ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit

S7160

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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I. INTRODUCTION

ApopTag® *In Situ* Apoptosis Detection Kits label apoptotic cells in research samples by modifying fragmented genomic DNA utilizing terminal deoxynucleotidyl transferase (TdT) for detection of positive cells by specific staining. This manual contains information and protocols for the ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit (Catalog number S7160).

Using this Manual

This manual accommodates both the novice and the experienced ApopTag® user. These protocols are presented in a streamlined manner. However, users are directed to sections which provide supplemental information by notations in the protocol.

The novice user is advised to read the Introduction, especially the section on sample fixation. Before beginning the protocol, reading the assigned TECH NOTES is recommended. Directions for preparing some of the required reagents can be found in Sec. IV. *Appendix*. Should additional questions arise, assistance is available from Chemicon Technical Service at (800) 437-7500 or at techserv@chemicon.com.

Background

Apoptosis is a form of cell death that eliminates compromised or superfluous cells. It is controlled by multiple signaling and effector pathways that mediate active responses to external growth, survival, or death factors. Cell cycle checkpoint controls are linked to apoptotic enzyme cascades, and the integrity of these and other links can be genetically compromised in many diseases, such as cancer. There are many books in print and hundreds of recent review articles about all aspects of apoptosis (e.g. 7, 11, 19, 24, 39, 42) and the methods for detecting it (e.g. 10, 32, 36).

Of all the aspects of apoptosis, the defining characteristic is a complete change in cellular morphology. As observed by electron microscopy, the cell undergoes shrinkage, chromatin margination, membrane blebbing, nuclear condensation and then segmentation, and division into apoptotic bodies which may be phagocytosed (11, 19, 24). The characteristic apoptotic bodies are short-lived and minute, and can resemble other cellular constituents when viewed by brightfield microscopy. DNA fragmentation in apoptotic cells is followed by

cell death and removal from the tissue, usually within several hours (7). A rate of tissue regression as rapid as 25% per day can result from apparent apoptosis in only 2-3% of the cells at any one time (6). Thus, the quantitative measurement of an apoptotic index by morphology alone can be difficult.

DNA fragmentation is usually associated with ultrastructural changes in cellular morphology in apoptosis (26, 38). In a number of well-researched model systems, large fragments of 300 kb and 50 kb are first produced by endonucleolytic degradation of higher-order chromatin structural organization. These large DNA fragments are visible on pulsed-field electrophoresis gels (5, 43, 44). In most models, the activation of Ca^{2+}-and Mg^{2+}-dependent endonuclease activity further shortens the fragments by cleaving the DNA at linker sites between nucleosomes (3). The ultimate DNA fragments are multimers of about 180 bp nucleosomal units. These multimers appear as the familiar "DNA ladder" seen on standard agarose electrophoresis gels of DNA extracted from many kinds of apoptotic cells (e.g. 3, 7,13, 35, 44).

Another method for examining apoptosis via DNA fragmentation is by the TUNEL assay, (13) which is the basis of ApopTag® technology. The DNA strand breaks are detected by enzymatically labeling the free 3'-OH termini with modified nucleotides. These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei, which have relatively insignificant numbers of DNA 3'-OH ends, usually do not stain with the kit. ApopTag® Kits detect single-stranded (25) and double-stranded breaks associated with apoptosis. Drug-induced DNA damage is not identified by the TUNEL assay unless it is coupled to the apoptotic response (8). In addition, this technique can detect early-stage apoptosis in systems where chromatin condensation has begun and strand breaks are fewer, even before the nucleus undergoes major morphological changes (4, 8).

Apoptosis is distinct from accidental cell death (necrosis). Numerous morphological and biochemical differences that distinguish apoptotic from necrotic cell death are summarized in the following table (adapted with permission from reference 39).

Table 1: Types of Cell Death: Differential Characteristics

Apoptosis	Necrosis
Morphologic Criteria	
Deletion of single cells	Death of cell groups
Membrane blebbing, but no loss of integrity	Loss of membrane integrity
Cells shrink, ultimately forming apoptotic bodies	Cells swell and lyse
No inflammatory response	Significant inflammatory response
Phagocytosis by adjacent normal cells, and some macrophages	Phagocytosis by macrophages
Lysosomes intact	Lysosomal leakage
Compaction of chromatin into uniformly dense masses	Clumpy, ill-defined aggregation of chromatin
Biochemical Criteria	
Onset tightly regulated by physiological homeostasis	Onset incidental to nonphysiological trama
Specific enzyme cascades for signal transduction and execution	Enzyme cascades altered or inactive
Metabolically viable during execution	Non-viable during execution
Macromolecules may be newly synthesized	Macromolecules not synthesized
Phosphatidyl serine exposure signals death	Nonspecific lytic effusion indicates death
Nonrandom, oligonucleosomal fragment lengths (DNA ladder)	Random DNA fragment lengths (DNA smear)

ApopTag® *In Situ* Apoptosis Detection Kits distinguish apoptosis from necrosis by specifically detecting DNA cleavage and chromatin condensation associated with apoptosis. However, there may be some instances where cells exhibiting necrotic morphology may stain lightly (14, 29) or, in rare instances, DNA fragmentation can be absent or incomplete in induced apoptosis (11). It is, therefore, important to evaluate ApopTag® staining results in conjunction with morphological criteria. Visualization of positive ApopTag® results should reveal focal *in situ* staining inside early apoptotic nuclei and apoptotic bodies. This positive staining directly correlates with the more typical biochemical and morphological aspects of apoptosis.

Since an understanding of cell morphology is critical for data interpretation and because of the potential for experimentally modifying or overcoming normal apoptotic controls, the following strategy is advised. When researching a new system, the staging and correlation of apoptotic morphology and DNA fragmentation should be characterized. In some tissues, cytoplasmic shrinkage may be indicated by a clear space surrounding the cell. The nuclear morphology of positive cells should be carefully observed at high magnification (400x-1000x). Early staged positive, round nuclei may have observable chromatin margination. Condensed nuclei of middle stages, and apoptotic bodies, usually are stained. Apoptotic bodies may be found either in the extracellular space or inside of phagocytic cells. It is highly recommended that less experienced observers should refer to illustrations of dying cells for comparison with new data (e.g. 11, 19, 24).

