows one to prepare and collect cells independently of the timing of their analysis. The methods presented utilize the two most widely used DNA fluorochromes, propidium iodide (PI; see Basic Protocol 1) and 4′,6-diamidino-2-phenylindole (DAPI; see Alternate Protocol 1).

The second set of methods presented (see Basic Protocol 2 and Alternate Protocol 2) utilize detergents and/or hypotonic solutions to permeabilize cells; these methods generally provide more accurate estimates of DNA content compared to measurement of fixed cells. The approach presented in Basic Protocol 2 combines detergent treatment with use of proteolytic enzymes; it is widely used for clinical material, especially for DNA analysis in samples of solid tumors. Alternate Protocol 2 is a simpler method designed for uniform populations (e.g., tissue culture cells).

A third approach is required for DNA content measurements in live cells (see Basic Protocol 3). The primary application of this method is for cell sorting, where cells selected on the basis of differences in DNA content can be subcultured for the purpose of analyzing their growth characteristics, testing their sensitivity to drugs, cloning, or expanding their number. On the other hand, archival samples of paraffin-embedded tissues can be analyzed by flow cytometry following nuclear isolation (see Basic Protocol 4). This methodology is widely used in retrospective studies probing the prognostic value of DNA content in tumors.

Univariate DNA content measurement can also discriminate apoptotic cells, which are characterized by fractional DNA content due to DNA degradation by the apoptosis-associated endonuclease(s) (Wyllie, 1992; Darzynkiewicz et al., 1997). Apoptotic cells can therefore be identified within a population as the cells that evidence fractional DNA content following extraction of the degraded DNA and subsequent cell staining with PI or DAPI (see Basic Protocol 5). This approach is combined with analysis of DNA degradation by gel electrophoresis (see Support Protocol). The characteristic pattern of DNA degradation, preferentially producing internucleosomal DNA sections and generating so-called DNA laddering on the gels, is considered to be a hallmark of apoptosis. An alternative approach to detecting apoptotic cells by simultaneously measuring DNA content and DNA strand breaks, in which formaldehyde-fixed cells are enzymatically labeled with 5-bromodeoxyuridine triphosphate (BrdUTP) and then costained with

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FITC-conjugated antibody to BrdU (to detect DNA strand breaks) and with PI (to analyze DNA content), is presented in *UNIT 7.4*.

Discrimination of cells in particular phases of the cell cycle on the basis of differences in DNA content is helped by computer analysis. The software used to deconvolute the histograms often allows one to measure the cell cycle distribution of both diploid normal cells (host infiltrating and stromal cells) and aneuploid cell populations in aneuploid tumors.

The protocols presented in this unit can easily be modified for use of other DNA fluorochromes. For example, PI, which is used in Basic Protocols 1, 2, and 5, can be replaced by DAPI (e.g., see Alternate Protocols 1 and 2; see Basic Protocol 4), which then is applied at lower concentration and without the need to incubate cells with RNase.

BASIC PROTOCOL 1

DNA CONTENT ANALYSIS OF FIXED CELLS WITH PROPIDIUM IODIDE

This protocol uses ethanol to fix and permeabilize cells, aiding access of dye to DNA in intact cells and allowing DNA content analysis of stained cells by flow cytometry. The use of a fixation step makes this protocol applicable in situations when samples have to be stored before the analysis.

The fixed cells are rinsed with PBS and then stained with the DNA fluorochrome PI in a solution containing Triton X-100 as well as RNase A. Flow cytometry requires excitation with blue light and detection of PI emission at red wavelengths. Alternate Protocol 1 uses DAPI (which requires UV excitation) instead of PI.

Materials

70% ethanol
Cells to be stained
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Propidium iodide (PI)/Triton X-100 staining solution with RNase A (see recipe), freshly made

12 × 75–mm centrifuge tubes, preferably polypropylene or silanized
Beckman TJ rotor or equivalent
Flow cytometer with 488-nm argon ion laser or mercury arc lamp as fluorescence excitation source

Additional reagents and equipment for preparing cell suspensions for fixation (*UNIT 7.4*)

Fix cells with ethanol

1. Prepare the fixative by filling 12 × 75 mm–centrifuge tubes with 4.5 ml of 70% ethanol. Keep tubes on ice.
2. Collect cells (*UNIT 7.4*) and suspend 10^6 to 10^7 cells in 5 ml PBS in a centrifuge tube.
3. Centrifuge cells 6 min at ~200 × *g* (e.g., 1000 rpm in Beckman TJ rotor).
4. Using a Pasteur pipette thoroughly resuspend cells in 0.5 ml PBS.

   It is important to achieve a single-cell suspension. Fixation of cells that are in aggregates while suspended in PBS stabilizes the aggregates, which are then impossible to disperse. It is essential, therefore, to have a monodisperse cell suspension at the time of mixing cells with ethanol.

5. Transfer the cell suspension into the tubes containing 70% ethanol. Keep cells in fixative ≥2 hr.

   Cells suspended in 70% ethanol can be stored at 0° to –40°C for several months if not years.

Stain cells with PI

6. Centrifuge the ethanol-suspended cells 5 min at 200 × *g*. Decant ethanol thoroughly.
7. Suspend the cell pellet in 5 ml PBS, wait 60 sec, and centrifuge 5 min at 200 × *g*.
8. Suspend cell pellet in 1 ml PI/Triton X-100 staining solution with RNase A. Keep either 15 min at 37°C or 30 min at room temperature.

Perform flow cytometry

9. Set up and adjust the flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

   For excitation, the 488-nm argon ion laser line may be used. Alternatively, use a BG 12 optical filter when the source of illumination is mercury arc or xenon lamp. A long-pass (>600 nm) filter is recommended for detecting PI emission.

10. Measure cell fluorescence in the flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data (Fig. 7.5.1) using DNA content frequency histogram deconvolution software.

DNA CONTENT ANALYSIS OF FIXED CELLS WITH DAPI

ALTERNATE PROTOCOL 1

This protocol is similar to Basic Protocol 1, except the cells are stained with DAPI rather than PI. Thus, there is no need for cell treatment with RNase. Excitation of DAPI, however, requires a UV light source, which is not universally available. Emission of DAPI is measured at blue wavelengths.

Additional Materials *(also see Basic Protocol 1)*

Cells to be stained
DAPI/Triton X-100 staining solution (see recipe), freshly made
Flow cytometer with UV light illumination source (e.g., mercury arc lamp, laser tuned to 340 to 380 nm)

Fix cells with ethanol

1. Fix cells in 70% ethanol (see Basic Protocol 1, steps 1 to 5).

Stain cells with DAPI

2. Centrifuge the ethanol-suspended cells 5 min at 200 × *g*. Decant ethanol thoroughly.
3. Suspend the cell pellet in 5 ml PBS, wait 60 sec, and centrifuge again, 5 min at 200 × *g*.
4. Suspend cell pellet in 1 ml DAPI/Triton X-100 staining solution. Keep 30 min in the dark.

Perform flow cytometry

5. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths.

   For excitation, an UG 1 optical filter (short-pass, 390 nm) may be used when the light source is a mercury arc or xenon lamp. For detecting DAPI, a band-pass filter at 470 ± 20 nm is recommended.

6. Measure cell fluorescence in the flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data (Fig. 7.5.1) using software that deconvolutes DNA content frequency histograms.

BASIC PROTOCOL 2

DNA CONTENT ANALYSIS OF SAMPLES UTILIZING DETERGENTS AND TRYPSIN

This protocol uses detergent-based lysis and staining solutions, which improve DNA content analysis by flow cytometry compared to staining of intact cells as in Basic Protocol 1 and Alternate Protocol 1. This protocol is suitable for tissue samples, whereas Alternate Protocol 2 provides a simplified detergent-based method designed for fixed cells or cells from suspension cultures.

Cells are collected via aspiration from tissue samples into a sucrose-citrate buffer that contains DMSO, which allows for long-term storage of samples if needed. After samples are supplemented with an internal DNA standard (a mixture of chicken and trout erythrocytes), cells are lysed, digested with trypsin, and stained with PI. The stained nuclei are then subjected to flow cytometry.

Materials

Tissue sample with cells to be stained (e.g., resected tumor)
Citrate/DMSO buffer (see recipe)
Internal DNA content standard (e.g., chicken or trout erythrocytes; see recipe)
Cell lysis solution with trypsin (see recipe)
Trypsin-inactivating solution (see recipe), ice cold
Propidium iodide (PI)/spermine staining solution (see recipe), ice cold

0.5 × 25–mm needles (25 G × 1 in.)
10-ml disposable syringes
38 × 12.5–mm polypropylene tubes with caps
24- to 34-µm nylon mesh
Flow cytometer with 488-nm argon ion laser fluorescence excitation source

Collect cells from tissue samples

1. Insert the needle into the tissue sample and apply suction by syringe. Move the needle several times forward and back in different directions. Do not draw the aspirate into the syringe: keep the material within the needle.

   The 1-in. needle should be adequate for collecting cells in vitro from resected tumors. Longer needles may be needed for aspirating cells in vivo. Alternatively, specimens may be frozen in dry ice, stored at –60° to –80°C, thawed to room temperature, and then aspirated.

2. Flush the aspirate plug with 200 µl citrate/DMSO buffer into a 38 × 12.5–mm polypropylene tube.

   The tubes may be capped and mechanically shaken to further disperse the cells.

3. Count the cells in a hemacytometer. Repeat aspirations (steps 1 and 2) until 10^6 cells per sample are obtained.

   The samples may be analyzed the same day (steps 5 to 11) or stored at –40° to –80°C for up to several years. The DMSO in the citrate cell collection buffer protects cells during prolonged storage.

Lyse cells and stain with PI

4. Thaw stored samples in a 37°C water bath and transfer to room temperature.

5. Add the internal DNA content standard.

   The mixture of chicken and trout erythrocytes used as the internal DNA content standard (chicken-to-trout cell ratio 3:5), which may be thawed to room temperature in a 37°C water

bath after retrieval from −40° to −80°C storage, if necessary, is added to the 200 µl sample of tumor cells in a proportion of ~1:5 with respect to the number of studied cells.

