



Monoclonal Antibody to ssDNA (F7-26)

ALX-804-192-L001

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→ For Prod. No. ALX-804-192-R200 please use this Manual and adjust quantities correspondingly!

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Apoptosis Assay With Monoclonal Antibodies to Single-Stranded DNA

New protocol for formalin-fixed tissues

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1. Principles of the procedure

Apoptosis assay described herein is based on the increased sensitivity of DNA in apoptotic cells to thermal denaturation. In this method DNA is denatured by heating in the presence of formamide and stained with monoclonal antibody (MAb) F7-26 specific to single-stranded DNA (ssDNA).

The protocols presented below are based on the **modification** of the previously described procedure for the detection of apoptotic cells with MAb specific to ssDNA (1-3). The main part of this modification is the heating cells and tissue sections in the presence of **formamide**, instead of PBS-MgCl₂ solution (4,5). The **formamide-MAb** procedure has the following advantages:

1. Selective denaturation of DNA in apoptotic cells after the heating at a relatively low temperature (56-75°C instead of 100°C).
2. Specific staining of apoptotic cells in thin sections of **formalin-fixed tissues** prepared with routine histological techniques (previously described procedure produced best results after methanol fixation). Indeed, this new protocol produced best and stable results after formalin fixation (see **color micro-photographs** below).

Groos et al. (6) evaluated different techniques for the in situ detection of apoptosis in human and rat small intestine and concluded that the antibody detecting formamide-denatured DNA was both suitable and reliable. In comparison with this the TUNEL assay was less reliable and had to be adjusted for each specimen on the basis of the finding produced by other techniques.

This procedure specifically **stains condensed chromatin** of apoptotic cells. Since chromatin condensation is a definite marker of apoptosis (7), the histochemical identification of condensed apoptotic chromatin by anti-ssDNA MAb makes possible specific detection of apoptotic cells. The specificity of the assay is based on the high sensitivity of DNA in condensed chromatin of the apoptotic cells to thermal denaturation (1,8). In the presence of formamide, only in apoptotic nuclei DNA denatures and binds the **anti-ssDNA MAb F7-26** after heating at 56-75°C (4,5).

Importantly, formamide induces DNA denaturation in the apoptotic cells, and did not affect the stability of DNA with single- or double-stranded DNA breaks in the absence of apoptosis (4,5). As a result, formamide-MAb procedure specifically stains apoptotic cells and clearly **distinguishes apoptosis from necrosis**. Independence of DNA breaks is critical, because techniques that detect double-stranded DNA breaks, such as TUNEL, are not specific for apoptosis (9-17). Indeed, necrotic cells in three models of necrosis were brightly stained by TUNEL, but were completely negative by formamide-MAb staining (4,5). (see Figure 2 below).

The following facts demonstrate that selective denaturation of DNA in apoptotic nuclei in the presence of formamide is **not related to DNA breaks**: 1. Necrotic cells with a high level of double-stranded DNA breaks, demonstrated by TUNEL,

do not react with the anti-ssDNA MAb. 2. Apoptotic cells with very low and high levels of DNA fragmentation are stained by formamide-MAb technique with a similar intensity.

Nuclei with single-stranded DNA breaks induced by oxygen radicals do not bind the MAb after the heating in formamide, although alkaline denaturation detects DNA breaks in these nuclei (4,5).

These data indicate that DNA denaturation in apoptotic cells in the presence of formamide is induced by the changes in chromatin associated with the condensation per se independently of DNA breaks. As histones are known to stabilize DNA against thermal denaturation (18), it is possible that changes in DNA-histone **interactions** in condensed chromatin of the apoptotic nuclei may be responsible for the increased DNA sensitivity to thermal denaturation. Indeed, digestion of histones occurs during apoptosis (19,20) and increased immune-reactivity and extractability of histones precedes appearance of DNA breaks (21).

Monoclonal antibody F7-26 used in this procedure was generated against calf thymus ssDNA and selected on the basis of reactivity with mouse apoptotic cells. This antibody specifically reacts with **deoxycytidine** and requires for its binding ssDNA of at least 25-30 bases in length (2,22). MAb F7-26 is **not species-specific** and could be used to detect apoptotic cells in various species (mouse, rat and human cells were tested).