An additional, although far less sensitive, method of confirming ApopTag® staining results is the detection of DNA fragmentation on agarose gels. If a large percent of the cells in the tissue are apoptotic, then electrophoresis of extracted total genomic DNA and standard dye staining can be used to corroborate the *in situ* staining. However, the single-cell sensitivity of ApopTag® histochemistry is far higher than this method. DNA laddering data of comparable sensitivity may be obtained in several other ways. These include methods for selectively extracting the low molecular weight DNA (15), for preparing radiolabeled DNA (30, 40) in combination with resin-bed purification of DNA (12), and for DNA amplification by PCR (35).

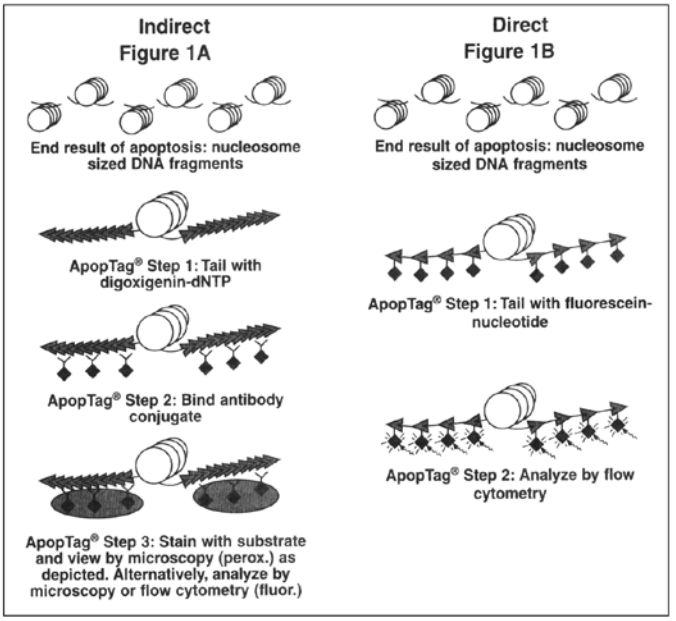
The *in situ* staining of DNA strand breaks detected by the TUNEL assay and subsequent visualization by light microscopy gives biologically significant data about apoptotic cells which may be a small percentage of the total population (13, 16). Apoptotic cells stained positive with ApopTag® Kits are easier to detect and their identification is more certain, as compared to the examination of simply histochemically stained tissues. Another feature of ApopTag® is that

quantitative results can be obtained using flow cytometry, since end-labeling methodology detects apoptotic cells with a >10-fold higher sensitivity than necrotic cells (14,17). In addition, the occurrence of DNA fragmentation with regard to the cell cycle phase of apoptotic cells can be examined using the TUNEL assay and flow cytometry (16,18).

Principles of the Procedure

The reagents provided in ApopTag® Kits are designed to label the free 3'OH DNA termini *in situ* with chemically labeled and unlabeled nucleotides. The nucleotides contained in the Reaction Buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT) (13, 31). TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double-stranded or single-stranded DNA. In the ApopTag Direct Methodology, the incorporated nucleotides form an oligomer composed of fluorescein nucleotide and unlabeled nucleotide in a random sequence. The ratio of labeled to unlabeled nucleotide in the kit is optimized to minimize fluorescein self-quenching. The exact length of the oligomer added has not been measured.

Figure 1: ApopTag® Methodology



The ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit (S7160) does not require the use of an antibody for detection, since the DNA fragments are directly labeled with a fluorescein nucleotide. Because the anti-digoxigenin antibody is not used, this system requires fewer applications of solutions (Figure 1B), which promotes good cell recovery due to fewer manipulations. The protocol also requires less time. There is little difference in the mean signal strength relative to the indirect method (32).

Results using ApopTag® Kits have been widely published (see Sec. V. *References, Publications Citing ApopTag® Kits*). The ApopTag® product line provides various options in experimental design. A researcher can choose to detect staining by brightfield or fluorescence microscopy or by flow cytometry, depending on available expertise and equipment. There are also opportunities to study other proteins of interest in the context of apoptosis when using ApopTag® Kits. By using antibodies conjugated with an enzyme other than peroxidase and an appropriate choice of substrate, it is possible to simultaneously examine another protein and apoptosis using ApopTag® Peroxidase Kits. There is also a choice of fluorophores (fluorescein and rhodamine) using ApopTag® technology. Flexibility exists in choosing antibody-fluor combinations to study other important proteins.

Kit Components

Table 2

Component	Part #	Vol/Qty	Storage
Equilibration Buffer	90416	3.0 mL	-15°C to -25°C
Reaction Buffer	90427	2.0 mL	-15°C to -25°C
TdT Enzyme	90418	0.64 mL	-15°C to -25°C
Stop/Wash Buffer	90419	20 mL	-15°C to -25°C
Plastic Coverslips	90421	100 ea.	Room Temp.

Number of tests per kit: Sufficient materials are provided to stain 40 tissue specimens of approximately 5 cm^2 each when used according to instructions. Reaction Buffer will be fully consumed before other reagents when kits are used for slide-mounted specimens.

Specificity and Reactivity

In some instances, certain tissues contain cell types which bind any fluorescein labeled nucleotide such as that contained in the ApopTag® Fluorescein Direct Kit (S7160). It is therefore recommended that this kit be used to stain cell suspensions, cytospins and cell cultures, but not tissue sections. Chemicon suggests the use of the ApopTag® Fluorescein Kits (S7110, S7111) or the ApopTag® Red Kit (S7165) for fluorescent staining tissue sections.

Precautions

1. The following kit components contain potassium cacodylate (dimethylarsinic acid) as a buffer: Equilibration Buffer (90416), Reaction Buffer (90427), and TdT Enzyme (90418). These components are harmful if swallowed; avoid contact with skin and eyes (wear gloves, glasses) and wash areas of contact immediately.
2. TdT Enzyme (90418) contains glycerol and will not freeze at -20°C. For maximum shelf life, do not warm this reagent to room temp. before dispensing.