6. Add 1.8 ml cell lysis solution with trypsin to 200 µl of tumor aspirate cells in citrate/DMSO buffer. Cap the tubes and incubate 10 min at room temperature, mixing gently by inverting the tubes five to ten times.
7. Add 1.5 ml ice-cold trypsin-inactivating solution. Mix gently 10 min at room temperature.
8. Add 1.5 ml ice-cold PI/spermine staining solution. Wrap tubes in aluminum foil or keep them in the dark, on ice. Mix gently and filter the sample through 24 to 34-µm nylon mesh to remove debris and cell aggregates.
9. Store cells on ice. Measure cell fluorescence between 20 min and 4 hr after adding the staining solution.

Perform flow cytometry

10. Set up and adjust flow cytometer to provide excitation with blue light and detection of PI emission at red wavelengths.

    For excitation, the 488-nm argon ion laser line can be used. Use a BG 12 optical filter (short-pass, 470 nm) when the source of illumination is mercury arc or xenon lamp. For detecting emission of PI, a long-pass (>600 nm) filter is recommended.

11. Measure cell fluorescence in a flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data (Fig. 7.5.1) using software that deconvolutes DNA content frequency histograms.

DNA CONTENT ANALYSIS OF SAMPLES UTILIZING DETERGENTS

ALTERNATE PROTOCOL 2

This protocol uses detergent only, rather than detergent with trypsin as in Basic Protocol 2, to lyse cells and aid staining of DNA for flow cytometric analysis. In this simplified method, cells in suspension are mixed with staining solution that contains DAPI, buffers, and Triton X-100; the DNA content of the stained nuclei is then measured by flow cytometry, using UV excitation. This method also allows simultaneous analysis of DNA and protein if the protein-specific dye sulforhodamine 101 is included in the staining solution.

Additional Materials *(see also Basic Protocol 2)*

Cells to be stained
DAPI/PIPES staining solution (see recipe)
Flow cytometer with UV light illumination source (e.g., mercury arc lamp, laser tuned to 340 to 380 nm)
Additional reagents and equipment for preparing cell suspensions from tissue cultures (*UNIT 7.4*)

Stain cells with DAPI

1. Mix 0.2 ml cell suspension (10^5 to 10^6 cells) with 2 ml DAPI/PIPES staining solution. Keep the sample ≥10 min on ice.

    Cells used in this protocol may be collected directly from tissue culture flasks or plates (UNITS 7.3 & 7.4) and suspended in PBS to ~5×10^6 cells/ml. In addition, cells may be fixed ≥2 hr in 70% ethanol (see Basic Protocol 1), then rinsed and resuspended in 0.2 ml PBS. The advantage of ethanol fixation is that it offers the possibility of sample storage or transport prior to analysis.

The DAPI/detergent staining solution may be supplemented with sulforhodamine 101 (0.02 mg/ml final concentration in the staining solution) to allow simultaneous measurement of DNA and protein. Fixed cells should be used for analysis of protein content. Regardless of the dye(s) used, cell fluorescence should be measured within 10 to 60 min after staining.

Perform flow cytometry

2. Set up the flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI fluorescence at blue wavelengths.

   For UV excitation, use an UG 1 optical filter when the source of excitation is mercury arc or xenon lamp. For detecting DAPI emission, a band-pass filter at 470 ± 20 nm is recommended. Fluorescence of sulforhodamine (which like DAPI also is excited with UV light) is at red wavelengths > 600 nm.

3. Measure cell fluorescence in a flow cytometer within 60 min of staining. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data (Fig 7.5.1) using software which deconvolutes DNA content frequency histograms (see Chapter 10).

BASIC PROTOCOL 3

SUPRAVITAL STAINING OF DNA

Supravital staining of DNA offers the possibility of sorting of live cells on the basis of differences in their DNA content.

This protocol uses Hoechst 33342 to measure DNA by flow cytometry in live cells. The actual procedure of cell staining is simple. Cells suspended in culture medium are incubated in the presence of 2.0 to 5.0 µg/ml Hoechst 33342 for 20 to 90 min. Cell fluorescence is then measured directly, without any additional treatments, centrifugations, etc.

Materials

Cells to be stained, 10^6 cells/ml suspended in tissue culture medium
1 mg/ml Hoechst 33342 staining solution (see recipe)
Flow cytometer with UV light illumination source

Stain cells with Hoechst 33342

1. Add Hoechst 33342 staining solution to cells suspended in tissue culture medium (10^6 cells/ml) to obtain a final dye concentration of 2 µg/ml. Incubate 20 min at 37°C.

Perform flow cytometry

2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of Hoechst 33342 at blue wavelengths.

   An UG 1 optical filter may be used when the source of excitation is a mercury arc or xenon lamp. For detecting the blue fluorescence of Hoechst 33342, a band-pass filter at 470 ± 20 nm is recommended.

3. Measure cell fluorescence in the flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter.

   When intensity of cell fluorescence or resolution of cells in the cell cycle phases is inadequate, prolong the staining time (up to 90 min) and/or increase the dye concentration in the medium (up to 5 µg/ml). The same sample may be reanalyzed after prolonged incubation and/or addition of more staining solution.

   This protocol is predominantly used for sorting live cells. However, because sensitivity of cells to Hoechst 33342 varies depending on the cell type, it is possible that viability and cell cycle sorted progression of cells may be affected by the staining procedure.

DNA CONTENT ANALYSIS OF PARAFFIN-EMBEDDED SAMPLES

This protocol describes DNA content analysis of archival samples embedded in paraffin blocks. The technique is based on preparation of thick microtome sections of the paraffin-embedded material, solubilization and extraction of paraffin from the sections, tissue rehydration in graded ethanols, and isolation of nuclei by proteolytic digestion of the tissue. Samples are then stained with DAPI and subjected to flow cytometry.

Materials

Paraffin-embedded tissue blocks
Xylene or xylene substitute (e.g., Histo-Clear, National Diagnostics)
100%, 95%, 80%, and 50% ethanol
Protease solution (see recipe), freshly made
0.15 M NaCl, for diluting nuclei if needed
DAPI/phosphate staining solution (see recipe), freshly made

Microtome
57-μm nylon mesh bags, 1 × 1 cm
1- to 2-mm-diameter glass beads
Phase-contrast or differential interference–contrast (Nomarski optics) microscope
Flow cytometer with UV light illumination source

Prepare paraffin sections

1. Cut a standard thin section (5 to 10 μm from the paraffin-embedded tissue block), adjacent to the subsequent section that will be subjected to nuclear isolation. Process by routine hematoxylin and eosin (HE) staining.

2. Examine the thin section by light microscopy and select the area (e.g., tumor site) to be processed by flow cytometry. On the basis of examination of the thin section, with a scalpel trim the block from the undesired tissue.

3. Mount the paraffin block on a microtome. Cut sections 50 to 100 μm thick.

   The sections may curl up as they come from the microtome knife. Depending on the size (area) of the section and the cell density in the tissue, one to four thick sections are generally adequate for DNA analysis.

Isolate cell nuclei from paraffin sections

4. With forceps transfer the tissue sections into 1 × 1–cm fine mesh (57 μm) nylon bags. Add one or two 1- to 2-mm glass beads to prevent floating of bags on the surface of solutions used in subsequent steps.

5. Immerse bags in 20 ml xylene or xylene substitute and mix 60 min on a slowly rotating shaker at room temperature.

   CAUTION: Xylene is toxic. Wear gloves and keep lids on jars. When possible, xylene should be substituted by less toxic reagents such as Histo-Clear.

   Keep xylene and ethanol solutions in aliquots of 20 ml in closed glass or plastic, xylene-resistant containers (e.g., Coplin jars or Erlenmeyer flasks).

6. Drain xylene and transfer the bags with sections to 20 ml of 100% ethanol. Keep 10 min at room temperature. Successively transfer the bags to 95%, 80%, and 50% ethanol, keeping the bags 20 min in each solution.

7. Transfer each bag to a separate 15-ml tube containing distilled water. Keep 30 min at room temperature. Repeat rinse with water.

   Separation of bags from one another at this stage is necessary because with rehydration the tissue becomes soft and breaks up, which may cause cross-contamination of samples. Cross-contamination may be avoided by using bags made of fine nylon mesh (<4 μm).

8. Transfer the bags into small (5 ml) tubes containing 1 ml protease solution. With scissors, cut through the nylon mesh and release the contents of the bag into the protease solution. Leave the opened bag in the tube to release nuclei from pieces of tissue that may remain attached to the bag. Incubate 30 min in water bath at 37°C on rotary shaker.

9. After incubation examine the nuclear suspension with a phase-contrast or differential interference–contrast (Nomarski optics) microscope. If isolation of nuclei is inadequate, prolong the incubation in protease solution up to 2 hr. Count the number of isolated nuclei with a hemacytometer. If the number of isolated nuclei per 1-ml sample exceeds 10^6, adjust the number by removing a portion of the sample and diluting the remainder to 1 ml with 0.15 M NaCl.

Stain cells with DAPI

10. Add 1 ml of DAPI/phosphate staining solution. Store ≥60 min on ice prior to the measurement; the nuclei can be stored overnight at 0° to 4°C. Filter through 57-µm nylon mesh before analyzing by flow cytometry.

    The final DAPI concentration is 2 µg/ml. Although DAPI is recommended for staining DNA in nuclei isolated from paraffin blocks, PI can be used instead. To stain with PI, add RNase A (100 µg/ml) at step 7 and add 1 ml PI solution (10 µg/ml in PBS), instead of DAPI, at step 10.

Perform flow cytometry

11. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and for detection of DAPI emission at blue wavelengths.

    For excitation, an UG 1 optical filter may be used when the source of excitation is a mercury arc or xenon lamp. For detecting the emission of DAPI, a band-pass filter at 470 ± 20 nm is recommended.

12. Measure cell fluorescence with the flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data using software which deconvolutes DNA content frequency histograms (Chapter 10).