The selective denaturation of apoptotic DNA at 56°C in the presence of formamide made possible adaptation of formamide-MAb assay for the detection of apoptotic cells in microtiter plates. The **ApoptosisELISA** involves growth of cells in 96-well plates, treatment of attached cells with formamide and one-step immunostaining of the denaturation DNA with a mixture of anti-ssDNA MAb and peroxidase-conjugated anti-mouse IgM (5). A near linear increase in absorbance was seen as the number of apoptotic cells per well increased from 500 to 5000.

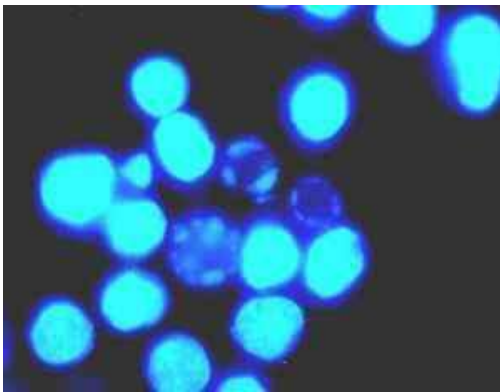
The utility of formamide-MAb ELISA for **drug screening** was demonstrated by its ability to distinguish anticancer drugs from toxic chemicals, to predict drug synergism and to detect selective toxicity to cancer cells (23,24). The **sensitivity** of apoptosis ELISA was similar to the sensitivity of long-term cell survival assay and significantly higher than that of MTT and SRB growth inhibition assays.

2. Applications

A. FLUORESCENCE MICROSCOPY

In cultures of breast cancer MB-MDA-468 cells treated with protein kinase inhibitor staurosporine, only cells with chromatin condensation typical of apoptosis were labeled with antibody to ssDNA. In contrast cells with dispersed chromatin in staurosporine-treated cultures or interphase nuclei of untreated cells did not react with the MAb (Fig 1A,B).

A. DNA fluorochrome DAPI



B. MAb to ssDNA

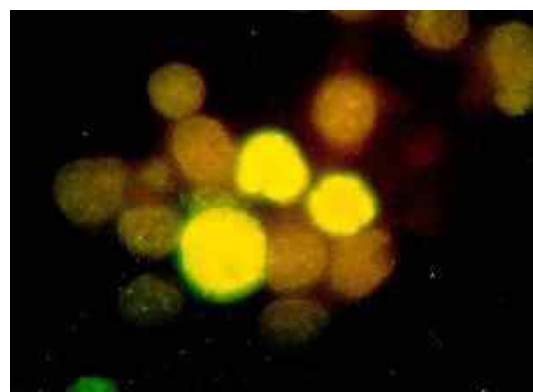
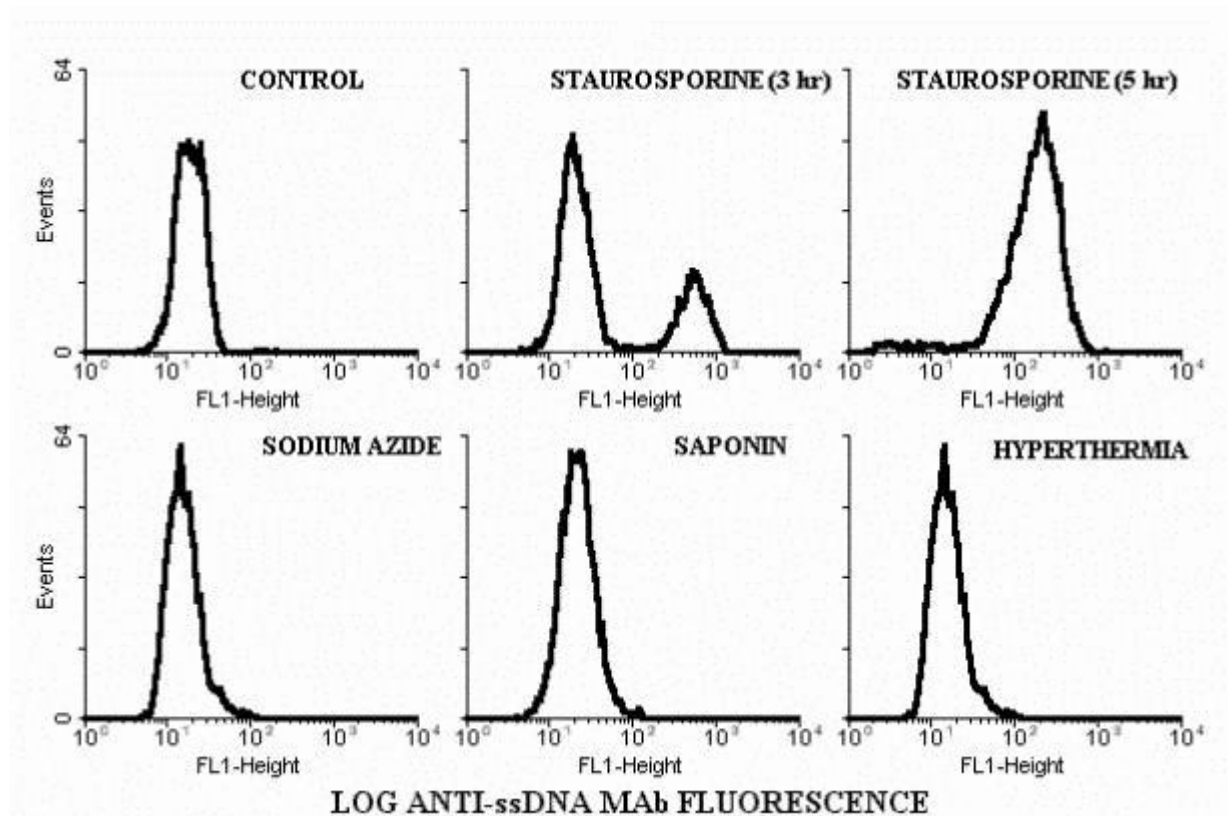