Storage and Shelf Life

1. Store the kit at -15°C to -25°C until the first use. After the first use, if the kit will be used within three months, store the TdT Enzyme (90418) at -15°C to -25°C and store the remaining components at 2°C to 8°C.
2. Protect the fluorescein nucleotide (90427) in the Reaction Buffer from unnecessary exposure to light.

II. IMMUNOCYTOCHEMISTRY METHODS

Materials Required But Not Supplied

Solvents and Media

a. Deionized water (dH_2O)

b. Xylene

c. Ethanol: absolute, 95%, 70%, diluted in dH_2O

d. Ethanol: acetic acid, 2:1 (v:v) (for tissue cryosection or cells protocols)

e. Slide mounting medium (Antifade)

Solutions

a. 1% paraformaldehyde in PBS, pH 7.4 (methanol-free formaldehyde for tissue cryosections or cells). See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

b. PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl)

c. Protein Digesting Enzyme or Proteinase K, Catalog No. 21627, (for paraffin-embedded tissue protocol).

d. 0.5-1.0 µg/mL Propidium Iodide in Antifade (S7112)

e 0.5-1.0 µg/mL DAPI (4'-6' diamino-2-phenylindole) in Antifade (S7113)

Materials

a. Silanized glass slides

b. Glass coverslips (for oil immersion objective, use 22 x 50 mm)

c. Adjustable micropipettors

d. Glass or plastic coplin jars

e. Forceps for handling plastic coverslips (optional)

f. Humidified chamber

g. 37°C covered water bath, or incubator at 37°C

Equipment

Light microscope equipped with brightfield optics (40x and 10x objectives) and also equipped for fluorescence. See Sec. IV. *Appendix, TECH NOTE #6: Required fluorescence filters.*

Experimental Preparation and Setup

Note: *Protect the Reaction Buffer containing the fluorescein nucleotide (90427) from light.*

Reagent Volumes

The following are suggested volumes of the reagents that will ensure adequate coverage of the specimen:

Table 3: Recommended Reagent Volumes

Reagent	Vol/cm^2	Vol/5 cm^2
Equilibration Buffer	13 µL	65 µL
Working Strength TdT	11 µL	55 µL

Working Strength TdT Enzyme

The concentrated TdT Enzyme provided in this kit is supplied in a stabilization buffer to preserve activity. It must be diluted with Reaction Buffer prior to use. Mix reagents in a ratio of 70% Reaction Buffer to 30% TdT Enzyme. To prepare, add in a fresh microcentrifuge tube:

77 µL	Reaction Buffer (90427)
33 µL	TdT Enzyme (90418)
110 µL	Total

Mix well by vortexing. This reagent may be prepared in advance and stored on ice for no more than 6 hours. This amount is sufficient to treat two 5 cm^2 tissue specimens.

Note: *Use of excessive Working Strength TdT will result in fewer tests per kit.*

Protein Digesting Enzyme or Proteinase K

Dilute the 200 µg/mL stock of Proteinase K (Catalog No. 21627) to 20 µg/mL in PBS just before use.

Coplin jar
If this step is to be performed in a coplin jar, adding 3.9 mL of the 200 µg/mL stock Proteinase K (Catalog No. 21627) to 35 mL of PBS will give sufficient volume of the appropriate dilution.

Direct slide application
If this step is to be done directly on the slide, 60 µL of diluted stock is required per 5 cm^2 specimen.

Working Strength Stop/Wash Buffer

Prepare working strength Stop/Wash Buffer by adding:

1 mL	Stop/Wash Buffer (90419)
34 mL	dH_2O
35 mL	Total

This amount is sufficient to treat 5 slides in a coplin jar. This reagent may be prepared in advance and stored in a glass or plastic container at 4°C for up to 1 year. Use a fresh aliquot for each experiment.

Nuclear Counterstain with Propidium Iodide or DAPI

Propidium Iodide (PI) or DAPI is used with fluorescein. The brightness of the counterstain should be equal to or less than that of the FITC. The apparent ratio will depend upon multiple factors. A general purpose concentration of either stain is in the range of 0.5-1 µg/mL in Antifade. For less intense initial counterstaining, these can be diluted by mixing with 2-4 parts Antifade. PI or DAPI counterstain can be removed from chromatin (destained) by extensive washing in PBS. See Sec. IV. *Appendix, TECH NOTE #6: Required fluorescence filters* and *TECH NOTE #15: Fluorescent counterstains.*

Plastic Coverslips

The purpose of the plastic coverslips is to spread reagents evenly by capillary action over a defined area. They can be omitted for a faster protocol. If the basic coverslip method described in TECH NOTE #8 will be used, note that each plastic coverslip must be cut to a size of ~5 cm^2 prior to use. Volumes of reagents that sufficiently cover a 5 cm^2 specimen are also appropriate when

using a ~5 cm^2 coverslip. Refer to TECH NOTE #8 for suggestions as to when the use of coverslips is appropriate in the protocol.

Humidified Container

See Sec. IV. *Appendix, TECH NOTE #7: Containers.*

Length of Assay

Allow for a total processing time of about 3 hours. With paraffin sections, or 2 hours with tissue cryosections or cultured cells.

Protocol

***Note**: Apoptosis in adherent cell cultures can result in detachment from the substrate. Supernatants should be tested, if possible, by using cytospin processing.*

It is recommended that the following sections be read prior to beginning this procedure:

TECH NOTE #4: *double-labeling methods*
TECH NOTE #6: *fluorescence filters*
TECH NOTE #8: *plastic coverslips*
TECH NOTE #9: *controls*
TECH NOTE #10: *other pretreatments*
TECH NOTE #11: *sample handling*
TECH NOTE #13: *optional stopping points*

DO NOT ALLOW SAMPLES TO DRY OUT DURING PROCESSING.

1. Fix Specimen According to Type

Adherent Cultured Cells

a. Fix in 1% PARAFORMALDEHYDE in PBS, pH 7.4 in a coplin jar (or cell culture vessel) preferably for 10 minutes at room temp., or for up to 15 hours at 4°C. Drain off excess liquid. See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

b. Wash in 2 changes of PBS for 5 minutes each wash.

c. Post-fix in precooled ETHANOL:ACETIC ACID 2:1 for 5 minutes at -20°C in a coplin jar. Drain, but do not allow to dry (this solvent permeabilizes cells).

d. Wash in 2 changes of PBS for 5 minutes each wash. Go to Step 2.