    Note that because cell fixation and paraffin embedding after DNA stainability, the external standard of DNA content (DI), e.g., normal lymphocytes or chick erthrocytes, cannot be used. A subpopulation of cells with distinctly lower fluorescence is generally considered to be representative of diploid cells (DI 1.0).

BASIC PROTOCOL 5

DNA CONTENT ANALYSIS FOR DETECTION OF APOPTOTIC CELLS

DNA content measurement with flow cytometry can be used to detect apoptotic cells, which have diminished DNA content. In this protocol, cells are fixed in ethanol before being subjected to mild extraction of low-molecular-weight DNA that leaks from the cells. Samples are then stained with PI in the presence of RNase A and analyzed by flow cytometry.

Analysis of apoptosis as presented in this protocol can be combined with electrophoretic analysis of low-molecular-weight DNA extracted from fixed cells by the phosphate-citrate buffer; for description of such a procedure for agarose gel electrophoresis, see Support Protocol. Combining the methods presented in this protocol and the Support Protocol enables one to simultaneously analyze the molecular weight of DNA extracted from the very same cells that are subjected to flow cytometry.

Materials

70% ethanol
Cells to be analyzed
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
DNA extraction buffer: 0.2 M phosphate-citrate buffer, pH 7.8 (see recipe)
Propidium iodide (PI) Triton X-100 staining solution with RNase A (see recipe), freshly made

15-ml polypropylene centrifuge tubes and caps
Flow cytometer with 488-nm argon ion laser fluorescence excitation source

Fix cells in ethanol

1. Distribute 10-ml aliquots of 70% ethanol into 15-ml polypropylene centrifuge tubes. Keep tubes in ice.

2. Suspend 1–5 × 10^6 cells in 1 ml PBS. Fix cells in suspension by rapidly admixing, with a Pasteur pipette, 1 ml cell suspension into 10 ml of 70% ethanol in centrifuge tubes on ice. Fix cells ≥2 hr.

   Cells can be stored in fixative at −20°C for several weeks.

Extract low-molecular-weight DNA from cells

3. Centrifuge cells 5 min at 200 × *g*. Thoroughly decant ethanol. Add 50 µl DNA extraction buffer. Transfer tubes to a 37°C water bath, cap, and incubate 30 min on the shaker.

   The volume of DNA extraction buffer may vary. If the extracted DNA will be subjected to gel electrophoresis (see Support Protocol), small volumes (~50 µl) should be used. This simplifies subsequent steps in the gel electrophoresis procedure: such small volumes may be directly incubated with RNase and proteinase K and loaded on the gel without the necessity of concentrating the DNA.

   The efficiency of DNA extraction in step 3 should be controlled for optimal separation of apoptotic cells. If DNA degradation within apoptotic cells is extensive or if the cells have already shed apoptotic bodies, there is no need to extract low-molecular-weight DNA in step 3, as the apoptotic cells will already have a significantly reduced DNA content and will be well resolved on DNA content frequency histograms. On the other hand, if DNA degradation is incomplete and sub-G_1 and G_1 peaks are not separated, try extending the rinsing times (e.g., up to 2 hr) and using greater volumes of DNA extraction buffer (e.g., up to 500 µl).

4. Centrifuge cells 10 min at 1500 × *g*. Reserve supernatant for analysis of low-molecular-weight DNA by agarose gel electrophoresis (see Support Protocol), if desired.

Stain cells with PI

5. Resuspend cells in 1 ml PI staining solution with RNase A. Keep 30 min at room temperature, protected from light.

Perform flow cytometry

6. Set up and adjust flow cytometer for excitation with blue light and detection of PI fluorescence at red wavelengths.

   For excitation, the 488-nm argon ion laser line may be used; a BG 12 optical filter is recommended when the source of illumination is a mercury arc or xenon lamp. For detecting PI emission, a long-pass (>600 nm) filter is recommended.

7. Measure cell fluorescence in a flow cytometer. Use the pulse width–pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter. Analyze the data as shown in Fig. 7.5.4.

AGAROSE GEL ELECTROPHORESIS OF DNA EXTRACTED FROM APOPTOTIC CELLS

In this protocol, low-molecular-weight DNA, extracted from the same cells that are subjected to flow cytometry (see Basic Protocol 5), is subsequently analyzed by agarose gel electrophoresis (Gong et al., 1994). Cell extraction is described in Basic Protocol 5 (i.e., cells are prefixed in 70% ethanol and, after removal of ethanol, DNA is extracted with a small volume of 0.2 M phosphate-citrate buffer, pH 7.8). In this protocol, the extract is sequentially treated with RNase A and proteinase K and then directly subjected to electrophoresis.

Additional Materials *(also see Basic Protocol 5)*

Cells to be studied
2 mg/ml DNase-free RNase A stock solution (*APPENDIX 2A*)
1 mg/ml proteinase K (Sigma)
6× gel loading buffer (*APPENDIX 2A*)
0.8% agarose gel (see recipe)
DNA molecular weight standards, 100 to 1000 bp
Electrophoresis buffer: 10× TBE buffer (*APPENDIX 2A*)
Ethidium bromide staining solution (*APPENDIX 2A*)

Extract low-molecular-weight DNA from cells

1. Fix cells in ethanol and extract low-molecular-weight DNA in DNA extraction buffer (see Basic Protocol 5, steps 1 to 3).
2. Centrifuge cells 10 min at 1500 × *g*. Withdraw 40 µl of supernatant and transfer to a 0.5-ml microcentrifuge tube.

Remove proteins and RNA from cell extract

3. Add 5 µl of 2 mg/ml DNase-free RNase A stock solution and incubate 30 min at 37°C. Cap tube to prevent evaporation.
4. Add 5 µl of 1 mg/ml proteinase K and incubate 30 min at 37°C.

Perform electrophoresis

5. Add 5 µl of 6× gel loading buffer and transfer the entire tube contents to one well of a 0.8% agarose horizontal gel.
6. Prepare and load a sample of DNA molecular weight standards in a total of 55 µl in 1× gel loading buffer.
7. Assemble gel electrophoresis apparatus, using electrophoresis buffer to fill the reservoir. Run electrophoresis 16 to 20 hr at 2 V/cm. Turn off the power when the bromphenol blue from the loading buffer migrates a distance sufficient for separation of DNA fragments.
8. To visualize the bands, stain the gel 20 to 30 min with ethidium bromide staining solution.

   CAUTION: Ethidium bromide is a potential carcinogen. Wear gloves when handling.

9. Transfer the gel onto a UV transilluminator. Observe after illumination.

   CAUTION: Ultraviolet light is dangerous to eyes and exposed skin. Wear protective eyewear and facewear.

10. Photograph the gel using a red or orange (e.g., Kodak Wratten no. 23A) emission filter and a clear UV light blocking filter (e.g., Kodak Wratten no. 2B).

REAGENTS AND SOLUTIONS

*Use distilled, deionized water for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Agarose gel, 0.8%

Dissolve 1.6 g agarose in 200 ml hot (boiling) electrophoresis buffer (10× TBE). Cool to 55°C and pour solution onto a 15 × 15–cm sealed gel-casting platform. Insert the gel comb. Cool to room temperature. After the gel has hardened, remove the seal from the gel-casting platform and remove the gel comb. Place into an electrophoresis tank containing sufficient electrophoresis buffer to cover gel to a depth of ~1 mm.

Cell lysis solution with trypsin

Dissolve 3 mg trypsin (Sigma type IX from porcine pancreas) in 100 ml detergent stock solution (see recipe) and adjust to pH 7.6. Store ≤1 year at −40° to −80°C in aliquots of 5 to 10 ml in tubes. Before use, bring to room temperature in a 37°C water bath. Avoid repeated thawings.

Citrate/DMSO buffer

85.50 g sucrose (0.25 M final)
11.76 g trisodium citrate dihydrate (40 mM final)
5 ml DMSO (Sigma; 0.5% final)
H_2O to 1000 ml
Adjust to pH 7.6
Store 1 month at 4°C

To prepare the buffer, dissolve the dry ingredients and DMSO in ~800 ml water and then dilute to 1000 ml.

DAPI/phosphate staining solution, for nuclei isolated from paraffin tissue blocks

Add 4 µl of 1 mg/ml DAPI to 80 ml water. Add 11.4 mg anhydrous Na_2HPO_4 (0.8 M final) and 0.82 g citric acid monohydrate (40 mM final), and mix to dissolve. The solution should be pH 7.4. Add water to 100 ml. Prepare freshly.

The final DAPI concentration in this staining solution is 2 µg/ml. A DAPI stock solution, made by dissolving 1 mg DAPI in 1 ml water, may be stored several months in dark or foil-wrapped bottles at or below −20°C.

DAPI/PIPES staining solution, for detergent-lysed cells

Add 100 µl of 1 mg/ml DAPI to 100 ml PIPES/Triton X-100 buffer (see recipe). Store several weeks in dark or foil-wrapped bottles at 0° to 4°C.

A DAPI stock solution, made by dissolving 1 mg DAPI in 1 ml water, may be stored several weeks in dark or foil-wrapped bottles at or below −20°C.

For simultaneous staining of DNA and protein, add 100 µl of 1 mg/ml DAPI to 100 ml PIPES/Triton X-100 buffer (see recipe) and then add 2 mg sulforhodamine 101 (Molecular Probes); stir to dissolve. Store several weeks in dark or foil-wrapped bottles at 0° to 4°C.

DAPI/Triton X-100 staining solution, for ethanol-fixed cells

To 10 ml of 0.1% Triton X-100 in PBS add 10 µl of 1 mg/ml DAPI (Molecular Probes). Prepare freshly.

A DAPI stock solution, made by dissolving 1 mg DAPI in 1 ml water, can be stored several months in dark or foil-wrapped bottles at −20°C.

Detergent stock solution

1 g trisodium citrate dihydrate (3.4 mM final)
1 ml Nonidet P-40 (Sigma; 0.1% final)
522 mg spermine tetrahydrochloride (1.5 mM final)
61 mg Tris (Sigma 7-9; 0.5 mM final)
H_2O to 1000 ml
Store ≤1 year at 0° to 4°C

Dissolve the dry ingredients and NP-40 in ~800 ml water and then dilute to 1000 ml.