FIGURE 1. Fluorescence microphotographs of MB-MDA-468 cells treated with staurosporine, heated in formamide, stained with MAb F7-26 and counterstained with DNA fluorochrome DAPI. The same field is shown after UV excitation for DAPI (left panel) and visible light excitation for fluorescein-labeled antibody (right panels). Note that only **3 apoptotic cells** with chromatin condensed at the nuclear periphery are stained with the MAb. Magnification, 1000x

B. FLOW CYTOMETRY

The ability of formamide-MAb technique to distinguish between apoptosis and necrosis is demonstrated by flow cytometric analysis of cells treated with apoptosis and necrosis inducing agents. Fluorescence profiles of cell heated in the presence of formamide and stained with MAb to ssDNA are demonstrated in Fig. 2A. Two subsets of cells were observed in cultures treated with staurosporine: cells with low fluorescence similar to that of the untreated cells and brightly fluorescent apoptotic cells. In contrast to the high fluorescence intensity of apoptotic cells, the necrotic cells in cultures treated with the inhibitor of respiratory chain sodium azide or with hyperthermia had fluorescence profile similar to that of untreated control cells. In contrast, TUNEL stained not only apoptotic, but also necrotic cells.

A. MAB TO ssDNA.



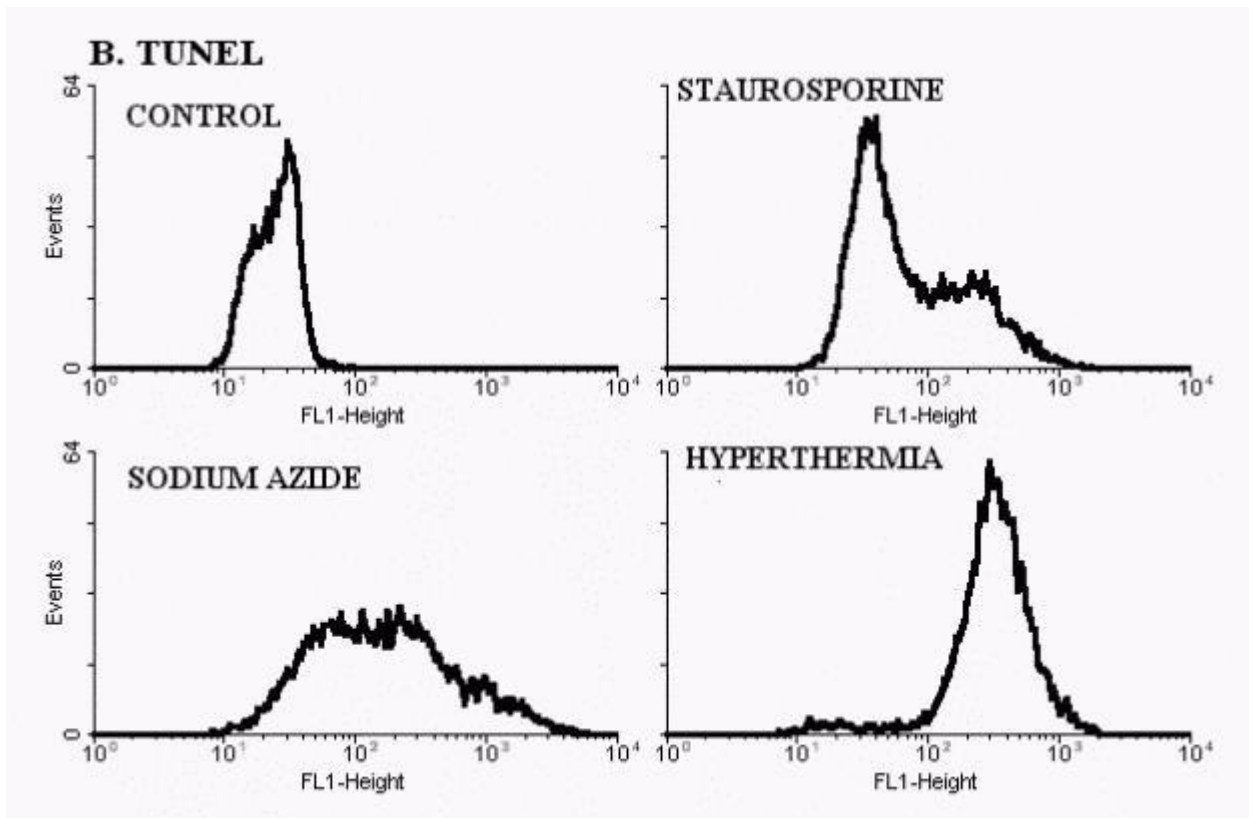


FIGURE 2.