Cell Suspensions for Microscopy

a. Fix the cells at at density of approximately 5 x 10^7 cells/mL in freshly diluted 1% PARAFORMALDEHYDE in PBS, pH 7.4 for 10 minutes at room temp. See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

b. Dry 50-100 µL of cell suspension on a microscope slide (optionally, cytospin cells).

c. Wash in 2 changes of PBS for 5 minutes each wash. Go on to Step 2.

2. Apply Equilibration Buffer

a. Gently tap off excess liquid and carefully blot around the section.

b. Immediately apply 75 µL/5 cm^2 of EQUILIBRATION BUFFER directly on the specimen.

c. Incubate for at least 10 seconds at room temp.

3. Apply Working Strength TdT Enzyme

a. Gently tap off excess liquid and blot or aspirate around the section.

b. Immediately pipette onto the section 55 µL/5 cm^2 of WORKING STRENGTH TdT ENZYME.

c. Incubate in a humidified chamber at 37°C for 1 hour. Avoid exposure to light.

4. Apply Stop/Wash Buffer

Put the specimen in a coplin jar containing WORKING STRENGTH STOP/WASH BUFFER, agitate for 15 seconds, and incubate for 10 minutes at room temp.

5. Counterstain and Mount After Fluorescein Staining

a. Gently tap off excess liquid and carefully blot or aspirate around the section.

b. Apply a mounting medium (preferably Antifade) containing 0.5-1.0 µg/mL Propidium Iodide. Use 15 µL for a 22 x 50 mm coverslip with an oil immersion objective.

c. Mount under a glass coverslip.

d. If storage is required, apply rubber cement to edges of the coverslip. Store at -20°C in the dark.

6. View Fluorescein and Counterstain

View by fluorescence microscopy using standard fluorescein excitation and emission filters. See Sec. IV. *Appendix, TECH NOTE #6: Required fluorescence filters* and *TECH NOTE #15: Fluorescent counterstains.*

III. FLOW CYTOMETRY METHODS

Materials Required But Not Supplied

Note: *See Sec. IV.* Appendix: Reagent Preparation *for specific instructions for preparing these reagents.*

Solvents and media

a. Deionized water (dH_2O)

b. 70% ice-cold ethanol

Solutions

a. 1% paraformaldehyde in PBS, pH 7.4 (methanol-free formaldehyde). See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

b. PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl)

c. PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) with 1% (w:v) BSA

d. Propidium Iodide

e. RNase A

f. Triton X-100 10% (w:v) stock solution

Materials

a. Adjustable micropipettors

b. 37°C covered water bath or incubator at 37°C

c. 15 mL screw-cap polypropylene centrifuge tubes

d. Microcentrifuge tubes

Equipment

Flow cytometer, equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters. See Sec. IV. *Appendix, TECH NOTE #7: Required fluorescence filters.*

Experimental Preparation and Setup

Working Strength TdT

The concentrated TdT Enzyme provided in the kit is supplied in a stabilization buffer to preserve activity. It must be diluted with Reaction Buffer prior to use. Mix reagents in a ratio of 70% Reaction Buffer to 30% TdT Enzyme. To prepare, add in a fresh microcentrifuge tube:

77 µL	Reaction Buffer (90427)
33 µL	TdT Enzyme (90418)
110 µL	Total

Mix well by vortexing. This reagent may be prepared in advance and stored on ice for no more than 6 hours. This amount is sufficient to treat two samples of 1-2 x 10^6 cells. See Sec. IV. *Appendix, TECH NOTE #1: Reagents* and *TECH NOTE #10: Controls.*

Working Strength Stop/Wash Buffer

Prepare working strength Stop/Wash Buffer by adding:

1 mL	Stop/Wash Buffer (90419)
34 mL	dH_2O
35 mL	Total

This reagent may be prepared in advance and stored in a glass or plastic container at 4°C for up to 1 year. Use a fresh aliquot for each experiment.

Controls for Flow Cytometry

Calibrators Needed

Three types of control samples are recommended for flow cytometry to aid in setting up electronic compensation and quadrant statistics. For bicolor experiments, these are: 1) cells stained with ApopTag® Fluorescein only; 2) cells stained with PI only; and 3) unstained cells. See Sec. IV. *Appendix, TECH NOTE #9: Controls.* These can be prepared by modifying the protocols below.

Preparation

Positive control cells for use in the recommended calibration tests can be prepared by fixing a cell suspension in 1% paraformaldehyde in PBS, pH 7.4 (See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation*) for 30 minutes on ice. Positive control samples can be prepared by inducing cells in suspension cultures, which should contain a mixture of viable, apoptotic and necrotic cells. Some examples are: 1) Jurkat cells treated with 300 ng/mL of anti-Fas monoclonal antibody CH-11 for 5 hours; 2) U-937 cells cultured with 2-4 ng/mL of TNF for 2-3 hours or with 4 µg/mL of camptothecin for 4 hours; and 3) murine thymocytes left in culture media for 1-3 hours.

Protocols

Fluorescent Staining of Cell Suspensions

Note: *Determination of cell count is important. The signal may be decreased if greater than 4 x 10^6 cells are used. Avoid moving cells between tubes: use of a single 15 mL screw-cap tube for all steps is recommended to prevent cell loss.*

Note: *Centrifugation steps should be performed at 400 x g for 5 minutes. Sperm cells should be centrifuged at 1200 x g for 10 min.*

See TECH NOTE #13: *Optional stopping points.*

See TECH NOTE #14*: Morphological confirmation of apoptosis.*

1. **Induce cells and sample at time points according to protocol. Count cells.**

2. **Fix Cells**
   a. Resuspend 1-2 x 10^6 cells in 0.5 mL of PBS.
   b. With a Pasteur pipette, add the suspension into 5 mL of 1% PARAFORMALDEHYDE in PBS, pH 7.4, on ice.
   c. Fix for 15 minutes on ice.
   d. Spin down the cells.
   e. Resuspend in 10 mL of ice-cold PBS.
   f. Spin down the cells.
   g. Resuspend in 70% ice-cold ETHANOL; keep at -20°C for a least 1-2 hours (up to 6 months).