DNA extraction buffer (0.2 M phosphate citrate buffer, pH 7.8)

192 ml 0.2 M Na_2HPO_4
8 ml 0.1 M citric acid
Store several months at 4°C

Hoechst 33342 staining solution, 1 mg/ml

Dissolve 1 mg Hoechst 33342 (Molecular Probes) in 1 ml water. Store in dark or foil-wrapped bottles several months at 0° to 4°C.

Internal DNA Content Standard

Chicken and trout erythrocytes are convenient internal DNA content standards. Chicken blood is acquired by heart puncture and collected with heparin (50 U/ml blood). The blood is diluted with citrate/DMSO buffer (see recipe) to obtain 1.5×10^6 cells/ml, as counted with a hemacytometer. Rainbow trout blood is obtained by caudal vein puncture of an anesthetized fish, immediately mixed with citrate/DMSO buffer, and adjusted to 2.5×10^6 cells/ml. These solutions are then mixed 1:1 (v/v) to obtain 2×10^6 cells/ml. The proportion of chicken to trout erythrocytes in such a mixture provides approximately similar height peaks on DNA frequency histograms. The standard cells can be kept frozen, in small aliquots, at −40° to −80°C.

PIPES/Triton X-100 buffer

3.02 g PIPES (Calbiochem; 10 mM final)
5.84 g NaCl (0.1 M final)
406 mg $MgCl_2 \cdot 6H_2O$ (2 mM final)
1 ml Triton X-100 (Sigma; 0.1% final)
H_2O to 1000 ml
Adjust to pH 6.8
Store ≤1 year at 0° to 4°C

Dissolve the dry ingredients and Triton X-100 in ~800 ml water, adjust pH with NaOH or HCl, and then dilute to 1000 ml.

Propidium iodide (PI)/spermine staining solution, for detergent-lysed cells

Dissolve 20 mg PI (Molecular Probes) and 116 mg spermine tetrahydrochloride (Sigma) in 100 ml detergent stock solution (see recipe) and adjust to pH 7.6. Store ≤1 year at −40° to −80°C in small aliquots in 5- to 10-ml foil-wrapped tubes. Before use, thaw in a 37°C water bath and then keep on ice, protected from light.

Propidium iodide (PI)/Triton X-100 staining solution with RNase A, for ethanol-fixed cells

To 10 ml of 0.1% (v/v) Triton X-100 (Sigma) in PBS add 2 mg DNase-free RNase A (Sigma) and 200 µl of 1 mg/ml PI (e.g., Molecular Probes). Prepare freshly.

A stock solution of PI, made by dissolving 1 mg PI in 1 ml water, can be stored several months at 0° to 4°C.

If the RNase is not DNase-free, boil a solution of 2 mg RNase A in 1 ml water for 5 min.

Protease solution

Dissolve 100 mg Sigma XXIV bacterial protease in 80 ml water. Add 1.58 g Tris·Cl (Sigma; 0.1 M final) and 0.41 g NaCl (0.7 M final) and dissolve. Adjust to pH 7.2 and add water to 100 ml. Prepare freshly.

The protease solution makes use of the "Carlsberg subtilisin."

Trypsin-inactivating solution

Dissolve 50 mg chicken egg white trypsin inhibitor (Sigma) and 10 mg RNase A (Sigma) in 100 ml detergent stock solution (see recipe) and adjust to pH 7.6. Store ≤1 year at −40° to −80°C in small aliquots in 5- to 10-ml tubes. Before use, bring to 0° to 4°C in a 37°C.

COMMENTARY

Background Information

Choosing a particular protocol among those presented in this unit depends primarily on the sample type (unfixed or fixed cells, paraffin-embedded tissue blocks) and the necessity for sample storage (or transport) between cell collection and analysis. The discussion below describes characteristics of each of the methods and its applicability to different material.

Analysis of fixed samples

In Basic Protocol 1 DNA content is measured in prefixed cell samples. The preference for analysis of fixed cells often is dictated by the need to store or transport samples (e.g., clinical samples of solid or hematologic tumors). Extended storage of unfixed cells, unless done at low temperatures following cell suspension in cryopreservative media, leads to cell deterioration and DNA degradation. Fixed cells, on the other hand, often can be stored for months if not years without much deterioration.

The fixative essentially has two functions: (1) it preserves the cells by preventing their lysis and autolytic degradation, and (2) it makes the cells permeable and their DNA accessible to the fluorochrome. Precipitating fixatives (ethanol, methanol, acetone) are preferred over cross-linking agents (formaldehyde, glutaraldehyde). This is because cross-linking of chromatin has deleterious effects on the stoichiometry of DNA staining. Precipitating fixatives, though inferior in terms of stabilization and preservation of the low-molecular-weight constituents within the cell, adequately stabilize undamaged DNA. It should be stressed, however, that damaged DNA, especially DNA having large numbers of double-strand breaks (e.g., as present in apoptotic cells, see Basic Protocol 5), leaks from the ethanol-fixed cells during their hydration and subsequent staining.

Although absolute alcohols or acetone, or a mixture of absolute ethanol and acetone (1:1), can be used and may be preferred for some applications (e.g., to obtain better stabilization and retention of particular proteins for their immunocytochemical detection), they induce more extensive cell aggregation, and the aggregates cannot be easily dissociated after hydration of the fixed cells. Fixation of cells in 70% to 80% ethanol (at 0° to 4°C), on the other hand, results in less cell clumping and is generally preferred in situations when the analysis is limited to DNA content alone. Sample storage at −20° to −40°C, especially when prolonged (months), appears to be more advantageous compared to storage at room temperature.

A variety of DNA fluorochromes (*UNIT 4.2*) can be used to stain DNA in the fixed cells. Staining with dyes that react with both DNA and RNA, such as PI used in Basic Protocol 1, requires preincubation of cells with RNase. For cytometry, PI requires blue light as the fluorescence excitation source, which is conveniently provided by the 488-nm line of the argon ion laser available on most flow cytometers.

Alternate Protocol 1 employs DAPI instead of PI for staining DNA in fixed cells. The advantage of DAPI is its greater specificity toward DNA, which often is reflected by lower coefficient of variation (CV) values of the mean DNA content of G_1 cell populations. A disadvantage of DAPI is the requirement for UV excitation, which may not be possible in all flow cytometers.

Analysis of detergent-lysed samples

The major advantage of detergent-based methods is better accuracy in DNA content estimates. Exposure of live cells to detergents results in rupture of the plasma membrane and leakage of cytoplasmic constituents. Thus, iso-

lated nuclei, rather than whole cells, are stained. Because several cytoplasmic constituents either are autofluorescent or nonspecifically interact with DNA fluorochromes, the specificity of DNA staining by methods based on cell permeabilization by detergents is superior compared to that of methods based on cell fixation. This is reflected by the high accuracy of DNA content estimates, which is represented by low values of the CV of the mean DNA content of cells having uniform DNA content, such as the G_1 cell population. It has to be taken into account, however, that lysis of mitotic cells, which lack a nuclear envelope, leads to dispersion of individual chromosomes. Thus, mitotic cells may not be detected by methods utilizing detergents or hypotonic solutions. Furthermore, the presence of isolated chromosomes or chromosome aggregates may contribute to an increased frequency of detection of objects with low fluorescence values, generally classified as debris or apoptotic cells. Likewise, the lysis of apoptotic cells, which have fragmented nuclei, releases numerous nuclear fragments from a single cell. This generally precludes application of detergent-based methods for analysis of the frequency of apoptotic cells on the basis of fractional DNA content, and an alternative method must be sought (see Basic Protocol 5).

Further improvement in the accuracy of DNA content analysis is seen after mild and controlled proteolysis of detergent-lysed cells. It is likely that the proteolytic step removes nuclear proteins known to restrict the accessibility of DNA to many fluorochromes, resulting in improved stoichiometry of DNA staining (Darzynkiewicz et al., 1984). This approach was perfected by Vindeløv (1983a,b,c,d; Vindeløv and Christiansen, 1994), who developed a highly accurate method of cellular DNA content analysis. These authors also pioneered in introducing internal DNA content standards as an intrinsic part of the staining protocol. Their methodology, presented in Basic Protocol 2, is now widely used, especially in clinical settings for DNA content analysis of tumor samples. Alternate Protocol 2 is a simplified detergent method that is more applicable to uniform cell populations such as tissue culture cells.

Vindeløv's procedure was designed for fine needle aspiration of normal tissue and tumor biopsies. The aspiration has two functions: collection of cells from the tissue and cell disaggregation. The aspiration can be done either in vivo (needles longer than 1 in. may be needed) or in vitro, from the resected tumor. In addition, the specimen may initially be frozen at dry ice temperature, stored at −60° to −80°C, thawed to room temperature, and then aspirated. The presence of the cryopreservative dimethylsulfoxide (DMSO) in the citrate buffer used for collecting cells in Basic Protocol 2 protects cells from damage if the samples are stored at low temperatures prior to staining.

Alternate Protocol 2 employs only detergent and is simpler than the detergent/proteolytic enzyme procedure of Basic Protocol 2. It also offers excellent resolution for uniform cell populations such as tissue culture cells (the CV of the mean fluorescence of G_1 populations is typically <2%). Its resolution for mixed cell samples (human tumors), however, is below that of the method employing a combination of detergent and proteolytic enzymes (see Basic Protocol 2).

Alternate Protocol 2 uses either unfixed or ethanol-fixed cells. Although the cells can be stained with DAPI alone, inclusion of sulforhodamine 101, which counterstains proteins (Stöhr et al., 1978), provides an attractive combination that allows for simultaneous bivariate analysis of DNA (DAPI, blue fluorescence) and protein (sulforhodamine 101, red fluorescence after UV excitation). Alternate Protocol 2, therefore, can be used for either univariate DNA content or two-parameter (DNA and protein content) analysis.