A. Fluorescence distributions of MB-MDA-468 cells heated in formamide and stained with MAb F7-26 generated on a flow cytometer. Apoptosis was induced by staurosporine and necrosis was induced by sodium azide or hyperthermia. Note that apoptotic cells are intensely stained with the MAb, while fluorescence profiles of necrotic and control cells are similar.

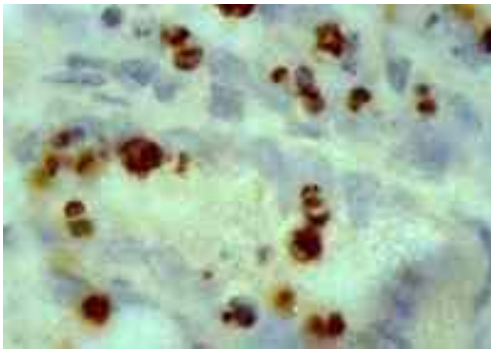
B. Fluorescence distributions of MB-MDA-468 cells stained with TUNEL generated on a flow cytometer. Apoptosis was induced by staurosporine and necrosis was induced by sodium azide or hyperthermia. Note that apoptotic and necrotic cells are stained.

C. DETECTION OF APOPTOSIS IN TISSUE SECTIONS

Small intestine of mice treated with DNA synthesis inhibitor, hydroxyurea and thymus of mice treated with hydrocortisone were fixed in formalin. Thin sections of tissues prepared with standard histological techniques were heated in formamide and stained with MAb F7-26.

Apoptotic cells with condensed chromatin and fragmented nuclei in the crypts of the small intestine from hydroxyurea-treated mice were stained with the MAb (Fig. 3A). There was no staining of cells in the crypts of untreated animals (Fig.3B). Since crypts contain large number of S-phase and mitotic cells these data indicate that this MAb does not stain normal proliferating cells.

A. Hydroxyurea



B. Control

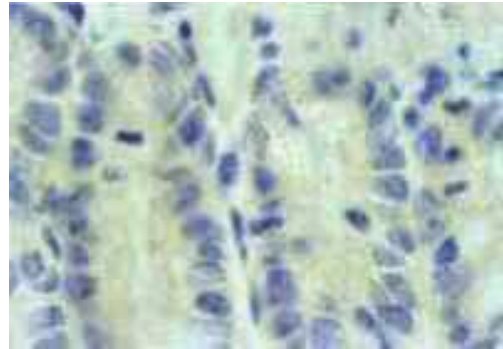
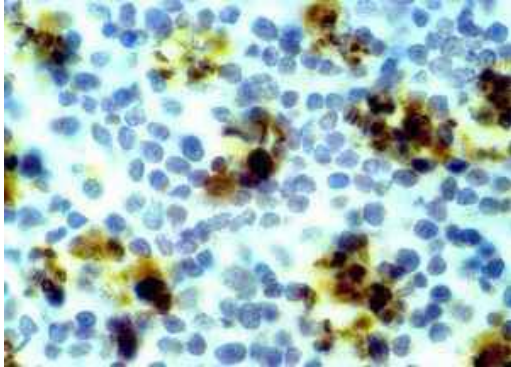