3. Assay Set-Up

a. Prepare an ice bath for holding WORKING STRENGTH TdT ENZYME.

b. Pre-warm an incubator to 37°C.

c. Prepare enough WORKING STRENGTH TdT ENZYME.

d. Prepare WORKING STRENGTH STOP/WASH BUFFER.

e. Prepare 0.1% (w:v) TRITON X-100 in PBS. (See Sec. IV. *Appendix: Reagent Preparation*)

f. Prepare PROPIDIUM IODIDE (PI) COUNTERSTAINING SOLUTION. (See Sec. IV. *Appendix: Reagent Preparation*)

g. Pretreat assay tubes with 5% BSA in PBS for 1 minute, and then drain well.

4. Wash Fixed Cells

a. Spin down 1-2 x 10^6 fixed cells per sample.

b. Add 1 mL PBS and vortex gently.

c. Spin down the cells and remove the supernatant.

d. Repeat steps b and c.

5. Apply Equilibration Buffer

Resuspend the cells in 75 µL/5 cm^2 of EQUILIBRATION BUFFER.

6. Apply Working Strength TdT Enzyme

a. Spin down the cells.

b. Remove the supernatant.

c. Resuspend the cells in 50 µL of WORKING STRENGTH TdT ENZYME.

d. Incubate in a water bath for 30 minutes at 37°C.

e. At 15 minutes of incubation, resuspend cells that have settled to the bottom of the tube.

Note: *Avoid exposure to light.*

7. **Stop/Wash**

   a. Add 1.0 mL of WORKING STRENGTH STOP/WASH BUFFER directly to the cell suspension.

   b. Spin down the cells and remove the supernatant.

   c. Resuspend cells in 1 mL of WORKING STRENGTH STOP/WASH BUFFER.

   d. Spin down the cells and remove the supernatant.

8. **DNA Staining**

   a. Add 1.0 mL of PROPIDIUM IODIDE STAINING SOLUTION.

   b. Incubate for 15 minutes at room temp.; avoid exposure to light.

9. **Collect Data (for example: using a Becton Dickinson FACScan flow cytometer equipped with a 15 mW argon ion laser). See Sec. IV. *Appendix, TECH NOTE #7: Required fluorescence filters.***

   a. Measure GREEN fluorescence of FLUORESCEIN.

   b. Measure RED fluorescence of PI.

   c. Generate a log FL1 vs linear FL2 dot plot (See Figure 2).

   d. The majority of the negatively stained cells will normally occur within the first log decade of the FL1 (Y) axis. Position the horizontal cursor in the gap between this population and the fluorescein positive cells. Events falling above the horizontal cursor should be counted as apoptotic cells.

   e. PI intensity variations on the FL2 axis are usually interpreted as several populations. These are, from left to right: the debris, the apparent sub-G1 (compromised) cells, the G0/G1 cells, the S cells and G2/M phase cells. The statistical analysis of events by cell cycle phase, if desired, is usually performed on a histogram of FL2 signals with the use of a special purpose software package.

Triple-labeling of Cell Suspensions

This protocol is adapted from reference 33 and R. Sgonc, personal communication. It requires a more advanced level of understanding of flow cytometry techniques. Calibration of a flow cytometer for triple color detection with proper compensation for spectral overlap will then be necessary. The scope

of this document does not allow for a thorough discussion of the relevant calibration methods and the interpretation of data for a tricolor experiment.

Note: *Determination of cell count is important. The signal may be decreased if greater than 4 x 10 ^{6} cells are used. Avoid moving cells between tubes: use of a single 15 mL screw-cap tube for all steps is recommended to prevent cell loss.*

Note: *Centrifugation steps should be performed at 400 x g for 5 minutes.*

Note: *Working concentrations of antibodies to surface marker 1 and surface marker 2 should be predetermined before running this protocol.*

See Sec. IV. *Appendix, TECH NOTE #6: Required fluorescence filters.*

1. Induce cells and sample at time points according to protocol. Count cells.

2. Wash Cells

a. Spin down the cells and resuspend in ice-cold PBS/1% BSA.

b. Repeat step a, adjusting to $1x10^{7}$ cells/mL.

3. Apply PE-Labeled Antibody to Surface Marker 1

a. Spin down 100 µL of cells and resuspend in 100 µL ice-cold PBS/ 1% BSA, containing the working concentration of a phycoerythrin conjugated antibody to first surface marker of interest.

b. Incubate on ice for 30 minutes.

c. Spin down the cells at 400 x g for 5 minutes and resuspend in 100 µL ice-cold PBS/1% BSA.

4. Apply Biotin-Labeled Antibody to Surface Marker 2, and then Avidin

a. Spin down the cells and resuspend in 100 µL ice-cold PBS/1% BSA, containing the working concentration of a biotin-labeled antibody to second surface marker of interest.

b. Incubate on ice for 30 minutes.

c. Spin down the cells and resuspend in 100 µL ice-cold PBS/1% BSA.

d. Spin down the cells and resuspend in 50 µL ice-cold PBS/1% BSA containing streptavidin-labeled Cy-Chrome. Incubate on ice for 30 minutes.

e. Spin down the cells and resuspend in 100 µL ice-cold PBS/1% BSA.

5. Fix Cells

a. Spin down the cells at 400 x g for 5 minutes and resuspend in 100 µL ice-cold PBS/1% BSA.

b. Add 100 µL of 1% PARAFORMALDEHYDE in PBS. See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

c. Incubate at room temp. for 30 minutes in a horizontal shaker.

d. Spin down the cells and resuspend in 100 µL ice-cold PBS/1% BSA.

6. Permeabilize Cells

a. Spin down the cells and resuspend in 100 µL 0.1% Triton X-100 in PBS.

b. Incubate on ice for 2 minutes.

c. Spin down the cells and resuspend in 100 µL ice-cold PBS/1% BSA.

7. Label with Working Strength TdT

Continue as in the Bicolor Fluorescent Staining Protocol performing steps 5-8.