Analysis of live cells

Staining of DNA in live cells (see Basic Protocol 3) is generally attempted for identification of DNA ploidy or cell cycle position of cells to be sorted on the basis of DNA content. Ideally, the fluorochrome used for supravital cell staining should be nontoxic and should not alter cell metabolism. Such a probe has yet to be developed. Although most DNA fluorochromes are charged molecules, which do not adequately penetrate the plasma membrane, some uncharged Hoechst dyes can pass through the membrane at limited rates. The most frequently used supravital DNA fluorochrome is Hoechst 33342. The procedure of staining cells with Hoechst 33342, followed by sorting of the stained cells, does not appear to induce immediate cytotoxicity to the sorted cells (Loken et al., 1980). Delayed toxicity attributed to Hoechst 33342, however, especially when stained cells were subsequently treated with antitumor drugs or radiation, has been observed. It also should be mentioned that Hoechst dyes photosensitize cells that have incorporated 5-bromodeoxyuridine (BrdU) into

their DNA, in particular to UV light at ~300 nm. Thus, the viability of sorted BrdU-labeled cells, counterstained with Hoechst dyes and illuminated with UV light, is expected to be impaired.

The intensity of cell staining and resolution of DNA content (i.e., the possibility of discriminating cells in different cell cycle phases) vary among different cell types. This variability, to a large degree, is due to a rapid efflux of Hoechst 33342 from the cell generated by the P glycoprotein pump. Cells characterized by rapid efflux mechanisms (e.g., multidrug resistant tumor cells or stem cells) stain poorly with Hoechst 33342. It has been observed, however, that agents which may impair the efflux function (e.g., calcium channel blocking drugs such as verapamil) improve stainability of some cell types with Hoechst 33342 (Krishan, 1987).

Analysis of paraffin-embedded samples

The method of isolating cell nuclei from paraffin-embedded tissues to retrieve archival samples for flow cytometric analysis (see Basic Protocol 4) was developed by Hedley et al. (1983). This methodology opened new possibilities for retrospective studies to determine the prognostic significance of DNA ploidy or cell cycle distribution. The method also can be used for prospective studies in situations when fresh material is not available or when the tumor sample is so small that the entire sample is required for histopathological examination. A variety of modifications of the original method involve different types of tissues, different fixatives, and other variables.

One of the advantages of this methodology is that the tissue fragments to be processed by flow cytometry can be selected on the basis of microscopic examination of a parallel tissue section. The blocks may then be trimmed to exclude, e.g., areas of noninvolved tissue (to diminish the proportion of stromal cells) or necrotic and hemorrhagic areas (to decrease the quantity of debris).

The accuracy of DNA content analysis of nuclei from paraffin blocks, as reflected by the CV of the mean DNA content of the G_1 population, is generally inferior compared to the methods that rely on either ethanol fixation (see Basic Protocol 1 and Alternate Protocol 1) or detergent treatment of fresh tissue (see Basic Protocol 2 and Alternate Protocol 2). This is due to the fact that tissues to be embedded in paraffin blocks frequently are prefixed in formaldehyde. Fixation with formaldehyde, by cross-linking DNA and proteins, impairs stoichiometric DNA stainability with most dyes. Compared with other fluorochromes, however, DAPI is the least affected by intercellular variability in chromatin structure (Darzynkiewicz et al., 1984) and therefore is preferred for staining nuclei isolated from paraffin blocks. Another factor that may decrease accuracy of identification of aneuploid cells or discrimination of cells in different phases of the cell cycle is the presence of debris, most of which may be due to the presence of transected nuclei with incomplete DNA content. Because probability of transecting a nucleus is directly proportional to thickness of the sections and to nuclear size, preparation of thicker sections (≥50 μm) may be advisable, especially for tumors with large nuclei (e.g., tetraploid and higher-ploidy stemlines).

Basic Protocol 4 presents the procedure as developed by Hedley et al. (1983) and modified by Heiden et al. (1991), which is applicable to most material. Further modifications, however, may be required for certain tissues or for different methods of cell fixation. Factors influencing the stainability of DNA in nuclei from paraffin blocks and approaches to optimize staining are discussed in detail by Hedley (1994) and Hitchcock and Ensley (1993).

Analysis of apoptotic cells

Apoptosis, frequently referred to as programmed cell death, is an active and physiological mode of cell death, in which the cell executes the program of its own demise and subsequent body disposal via shedding of "apoptotic bodies," which are engulfed by neighboring cells (reviewed by Wyllie, 1992; Darzynkiewicz et al., 1997). A complex multistep mechanism regulates the propensity of cells to respond to various stimuli by apoptosis. The possibility of intervention in regulatory mechanisms raised wide interest in apoptosis in many disciplines, particularly in oncology. Because of this wide interest, methods for identifying apoptotic cells are being routinely used in numerous laboratories. Most methods employ fluorochromes and are based on flow cytometric cell analysis.

One of the early and very characteristic events of apoptosis is activation of an endonuclease, which initially cleaves nuclear DNA at the sites of attachment to nuclear matrix to generate DNA fragments 50 to 300 kb in size, and subsequently at internucleosomal (spacer) sections to generate DNA fragments of 180 to 200 bp and multiples of such fragments (Oberhammer et al., 1993). The latter products are

visualized after agarose gel electrophoresis as a discontinuity (DNA "laddering") and are considered a hallmark of apoptosis.

Extensive DNA cleavage provided foundations for the development of flow cytometric assays to identify apoptotic cells. Two different approaches are frequently used. One is based on extracting low-molecular-weight DNA prior to cell staining, as described in Basic Protocol 5. It is also possible to analyze the extracted DNA by agarose gel electrophoresis (see Support Protocol). The other approach relies on fluorochrome labeling of DNA strand breaks in situ and is described in *UNIT 7.4*.

In the approach presented in Basic Protocol 5, cellular DNA is stained and measured following cell fixation in ethanol. Cell fixation in ethanol (or other precipitating fixatives, such as methanol or acetone) does not fully preserve the degraded DNA within apoptotic cells; this fraction of DNA leaks out during subsequent cell rinsing and staining. As a consequence, apoptotic cells have reduced DNA content and therefore can be recognized, following staining of cellular DNA, as cells with low DNA stainability. This peak (the "sub-G_1" peak) is located to the left of the peak representing G_1 cells on DNA content frequency histograms. Some cells represented by the sub-G_1 cell population may also have already lost their DNA by shedding apoptotic bodies—cellular fragments containing pieces of chromatin, mitochondria, and other organelles wrapped in the plasma membrane of the disintegrating apoptotic cell (Wyllie, 1992). Loss of DNA by such a mechanism characterizes cells at late stages of apoptosis. Regardless of the mechanism of DNA loss, apoptotic cells have generally lower DNA stainability. This is in contrast to cells dying by the alternative mode, necrosis, whose DNA content are little changed compared to live cells.

The degree of DNA degradation varies depending on the stage of apoptosis, cell type, and often the nature of the apoptosis-inducing agent. The extractability of DNA during the staining procedure (and thus separation of apoptotic from live cells) also varies. However, using a high-molarity phosphate-citrate buffer as the rinsing solution, as presented in Basic Protocol 5, enhances extraction of the degraded DNA. This approach can be used to control the extent of DNA extraction from apoptotic cells to the desired level to obtain maximal separation of apoptotic cells by flow cytometry (see Fig. 7.5.4). Because measurement of DNA content provides information about the cell cycle position of the nonapoptotic cell population, this protocol can be applied to investigate the cell cycle specificity of apoptosis.

The pattern of DNA cleavage during apoptosis, arising from preferential cleavage of internucleosomal ("spacer") sections, is reflected by the characteristic DNA "laddering" during gel electrophoresis. For many years, before flow cytometric methods became common in analysis of apoptosis, DNA gel electrophoresis was one of the most widely used assays (after microscopy) to detect apoptosis, and DNA laddering was considered to be a hallmark of the apoptotic mode of cell death (Compton, 1992; Wyllie, 1992). This methodology, which is still widely used, is presented in the Support Protocol.

The method is rapid and, unlike traditional biochemical techniques, uses nontoxic reagents to extract DNA (phenol, chloroform, etc., are avoided). The high molarity of the DNA extraction buffer (0.2 M Na_2HPO_4) weakens the electrostatic interaction between DNA and proteins in the cell, thereby allowing low-molecular-weight DNA to be released into the solution. In addition, the cells may be stored in 70% ethanol for months, prior to analysis, without any significant DNA degradation. Treatment with ethanol also inactivates many pathogens, thereby increasing the safety of sample handling. The method is applicable to clinical samples; cells can be fixed in ethanol and then stored and/or safely transported prior to analysis.

Critical Parameters and Troubleshooting

Fluorochrome concentration and cell density in samples

Several features of interactions between fluorochromes and nucleic acids are of importance in practical applications of DNA dyes in flow cytometry. First, as mentioned in the overview (*UNIT 7.1*) and discussed in more detail in *UNIT 4.2* on nucleic acid probes, the interactions, whether intercalative or involving DNA helix minor groove binding, are not covalent and thereby are reversible. The number of sites along a DNA molecule that interact with a particular fluorochrome, corresponding to the intensity of DNA staining, therefore, is expected to vary depending on the dye concentration. Variability is expected at low concentrations of the dye, up to a concentration when all potential binding sites are saturated. Stable levels of fluorescence, proportional to DNA con-

tent, thus require an equilibrium in which there is a significant excess of dye over number of binding sites. Under these conditions minor intersample variation in cell number does not significantly affect the concentration of the fluorochrome in the unbound, free form. One also has to remember, however, that even with the same number of cells, the number of binding sites may vary, for example, when the cells become hyperdiploid or arrested in G_2/M. According to the law of mass action, significant intersample variation in free (unbound) dye concentration and, consequently, in DNA stainability is expected when no excess of fluorochrome over number of binding sites exists. Given the above, how does the mass action law translate into practical conditions of equilibrium staining in flow cytometry?