FIGURE 3. Photomicrographs of small intestine crypts of a mouse treated with hydroxyurea (**A**) and small intestine crypts of a control animal (**B**), treated. Sections of formalin-fixed tissues were heated in formamide, stained with MAb F7-26 and counterstained with hematoxylin. Brown nuclei - antibody stained apoptotic cells with condensed chromatin and fragmented nuclei.

Multiple foci of MAb positive apoptotic cells were detected in the thymus of mice treated with the glucocorticoid, while only rare stained cells were seen in the thymus of untreated animals (Fig.4A,B).

A. Hydrocortisone



B. Control

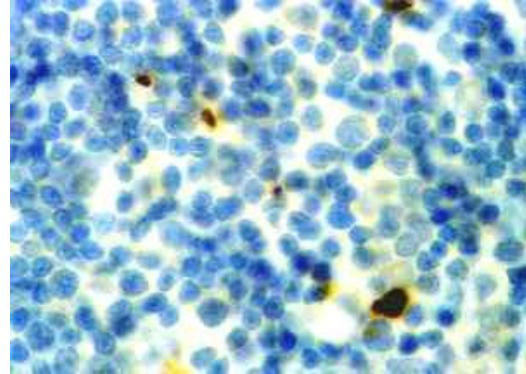


FIGURE 4. Photomicrographs of thymus of a mouse treated with hydrocortisone (**A**) and thymus of a control animal (**B**). Sections of formalin-fixed tissues were heated in formamide, stained with MAb F7-26 and counterstained with hematoxylin. Brown nuclei - antibody stained apoptotic cells with condensed chromatin and fragmented nuclei.

3. Protocols

A. STAINING OF TISSUE SECTIONS

I. Paraffin sections

Procedure includes the following steps:

1. Preparation of thin sections from **formalin-fixed tissues** using standard histological techniques.
2. Permeabilization in **saponin** and treatment with **Proteinase K**.
3. Heating in **formamide**.
4. Staining with anti-ssDNA monoclonal **antibody F7-26** and peroxidase conjugated **anti-mouse IgM**.
 1. Fix tissues in 4% or 10% neutral buffered formalin (or 4% paraformaldehyde in PBS) at +4°C for 24 hours, dehydrate and embed in paraffin. **Important: fix in cold formalin.**
 2. Cut 3-4 micron sections from paraffin blocks and attach sections to superfrost/plus slides. Stain freshly cut sections.
 3. Deparaffinize tissue sections in xylene, wash sequentially in 100%, 95%, 70% ethanol and PBS.
 4. Incubate slides in PBS containing 0.2 mg/ml saponin and 20 µg/ml Proteinase K at room temperature for 20 min.
 5. Wash slides in distilled water
 6. Transfer slides into coplin jar containing 40 ml of 50% formamide (v/v distilled H₂O) preheated in water bath to 56-60° C. Incubate slides in coplin jar with formamide for 20 min in water bath. **Important: temperature of formamide solution inside the jar should be 56° C.**
 7. After the heating, transfer slides into ice-cold PBS for 5 min.
 8. Quench endogenous peroxidase in 3% hydrogen peroxide for 5 min. Rinse in PBS.
 9. Treat sections with 3% non-fat dry milk (Carnation) for 20 min at 37 C to block non-specific antibody binding. Rinse in PBS. To prepare blocking solution dissolve non-fat dry milk powder in distilled water and preheat to 37 C.
 10. Apply 100 µl of monoclonal antibody F7-26 to the slide. Incubate at room temperature for 30 min and rinse in PBS. To prepare working concentration of the antibody, add 9 ml of 1% non-fat dry milk in PBS to the vial containing 100 µg of F7-26. The diluted antibody should be aliquoted and stored frozen at -20 C.
 11. Apply 100 µl of peroxidase-conjugated anti-mouse IgM, incubate 30 min and rinse with PBS. To prepare working concentration of the antibody, dilute peroxidase-conjugated rat monoclonal anti-mouse IgM (Zymed, cat # 04-6820) 1:100 in PBS. Use only **freshly prepared** solution. We recommend peroxidase-conjugated anti-IgM because biotin-avidin systems may produce non-specific cytoplasmic staining in

some tissues. Signal intensity was strong without biotin-avidin amplification. Sigma peroxidase-conjugated goat anti-mouse IgM (cat.#A8786) also could be used as a secondary antibody.