8. Collect Data (for example: using a Becton Dickinson FACScan Flow Cytometer equipped with a 15 mW argon ion laser). See Sec. IV. *Appendix, TECH NOTE #6: Required fluorescence filters.*

a Measure GREEN fluorescence of FLUORESCEIN.

b. Measure RED fluorescence of PI.

c. Measure ORANGE fluorescence of R-PHYCOERYTHRIN.

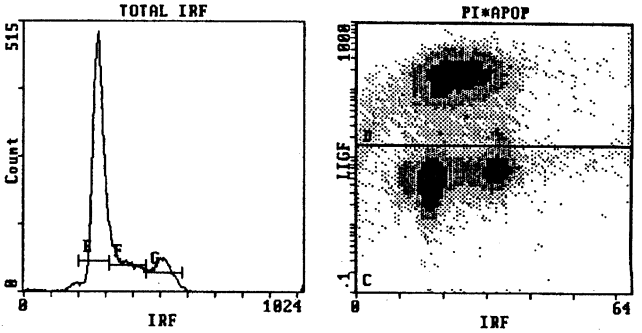
d. Measure VIOLET fluorescence of CY-CHROM.

Calibration Runs for Bicolor Flow Cytometry

Flow cytometric analysis of ApopTag® Fluorescein and PI counterstained cells is used to correlate the DNA content of cells with apoptosis. This allows for determination of the cell cycle phase of apoptosis in proliferating cells. Separate, single-labeled samples should be prepared and used for instrument calibration tests. The instrument must first be set up to achieve proper electronic compensation, so as to exclude any overlap of the two emission spectra.

However, optimal voltage settings will vary between instruments. We recommend that individually stained cells should be used in runs to calibrate these settings, so that electronic compensation can be used to adjust for any spectral overlap. The scope of this document does not allow for a thorough discussion of the relevant calibration methods. An example of a typical experiment is shown in Figure 2.

Figure 2: HL-60 Cells Labeled by the Bicolor Protocol



Apoptotic HL-60 cells were labeled with ApopTag Fluorescein and PI according to the Bicolor Fluorescent Staining Protocol. Exponentially growing HL-60 cells (of an inducible strain) were induced using the DNA topoisomerase I inhibitor, camptothecin. The cells were analyzed on a Coulter Epics Flow Cytometer. A histogram plot of PI fluorescene (left) indicated the G1 (labeled E), S (labeled F) and G2/M (labeled G) populations. A scatterplot of PI (X axis) vs. FITC (Y axis) on ungated cells (right) resolved apoptotic cells mostly in S phase. Thus, induction was specific to the S phase of the cell cycle (17).

IV. APPENDIX

Reagent Preparation

1. 1% Paraformaldehyde in PBS

Combine 1 mL of paraformaldehyde (methanol-free) and 15 mL of PBS, pH 7.4. See Sec. IV. *Appendix, TECH NOTE #2: Fixatives and fixation.*

2. 10X Phosphate Buffered Saline, pH 7.4 (PBS)

To make 1 L, dissolve the following in 800 mL dH_20:

Na_2HPO_4	55.0 g
NaH_2PO_4	13.5 g
NaCl	117.0 g

Adjust the pH to 7.4 using NaOH or HCl and add dH_20 to a final volume of 1000 mL.

3. PBS + 1% (w:v) BSA

Dissolve 1 g of BSA in PBS, pH 7.4, for a final volume of 100 mL.

4. DN buffer (30 mM Tris Base, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT)

a. Prepare 1 M Tris base, pH 7.2. Dissolve 12.1 g of Tris base in 80 mL of dH_2O. Adjust the pH to 7.2 with concentrated HCl and add dH_2O to a final volume of 100 mL.

b. Prepare 1.0 M $MgCl_2$. Dissolve 20.3 g of $MgCl_2 \cdot 6H_2O$ in sufficient dH_2O for a final volume of 100 mL.

c. Prepare DN buffer. Combine 3 mL of 1.0 M Tris, pH 7.2, 400 µL of 1.0 M $MgCl_2$, 1.54 mg of DTT and sufficient dH_2O for a final volume of 100 mL.

5. 0.1% (w:v) TRITON X-100 in PBS (bicolor or triple-labeling protocols)

To prepare an amount sufficient for 40 cytometry samples using either protocol, mix the following: 1 mL of Triton X-100 (10% solution) and 99 mL of PBS, pH 7.4. This reagent may be prepared in advance and stored at 4°C for up to one month.

6. PI Staining Solution

PI (Propidium Iodide) is used as the counterstain for flow cytometry of fluorescein stained cells. To make an amount sufficient for 10 cytometry samples, dissolve: 50 ug Propidium Iodide and 10 mg RNase A (700 Kunitz Units) in 10 mL of PBS, pH 7.4. This reagent should be freshly prepared and kept on ice until use.

Tech Notes

TECH NOTE #1: Reagents

- Reagent temperature:
  a. Do not warm the stock reagents before dispensing them from containers.
  b. After dilution, warm the reagents or mixtures to room temperature just before application to the specimen.
- TdT enzyme viscosity:
  Pipette TdT more slowly because of its viscosity, when removing the reagent from a container.

TECH NOTE #2: Fixatives and fixation

- Pure formaldehyde is prepared from solid paraformaldehyde polymer by hydrolysis in water to monomeric formaldehyde. It is then stabilized by packaging in sealed ampules under nitrogen. For example, a 1% (w:v) solution equals a 1:16 dilution of methanol-free, 16% (w:v) formaldehyde in PBS, pH 7.4. This is the preferred fixative for cell suspensions and cultured cells (for either microscopy or flow cytometry).

- Effects of different fixations and pretreatments for use on single cells were described by A. Negoescu, et al (28a) and by F. Labat-Moleur, et al (28b).

TECH NOTE #3: Reducing time spent performing the protocol

- For a faster protocol, after pretreatment and quenching, specimens can be washed in deionized water. This will remove the need to wash with Equilibration Buffer before labeling with TdT and will give comparable results with most samples.

TECH NOTE #4: Notes on double-labeling for microscopy

- TdT end-labeling has been used in combination with an *in situ* hybridization assay. In this method, hybridization was done following TdT labeling (45).