An individual human cell in the G_1 phase of the cycle contains 6×10^9 base pairs (bp) of DNA, and the minimal size of the intercalative binding site of free DNA is 6 bp (Le-Pecq, 1971). One expects, therefore, in a typical sample containing 10^6 diploid cells in a volume of 1 ml, that at 1 μM fluorochrome concentration (1 nmol/ml) there is an inadequate number of fluorochrome molecules to saturate all potential DNA binding sites (6×10^{14} molecules per 10^{15} binding sites). It should be remembered, however, that, due to DNA interactions with chromatin constituents in the cell nucleus, only a fraction of potential binding sites is accessible to the dye. The size of the inaccessible fraction is unknown. It has been observed, however, that exposure of cell nuclei to 0.1 M HCl, which dissociates histones from DNA, results in a manyfold increase in binding of most fluorochromes to nuclear DNA. The lowest increase (<50%) was seen in the case of DAPI, whereas the highest increase (>13-fold) was for 7-aminoactinomycin D (7-AAD; Darzynkiewicz et al., 1984). This indicates that DNA-histone interactions (i.e., nucleosomal chromatin structures) restrict the accessibility of DNA to different degrees, depending on chemical structure of the fluorochrome.

Another level of restriction, especially for intercalating dyes, is the presence of closed loops of DNA in chromatin (van Holde, 1988). Because accommodation of each intercalator molecule involves extension and unwinding of the DNA helix, the closed circular structures provide a topological restraint and prevent full unwinding. It is likely, therefore, that DNA topology in chromatin provides an additional, at least 2-fold restriction for binding the dyes that unwind DNA. It is not known to what extent direct DNA-nuclear matrix interactions (unrelated to stabilization of chromatin DNA loops) and other nuclear structures may additionally restrict DNA accessibility. Nonetheless, it is safe to assume that fewer than 10% of the potential DNA binding sites are actually accessible to most DNA fluorochromes in the nucleus. Thus, under typical staining conditions of 10^6 cells in 1 ml of 1 μM fluorochrome solution, there is an approximately 5-fold excess of dye molecules per accessible binding site.

The above calculations indicate the need to maintain a relatively constant number of cells per sample, if the intensity of cell fluorescence between samples is compared. When large numbers of cells (especially hyperdiploid cells) are present in samples and the dye is used at low concentrations, significant variation in DNA stainability is expected due to the intersample cell density variation.

The choice of optimal dye concentration is a compromise between the signal-to-noise ratio during the cell fluorescence measurement and the stoichiometric relations between dye and binding sites. Although the fluorescence of most DNA fluorochromes is increased manyfold on binding to DNA, a large excess of the dye (high concentration) in solution makes the solution itself fluorescent (noise), thereby decreasing the signal-to-noise ratio during measurement of the cell. Furthermore, at high concentrations of even the most specific DNA dyes (e.g., DAPI, Hoechst 33342), the nonspecific component of dye fluorescence, resulting from binding of dye to cell constituents other than DNA, is elevated. Optimal staining conditions, therefore, should rely on a minimal but adequate dye concentration, at which intersample variation in cell number does not significantly alter the free (unbound) dye concentration.

Stoichiometry of DNA staining

The accessibility of DNA to various fluorochromes also is modulated by tissue-specific differences in chromatin structure. The extreme case are cells undergoing erythropoiesis or spermatogenesis, in which DNA accessibility to most fluorochromes is markedly reduced during differentiation (Darzynkiewicz et al., 1984). The degree of reduction varies for individual fluorochromes. For example, binding of DAPI is the least influenced by chromatin structure, whereas binding of 7-AAD, an intercalating fluorochrome with a bulky substituent which locates at the minor grove of DNA helix, is affected to a large degree. Accessibility of the

dimeric cyanine fluorochromes such as TO-TO or YOYO also is significantly affected by differences in chromatin structure. In practical terms, therefore, one may expect intercellular variation in DNA stainability when mixed cell types are measured in the same sample. This can be manifested on DNA content frequency histograms as the presence of pseudo-aneuploid populations, or widening of the G_1 peak (increased CV value). For example, monocytes, showing higher DNA stainability with PI compared to lymphocytes or granulocytes, often form a typical pseudo-hyperdiploid peak on DNA frequency histograms. However, subjecting cells to the detergent methods, and in particular the combination of detergent and proteolytic treatment (as in the Vindeløv procedure; Basic Protocol 2), appears to diminish the effect of chromatin structure on DNA stainability, thereby improving the stoichiometric relationship between DNA content and cell fluorescence.

Cell fixation with a cross-linking reagent such as formaldehyde or glutaraldehyde stabilizes DNA-protein interactions in chromatin and therefore amplifies the chromatin structure–related differences in the accessibility of DNA to fluorochromes. This is manifested by a high CV of the mean DNA content of the G_1 population. Cell fixation in formaldehyde is thus the primary reason why nuclei isolated from paraffin blocks (Basic Protocol 4) generally yield rather DNA poor resolution (high CV) compared to cells permeabilized with detergents (Basic Protocol 2 and Alternate Protocol 2) or fixed in ethanol (Basic Protocol 1 and Alternate Protocol 1). One has to remember, however, that most cross-linking reactions are reversible. Therefore, if cross-linking itself is the major culprit for poor DNA resolution, prolonged incubations of deparaffinized and rehydrated cells in aqueous media are expected to improve DNA stainability. Furthermore, DAPI, being the least sensitive to differences in chromatin structure, is preferred for stoichiometric staining of DNA.

There are several ways to estimate the stoichiometry of DNA staining. For uniform cell populations such as cell lines in tissue culture, a comparison of the intensity of staining of the cell populations represented by the G_2/M versus G_1 peaks, which is expected to approach 2.0, provides a crude practical measure of linearity (Crissman and Steinkamp, 1990). Normal hepatocytes grow at different DNA ploidy levels and therefore may also serve as markers of linearity in DNA measurements. Likewise, incubation of cells in culture with cytochalasin B (5 µg/ml) induces polyploidization, making such cells useful as linearity standards. Inclusion of internal standards such as chicken or trout erythrocytes (Basic Protocol 2) provides still another marker of the stoichiometry of DNA measurement (Vindeløv et al., 1983d). These standards are discussed in more detail in *UNIT 7.2*.

There are situations, however, even under optimal conditions of cell staining, when stoichiometry in DNA analysis cannot be attained. This is often the case if cells have been pretreated with antitumor drugs that modify DNA and chromatin structure. Intercalating drugs, drugs with chromophores able to interact with the dyes via fluorescence resonance energy transfer, and drugs damaging DNA structure can all alter DNA stainability, often in unpredictable ways.

Base specificity of DNA fluorochromes

The base specificity of the fluorochrome plays a major role in analysis of individual chromosomes, which differ in GC/AT ratios (Gray and Cram, 1990). Thus a combination of chromomycin, which has preference for CG base pairs, and Hoechst 33342, which reacts with AT, makes it possible to discriminate nearly all human chromosomes, which otherwise cannot be distinguished on the basis of DNA content alone. Base specificity is also of importance in analysis of cells that differ in base composition, such as microorganisms of different species or plant cells. Ratiometric analysis of fluorescence intensities of fluorochromes reacting with GC and AT base pairs, respectively, can be used to estimate the relative ratios of these bases (e.g., for identification of particular bacterial species). Still another situation where the base composition of DNA plays a role in DNA stainability is the S phase of the cell cycle. Namely, replication of the DNA associated with heterochromatin which is rich in AT occurs at the end of S phase. This should be taken into account when analyzing DNA replication rates in relation to the degree of cell progression through S phase (cellular DNA content) based on the pattern of the S phase cell labeling with base-specific fluorochromes.

Accuracy of DNA content measurements

The most critical issue in DNA content analysis is the accuracy of the DNA content measurement. The accuracy, as mentioned, is reflected by the extent of variation in cellular

fluorescence between cells with identical DNA content, such as $G_0/G_{/1}$ cells. The CV of the DNA-associated mean fluorescence of G_1 cells, therefore, is considered an index of the accuracy of DNA content measurements. High accuracy is required, in particular, in studies of DNA ploidy, to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. The possibility of discriminating aneuploid cell populations is directly related to the CV values of DNA-associated fluorescence of the measured diploid and aneuploid cell populations.

Highly accurate DNA content measurement is also critical in analysis of cell cycle distributions. Regardless of the type of software used to deconvolute DNA content, frequency histograms (see Chapter 10 for further information on data analysis), the accuracy of estimates of cell proportions in respective phases of the cell cycle directly correlates with the accuracy of DNA content measurements. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of the G_0/G_1 cell population (i.e., acceptable maximal error in cellular DNA content estimates). Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of G_0/G_1 populations of normal, nontumor cells exceed 6%. An exception is analysis of the DNA content of cell nuclei isolated from paraffin blocks (Basic Protocol 4), where by the nature of the sample, which generally is prefixed in formaldehyde and contains nuclei with fractional DNA content (partially cut during sectioning), good accuracy is difficult, if not impossible, to achieve.

A variety of factors can contribute to poor accuracy in DNA content analysis. The most common is suboptimal optical and sample flow adjustment of the flow cytometer. Proper maintenance of the instrument and its careful adjustment prior to analysis of the experimental samples, to maximize the electronic signal intensity and minimize variability of the measurement of the uniformly stained cells or fluorescent beads, are required to achieve accurate DNA measurements (see *UNIT 1.3* for discussion of standardization, calibration, and control in flow cytometry). Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, represent another common cause of poor resolution in DNA analysis. As discussed above, an excessively large number of cells (DNA) in the sample, which leads to significant depletion of the free, unbound fluorochrome in the solution and changes the conditions of equilibrium staining, may be still another source of the problems that prevent accurate DNA content analysis. Diluting samples to achieve a lower cell concentration may improve results.

DNA content analysis in living cells (Basic Protocol 3) is expected to produce variable results, depending on the ability of cells to remove the Hoechst 33342 dye by efflux mechanisms. Depending on the cell type, the CV of the mean DNA content of the DNA-uniform cell populations (G_0/G_1, G_2/M) may vary from 5% to 10%, or even more. If the intensity of cell fluorescence is low, or if resolution of cells in various phases of the cell cycle is inadequate, staining times can be prolonged and/or the dye concentration can be increased. To improve staining (i.e., fluorescence intensity and stoichiometry) one may also attempt to use inhibitors of the P glycoprotein efflux pump (Krishan, 1987).