12. Apply chromagen solution (DAB), counterstain with hematoxylin and mount.

II. Frozen Sections

Two protocols are recommended for frozen sections:

I.

1. Fix tissue in **cold** 4% paraformaldehyde in PBS for 18-24 hours.
2. Cut frozen sections.
3. Immerse slides with sections into 80% methanol in PBS for 30 min
4. Air-dry fixed sections and store dried at room temperature.
5. Immerse dried sections in saponin / Proteinase K solution as in step 4 for paraffin sections and proceed as protocol described above.

II.

1. Cut frozen sections from fresh tissue
2. Fix sections in 4% paraformaldehyde in PBS for 10 min
3. Rinse in PBS
4. Immerse slides with sections into 80% methanol in PBS for 30 min
5. Air-dry fixed sections and store dried at room temperature.
6. Incubate slides with dried sections in PBS containing 0.2 mg/ml saponin for 20 min (Proteinase K is not used in this protocol)
7. Proceed to step 5 for paraffin sections.

B. STAINING OF ARCHIVAL FORMALIN-FIXED TISSUES

The standard protocol described above provides optimal results after fixation in cold formalin or paraformaldehyde. Fixation at room temperature or prolong fixation may inhibit staining, because excessive DNA-protein cross-links prevent formamide-induced DNA denaturation in apoptotic cells.

Additional protease (Pronase E) treatment is recommended for archival material if weak or negative staining is obtained with the standard protocol.

1. After Saponin/Proteinase K treatment rinse slides in PBS and Tris buffer (10 mM, pH 7.6)
2. Incubate slides in Pronase E (20 microg/ml Tris buffer) at room temperature for 20 min in Coplin jar. *Pronase E (Sigma Chemicals, cat.#P6911) is prepared as a stock solution 1 mg/ml Tris buffer and stored frozen.*
3. Rinse slides in distilled water and proceed to the heating in formamide as in standard protocol.

C. STAINING OF CELL SUSPENSIONS FOR FLOW CYTOMETRY AND FLUORESCENCE MICROSCOPY

Procedure includes the following steps:

1. Fixation in **methanol**.
 2. Heating in **formamide**.
 3. Staining with anti-ssDNA monoclonal **antibody F7-26** and fluorescein-conjugated **anti-mouse IgM**.
-
1. Resuspend cell pellets in 1 ml of cold PBS and slowly add 6 ml of methanol precooled to -20°C while vortexing.
 2. Store fixed cell at 15-20° C for 1-3 days before staining.
 3. Transfer 5×10^5 of fixed cells into 15 ml plastic tubes (Falcon 2096), centrifuge, remove supernatant.
 4. Resuspend pellet in 0.25 ml of formamide. Keep 5 min at room temperature.
 5. Immerse rack with tubes into water bath preheated to 75 degrees C for 10 min. *Circulating water baths are recommended for optimal heating.*
 6. After the heating, immediately transfer rack with tubes into room temperature water
 7. 2 ml of 1% non-fat dry milk in PBS to the tubes containing formamide, vortex and keep tubes at room temperature for 15 min.
 8. Centrifuge, remove supernatant, resuspend pellet in 100 microL of monoclonal antibody F7-26 and incubate at room temperature for 15 min. *To prepare working concentration of the antibody, add 9 ml of 5% fetal bovine serum in PBS to the vial containing 100 micro g of F7-26. The diluted antibody could be aliquoted and stored frozen at -20°C.*
 9. Add 1 ml of PBS, centrifuge and resuspend pellet in 100 microL of fluorescein-conjugated anti-mouse IgM. Incubate 15 min at room temperature. *To prepare working concentration of the antibody, dilute fluorescein-cojugated goat anti-mouse IgM (Sigma, Cat. # F-9259) in 1% non-fat dry milk in PBS. The diluted antibody could be aliquoted and stored frozen at -20° C.*
 10. For flow cytometry, add 1 ml of PBS, centrifuge and resuspend pellet in 0.5 ml of propidium iodide solution (1 microg/ml in PBS). For fluorescence microscopy, rinse cells with PBS, prepare cytopspins and stain slides with DNA fluorochrome DAPI (0.1 microg/ml in PBS for 10 min).

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