- A double-labeling fluorescence technique is possible. Choose a fluorescent detection system for an antibody that recognizes an antigen of interest, which provides good contrast with fluorescein or rhodamine.

TECH NOTE #5: Silanized slides

- In order to avoid detachment of tissue sections during processing, we highly recommend the use of silanized glass slides.

TECH NOTE #6: Required fluorescence filters

(Also see TECH NOTE #15: Fluorescent counterstains).

- For (Immuno)fluorescence microscopy
  a. Both the FITC signal and the Propidium Iodide (PI) counterstain can be viewed with a "long pass" filter for FITC (ex. 490 nm & em. 520 nm); this filter allows sufficient PI signal to "bleed through". A "dual pass" filter, designed for viewing both FITC and PI, would allow more red light through, possibly competing with and decreasing the FITC signal. As PI binds reversibly to DNA, a PI signal can be modulated up or down by washing the sample and reapplying PI at another concentration.

  b. Photobleaching will cause the signal to fade in proportion to the time and intensity of exposure to excitation light.

- For flow cytometry
  a. In the bicolor protocol, measure red fluorescence of PI at >620 nm using linear amplification.

  b. In both flow cytometry protocols, measure FITC fluorescence as a green signal (530 nm peak fluorescence) by the FL1 detector through a band pass filter (530 +/- 15 nm) using logarithmic amplification.

  c. In the triple-labeling protocol, measure R-phycoerythrin as an orange signal (575 nm peak fluorescence) by the FL2 detector through a band pass filter (585 +/-21 nm) using logarithmic amplification.

d. In the tricolor protocol, measure Cy-Chrom as a violet signal (peak fluorescence 670 nm) by the FL3 detector through a long pass filter (>650 nm) using logarithmic amplification.

TECH NOTE #7: Containers

- Wash and solvent exchange steps are best performed in coplin jars.

- A humidified chamber is required for the incubation steps. One can be constructed as follows using a clear plastic tray with a lid. Soak several paper towels in water and place them at the bottom of the tray. Place two pipettes across the towels. Place the slides across pipettes. Put the lid on top and place the chamber in a 37°C incubator.

TECH NOTE #8: Plastic coverslips

- Plastic coverslips can be used to assure that a constant volume of solution is applied per unit of specimen area. However, their handling time slows down the protocol.

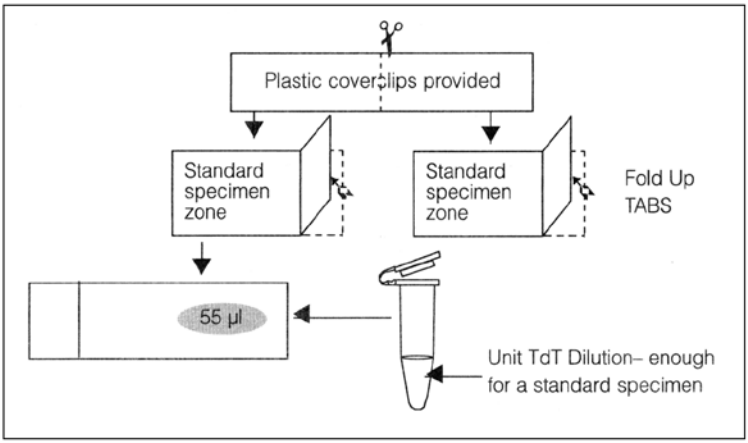
- Each square centimeter of plastic coverslip will require the volume of reagent indicated in Table 3, so that the reagent volume applied per unit of tissue area can be held constant. The surface to be covered is always equal to the area of the plastic coverslip, not the area of the specimen.

- Plastic coverslips may be trimmed to any desired size and shape. The kit's yield of specimens will be reduced if larger than standard coverslips are used.

- The plastic coverslips can be used during incubation steps with WORKING STRENGTH TdT.

- A basic coverslip method is described as follows:
  To make a pair of "standard area" (~ 5 cm^2) specimen coverslips, cut a plastic coverslip (provided) into two equal halves, and fold up a 1 cm handling tab across the width, then crease sharply (See Figure 3).

Figure 3: Unit TdT Dilution and Plastic Coverslips Use



- Drain one slide for approximately 10 seconds, and then tap off drops on a paper towel on the benchtop. Blot back and sides of the slide with a folded wipe. Carefully blot the area around the tissue section or cells, or else vacuum up solution using a pipette attached to an aspirator vacuum.

- Apply reagent solution to one end of the area to be covered, using a dropper bottle or pipette as required.

- Grasp the plastic coverslip by the handling tab and touch its opposite end to the droplet of reagent on the slide. Slightly arching the coverslip, roll it slowly downward, causing the solution to spread by capillary action. If solution does not spread evenly, tilt the slide until the flow reaches all edges.

- Apply plastic coverslips to microscope slides so as to minimize trapped air bubbles, which may cause variable enzyme reaction or detection.

- Place the slide across the pipettes, face-up and level, inside the humidified chamber. The slide edges should not touch anything so as to prevent drainage of the reagent.

TECH NOTE #9: Controls

- A positive control sample can be prepared from any sample by treating with DNase I by (3, 13), as follows.
    1. Pretreat section with DN Buffer (30 mM Trizma base, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT) at room temp. for 5 minutes.

2. Dissolve DNAse I in DN Buffer to a final concentration of 1.0-0.1 μg/mL (specific activity is 10,000 U/mL - 1,000 U/mL).

3. Apply DNAse solution and incubate for 10 minutes at room temp.

4. Rinse with 5 changes of dH_2O for 3 minutes each change.

- Chemicon recommends using DNase I from Sigma (D7291) or Worthington Biochemical (LS06333). As the consistency and prior processing of tissues will differ, testing a range of conditions including proteinase K digestion is recommended.

- Negative Controls
  a. A negative control or sham staining can be performed without active TdT but including proteinase K digestion to control for nonspecific incorporation of nucleotides or for nonspecific binding of enzyme-conjugate. Water or Equilibration Buffer can be substituted for the volume of TdT ENZYME reagent.

  b. Inactive WORKING STRENGTH TdT can be prepared by adding to the regular TdT mixture, a 5% (v:v) dilution from the bottle of Stop/ Wash Buffer concentrate, to chelate the divalent cationic enzyme cofactor.