It should be stressed that there are situations when, despite good accuracy of DNA content measurements (in terms of proper instrument adjustments and sample staining), the CV of the mean DNA content of G_1 cell populations may be relatively large. This may be the case when significant numbers of dead or dying cells are present in the sample or when the cells were treated with drugs that interact with DNA. Furthermore, because of the nature of the tumor, which may either be polyclonal or have developed drug resistance by gene amplification mechanisms (e.g., as reflected by the presence minute chromosomes), tumor cell populations may have variable DNA content and therefore intrinsically high CV values for the G_0/G_1 cell populations.

Detection of apoptotic cells

For optimal detection of apoptotic cells (Basic Protocol 5), the sub-G_1 peak should be positioned at the midpoint between the G_1 peak and the channel zero. It is also recommended to gate out objects with fluorescence values <10% of that of the G_1 peak (e.g., by positioning the G_1 peak at 100 channels and gating out all objects with fluorescence intensity lower than ten channels). In this way objects with minimal fluorescence, and therefore less likely to be apoptotic cells, will be excluded from the counts. Although very late apoptotic cells may be gated out by such a strategy, the consistent underestimate of apoptosis is of lesser significance than erroneous classification of the nonapoptotic events as apoptotic cells.

Extraction of the degraded low-molecular-weight DNA by 0.2 M phosphate-citrate buffer (DNA extraction buffer) is a result of weakening of electrostatic interactions between DNA and cellular proteins at the high ionic strength of this buffer; such interactions restrict elution of DNA from ethanol-prefixed cells (Gong et al., 1994). Thus, optimal positioning of apoptotic cells on DNA frequency histograms can be done by controlled DNA extraction with this buffer. In other words, if DNA degradation within apoptotic cells is extensive or if the cells have already lost a fraction of their DNA by shedding apoptotic bodies, there is no need for step 3 in Basic Protocol 5. Conversely, extended rinsing with higher volumes of DNA extraction buffer is needed in situations when DNA degradation is incomplete and the sub-G_1 and G_1 peaks are not separated.

It should be stressed that detection of apoptotic cells by DNA content analysis is incompatible with methods which rely on cell lysis (i.e., employing detergents and proteolytic enzymes). The nucleus of an apoptotic cell is fragmented, and therefore a single cell may contain numerous nuclear fragments separated from one another. Lysis of such a cell releases these fragments, which following DNA content measurement will be located at the sub-G_1 peak position. Individual nuclear fragments, therefore, may be mistakenly identified as single apoptotic cells. Other critical factors in detection of apoptotic cells are presented in UNIT 7.4 and discussed elsewhere (Darzynkiewicz et al., 1997).

Critical factors pertaining to DNA content analysis which are more specific to particular methods and are discussed above (see Background Information).

Anticipated Results

DNA content analysis of fixed cells

Figure 7.5.1 presents DNA frequency histograms of HL-60 cells stained with PI (panel A) and DAPI (panel B) according to Basic Protocol 1 and Alternate Protocol 1, respectively. On the basis of differences in DNA content, one can identify a population of G_0/G_1 cells with uniform, low DNA content values, G_2/M cells with DNA content twice that of G_0/G_1 cells, and S phase cells with intermediate DNA content. The DNA frequency histograms were deconvoluted using Multicycle software (Phoenix Flow Systems).

DNA content analysis utilizing trypsin and/or detergent

Figure 7.5.2 presents DNA content distribution histograms for cells obtained from a fine-needle aspirate of a surgical biopsy of human breast cancer and stained with PI according to Basic Protocol 2. The peaks from left to right represent chicken (C) and trout (T) erythrocytes, diploid normal nuclei (D), and a hyperdiploid (DI > 1.0) population of tumor cell nuclei. Under conditions of stoichiometry of DNA stainability, the ratio of the mean DNA content of diploid human cells to chicken erythrocytes is 2.857, the ratio to trout erythrocytes is 1.258, and the ratio of mean DNA in trout versus chicken erythrocytes is 2.28 (Vindeløv et al., 1983d). Another landmark of linearity in DNA content analysis is the ratio of G_2 to G_1 peaks, which is expected to approach 2.0.

Figure 7.5.3 presents DNA histograms of HL-60 cells stained with DAPI according to Alternate Protocol 2.

DNA content analysis of paraffin-embedded samples

Accuracy of DNA content analysis in cell nuclei isolated from paraffin blocks (Basic Protocol 4)—especially of tissues that were fixed in formaldehyde—is generally inferior to that of cells permeabilized with detergents or fixed by suspension in ethanol or methanol. Thus, the CV of the mean DNA content of the DNA-uniform cell populations (e.g., G_0/G_1 cells) rarely can be <4% and is generally within the range of 5% to 8%. Furthermore, there is often a high frequency of events with fractional DNA content (sub-G_1 peak) on the DNA content frequency histogram. In contrast to intact cells that are fixed in suspension, where such events are recognized as apoptotic cells (see Basic Protocol 5), in the case of nuclei isolated from paraffin blocks these events represent predominantly nuclear fragments resulting from sectioning through the nucleus. The frequency of such mechanically fragmented nuclei is inversely proportional to the thickness of the section and to the nuclear diameter.

It is difficult to find appropriate external or internal standards for DNA content (DI) for nuclei isolated from paraffin blocks. One has to be cautious, therefore, in interpreting differences in DNA stainability between cells of different lineages (or normal versus tumor cells) in different samples or even within the same sample. Chemical reactions of formaldehyde with nuclear constituents during fixation

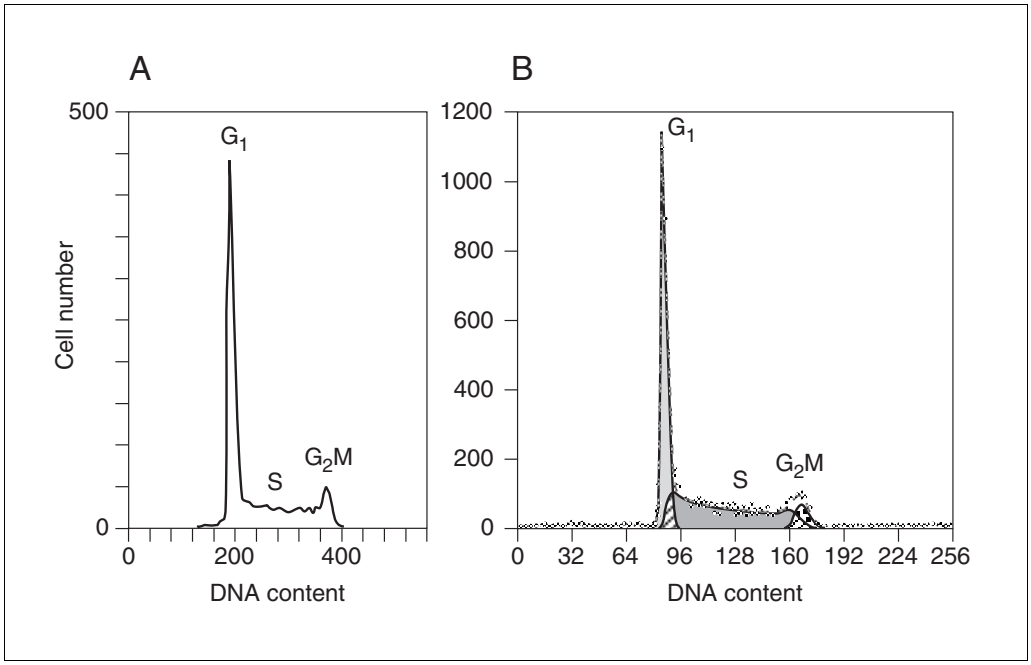


Figure 7.5.1 Flow cytometric DNA content analysis of HL-60 cells stained with PI (**A**) and DAPI (**B**) following ethanol fixation according to Basic Protocol 1 and Alternate Protocol 1, respectively. The DNA frequency histogram **(B)** was deconvoluted using Multicycle software (Phoenix Flow Systems).

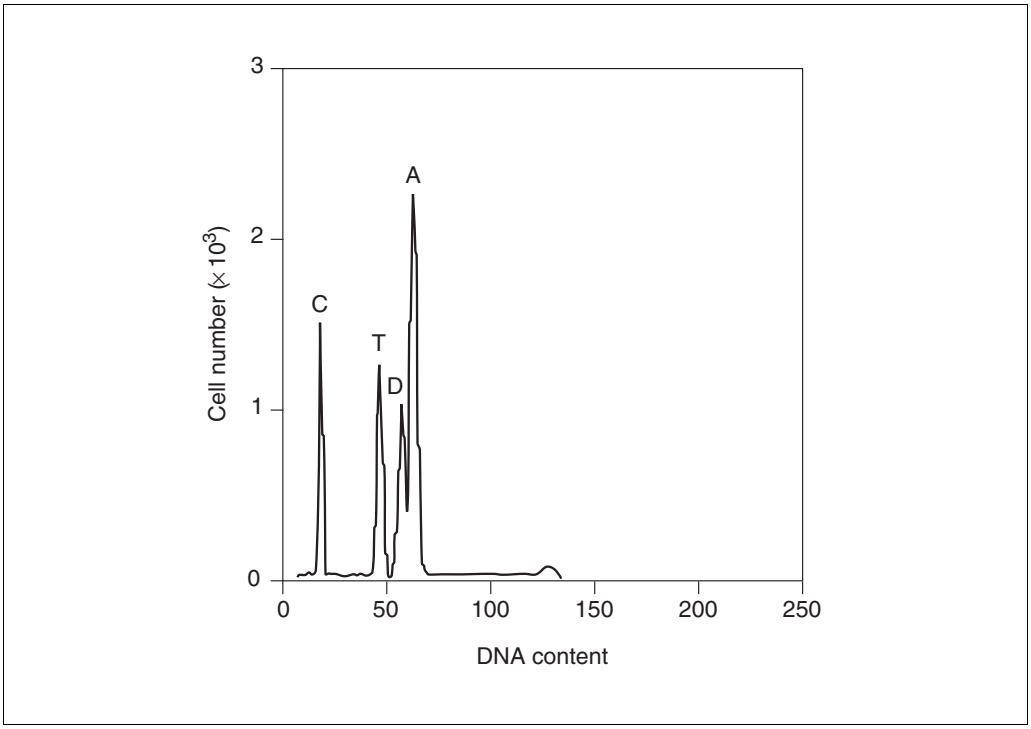


Figure 7.5.2 Flow cytometric DNA content analysis of biopsied human breast cancer cells stained with PI following detergent/trypsin treatment according to Basic Protocol 2. DNA analysis revealed chicken erythrocytes (C), trout erythrocytes (T), diploid normal nuclei (D), and hyperdiploid (aneuploid) tumor cell nuclei (A). Reprinted from Vindeløv and Christensen (1990).

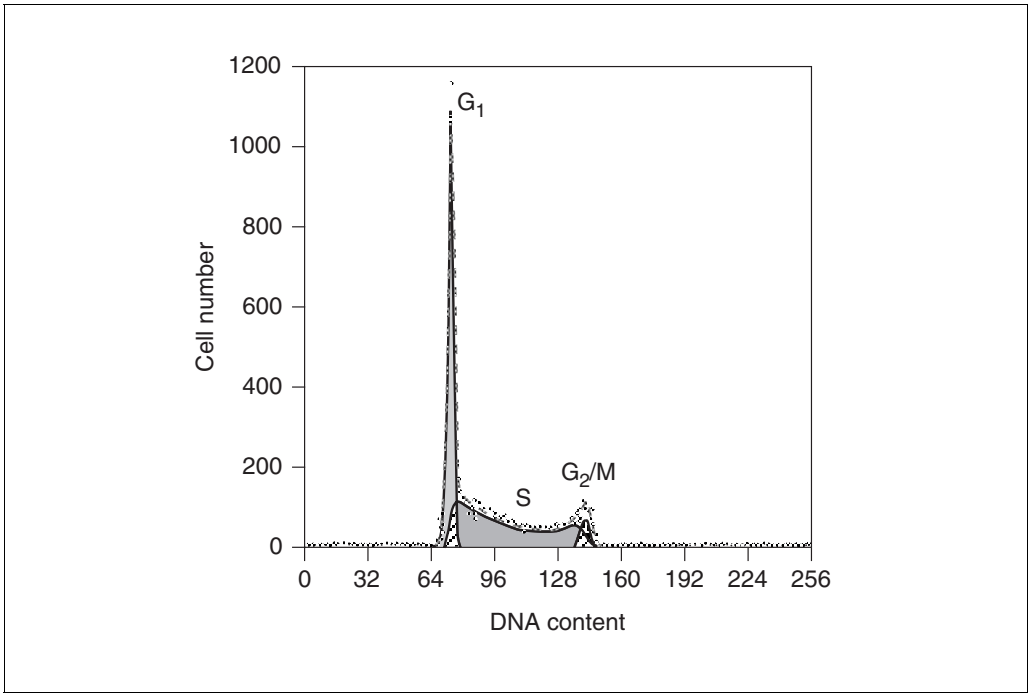


Figure 7.5.3 Flow cytometric DNA content analysis of detergent-permeabilized HL-60 cells stained with DAPI according to Alternate Protocol 2.

affect subsequent in situ DNA stainability in proportion to the time of cell exposure to formaldehyde, and temperature. The extent of chemical modification is also related to size of the fixed tissue (i.e., rate of formaldehyde penetration), intracellular ionic conditions, and the chemical nature (tissue specificity) of nuclear constituents. Isolation of nuclei from paraffin blocks and their rehydration cannot fully restore the original DNA stainability. Thus, the stoichiometry of DNA detection may be different in normal cell nuclei (used as DI standard) and tumor cell nuclei, even if the tissues were fixed identically.

DNA content analysis for detection of apoptotic cells

Populations of apoptotic cells are discriminated by Basic Protocol 5 on the basis of reduced DNA content (stainability) in these cells. On DNA content frequency histograms, apoptotic cells are thus represented by the sub-G_1 peak, which should be separated from the G_1 peak (Fig. 7.5.4). Depending on the degree of DNA degradation, however, or the prior loss of cellular DNA via shedding apoptotic bodies, the position of the sub-G_1 peak may vary, from near overlap with the G_1 peak to very low channels. When apoptotic cells are measured at very low fluorescence channels, it is difficult to distinguish them from individual apoptotic bodies, fragments of chromatin, broken nuclei, chromosomes, cell debris, etc.

The procedure for detecting apoptotic cells presented in Basic Protocol 5 is simple and inexpensive. It allows one to enumerate apoptotic cells and, in addition, to reveal DNA ploidy and/or the cell cycle distribution of the nonapoptotic cell population. Another advantage of this method is its applicability to any DNA fluorochrome (see Background Information) or instrument. Degraded DNA extracted from ethanol-fixed apoptotic cells can be directly analyzed by gel electrophoresis. Additional issues pertaining to the identification of apoptotic cells are discussed in *UNIT 7.4*.

In the Support Protocol, the presence of apoptotic cells in the suspension of cells extracted with the phosphate-citrate buffer is manifested as a DNA "ladder" on the gels (Fig. 7.5.4C). The sensitivity of the method (i.e., the minimal number of apoptotic cells that can be detected per sample) varies depending on the degree of DNA degradation in individual cells (advancement of apoptosis) and efficiency of DNA extraction. The method, however, is more sensitive than traditional DNA extraction using phenol. This is because the degraded, low-molecular-weight DNA (i.e., DNA which is responsible for generating the ladder) is selectively extracted from the sample, whereas high-molecular-weight DNA remains in the cells.

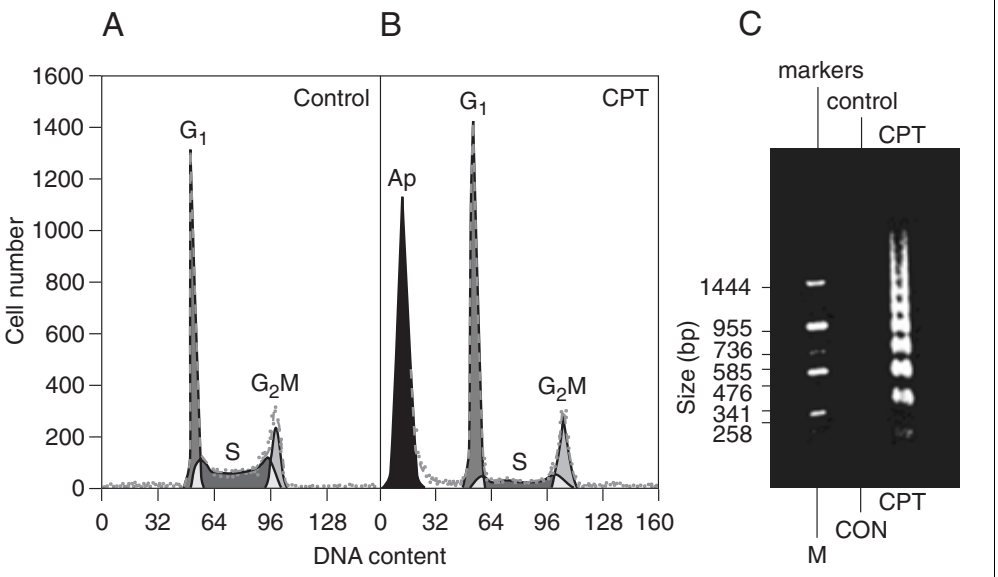


Figure 7.5.4 Flow cytometric DNA content analysis of control (**A**) and camptothecin-treated cells (**B**) subjected to extraction of low-molecular-weight DNA and stained with PI according to Basic Protocol 5, and electrophoretic analysis of the low-molecular-weight DNA (**C**) according to the Support Protocol. Cells treated with camptothecin (CPT) show the sub-G_1 population of apoptotic cells (AP). The gel shows the DNA laddering characteristic of apoptotic cells (lane CPT), whereas control cells (lane CON) show no loss of low-molecular-weight DNA. Lane M shows DNA markers.

Thus, relatively small fractions of apoptotic cells (e.g., 1% to 2%) can be detected if DNA is extracted from a large number (>10^6) of cells.

It should be stressed that not always is DNA cleared to fragments of 180 to 200 bp during apoptosis (Oberhammer et al., 1993). The methods described in this chapter (see Basic Protocol 5 and *UNIT 7.4*) cannot defect such apoptosis, and other means should then be used (Darzynkiewicz et al., 1997).

Time Considerations

For Basic Protocol 1, cell fixation takes ~10 min, but cells have to be kept in the fixative ≥2 hr; the cell staining procedure takes up to ~45 min. Alternate Protocol 1 requires identical times for cell fixation and staining.

The detergent-based cell staining procedure of Basic Protocol 2 takes ~60 min, but collecting cells from tissues may require up to 1 hr. For Alternate Protocol 2, on the other hand, which is much simpler, cell staining takes ~15 min.

Supravital staining of cells (Basic Protocol 3) requires 20 min for staining, although extended staining times (up to 90 min) may be needed.

For Basic Protocol 4, the whole staining procedure (sectioning and deparaffinizing tissue, isolating nuclei, and staining) takes ~7 hr.

Cell staining to detect apoptotic cells (Basic Protocol 5) takes ~1 hr. Ethanol prefixation of cells, however, adds ≥2 hr to the time requirements. In the Support Protocol, preparing DNA for gel electrophoresis takes ~1.5 hr. Gel electrophoresis takes up to 20 hr. Staining and visualizing the DNA on gels takes ~30 min.

The time required for flow cytometric measurements of cell fluorescence may vary from 1 to 10 min per sample, depending on cell concentration, flow rate, number of cells to be measured, etc. The time for data analysis may also vary, from 1 to 10 min per sample, depending on sample complexity (e.g., presence of aneuploid populations, debris, etc.; see Chapter 10).

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