TECH NOTE #10: Additional pretreatment procedures

- Besides protease, there are two other possible pretreatments for exposing the DNA.

- In the heating method, the slide is placed in 10 mM citrate buffer, pH 3.0 - 6.0, in a coplin jar, and gently boiled for 3-5 cycles of 3 minutes each in a microwave oven (28b, 37). Refill with fresh buffer between cycles. Do not let the sample dry out. A pressure cooker or an autoclave can be used instead of a microwave. Let the solution sit on the bench until it reaches a warm but not room temp. before proceeding.

- In the detergent pretreatment method, 0.5% TRITON X-100 can be applied for 10 minutes. (41).

TECH NOTE #11: Sample handling

- Do not let the specimen go dry by evaporation when changing solutions. Remove the slides from the final wash and tap off excess water, then blot or aspirate around the section, and promptly apply the next reagent. If there are

many samples to be processed, slides can be treated at fixed time intervals (e.g. every 20-30 seconds) and immediately placed in a humid chamber. Incubations can then be terminated at similar intervals to maintain a constant incubation time.

TECH NOTE #12: Use of xylene

- Keep organic solvents tightly capped when not in use.

TECH NOTE #13: Optional stopping points

- There are several optional stopping points for temporary storage during sample processing. These are:

  In the microscopy protocols:

  a. Slides may be left in EQUILIBRATION BUFFER or water for up to 60 minutes at 4°C to room temp.

  b. After incubating in working strength TDT ENZYME, slides can be washed for 5 minutes in STOP-WASH SOLUTION, and then immersed in 70% EtOH in a coplin jar and stored at -20°C for at least 3 days. Before continuing with the protocols, samples should be washed with three changes of PBS for 2 minutes per change.

  In the flow cytometry protocols:

  a. After placing the cells in 70% ethanol, they can be stored at -20°C for at least 3 months.

  b. After PI is added, the tube containing the cells can be wrapped in foil and stored at 4°C for 2-3 days.

TECH NOTE #14: Morphological confirmation of apoptosis

- To confirm morphological apoptosis, a sample of unsorted live positive cells can be checked in a phase contrast microscope. Apoptotic cells appear phase-dark and have pyknotic nuclei. Using a fluorescence microscope, live cells can be stained for phosphatidylserine externalization on membrane blebs with the Annexin V FITC protein; or they can be stained to examine for marginated or segmented chromatin morphology with a membrane permeant DNA-binding dye such as Hoechst 33342 (10).

TECH NOTE #15: Fluorescent counterstains

- PI or DAPI staining intensity, as visualized by microscopy, is affected by variations in these factors: the tissue type, the fixation method (type, concentration, freshness and time), tissue pretreatments (proteinase or other), the stain concentration, the light filter used, and photobleaching during imaging. The optimal counterstain concentration will result in fluorescence intensity nearly equal to that of the primary stain. In addition, the fluorescence signal per cell may be less intense when more concentrated samples are tested (i.e. more cells/mL) by flow cytometry.

TECH NOTE #16: Fixation using plastic supports

a. If adherent cells do not remain on the support during the procedure, the cells may be air dried onto the support prior to fixation in 1% PARAFORMALDEHYDE. However, it is important to remember that apoptosis in adherent cell cultures can result in detachment from the substrate.

Related Products

Table 4: ApopTag® TUNEL Apoptosis Detection Kits

Cat #	Product	Quantity
S7100	ApopTag® Peroxidase In Situ Apoptosis Detection Kit	40 tests
S7101	ApopTag® Peroxidase Plus In Situ Apoptosis Detection Kit	40 tests
S7110	ApopTag® Fluorescein In Situ Apoptosis Detection Kit	40 tests
S7111	ApopTag® Fluorescein Plus In Situ Apoptosis Detection Kit	40 tests
S7165	ApopTag® Red In Situ Apoptosis Detection Kit	40 tests

Table 5: Apoptosis Reagents

Cat #	Product	Quantity
S7114	Antifade Solution	1 mL
S7106	ApopTag® Equilibration Buffer	15 mL
S7115	ApopTag® Positive Control Slides	5 slides
S7105	ApopTag® Reaction Buffer	1 mL
S7108	ApopTag® Stop/Wash Buffer	20 mL
S7107	ApopTag® TdT Enzyme	300 mL
S7113	DAPI/Antifade Solution	1 mL
S7112	Propidium Iodide/Antifade Solution	1 mL
S7109	Propidium Iodide Solution	1 mL

Positive Control Slides are supplied in the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (S7101), ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (S7111), and by the package (S7115). The slides contain unstained rat mammary glands obtained at the fourth day after weaning (36), which were fixed for 18 hours in 10% neutral buffered formalin. After embedding in paraffin, 5 micron thick sections were cut from the middle of the tissue and mounted on silanized slides.

Table 6: Caspase Assays

Cat #	Product	# of Tests
APT403	CaspaTag™ Caspase 3 *In Situ* Assay Kit, Fluoresein	100 tests
APT400	CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluoresein	100 tests
APT500	CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Sulforhodamine	100 tests
APT503	CaspaTag™ Caspase 3 *In Situ* Assay Kit, Sulforhodamine	100 tests

Table 7: DNA Fragmentation Analysis (Ligation)

Cat #	Product	Quantity
S7200	ApopTag® Peroxidase *In Situ* Oligo Ligation (ISOL) Apoptosis Detection Kit	40 tests

Table 8: Mitochondrial Membrane Permeabilization

Cat #	Product	Quantity
APT142	MitoLight® Mitochondrial Apoptosis Detection Kit	25 tests
APT242	MitoLight® Mitochondrial Apoptosis Detection Kit	100 tests

V. REFERENCES

Internet Sites

Chemicon Corporation: www.chemicon.com

APOPTOSIS Online: The Apoptosis Information & Communication Center at www.apopnet.com

Purdue Cytometry Mailing List: www.cyto.purdue.edu/hmarchive/Cytometry/index.html

PubMed: www.ncbi.nlm.nih.gov/pubmed

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