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Preparation of Nuclei from Paraffin-Embedded Tissues for DNA Analysis

SCOPE

This procedure describes a method for the preparation of a nuclear suspension from paraffin-embedded tissue optimized for flow cytometric analysis using a BD FACScan™ or BD FACSort™ flow cytometer equipped with a doublet discrimination module (DDM). CellFIT software on the Hewlett-Packard (HP) platform may be used to acquire and analyze DNA samples or BD CellQuest™ software and the DNA Experiment Document may be used to acquire DNA samples for analysis with an appropriate DNA analysis software package.

This procedure uses formalin-fixed, paraffin-embedded tissue and is a modification of a procedure that has been previously described.[1,2] The specimen is sectioned from a paraffin-embedded block, deparaffinized, rehydrated through a sequence of ethanol wash steps, and stored overnight in water to ensure complete hydration. The following day, the tissue is dissociated by a pepsin treatment, the nuclear proteins are stabilized with spermine, the RNA is digested, and finally the DNA is labeled with propidium iodide (PI) for flow cytometric analysis.

REAGENT

- BD CycleTEST™ PLUS Reagent Kit (BD Cat. No. 340242). Refer to the BDCycleTEST PLUS Reagent Kit package insert for precautions.
- Phosphate-buffered saline (PBS) (1X) (Dulbecco's PBS without calcium, magnesium, or phenol red, pH 7.2 ±0.2). Store 1X PBS at 2° to 8°C. Filter through a 0.2-µm filter prior to use.
- AmeriClear histology clearing solvent (Scientific Products Cat. No. C4200-1)
- Ethanol, 100%, 95%, 70%, and 50% solutions (Scientific Products Cat. No. 7018-4)
- 1 N HCl (Scientific Products Cat. No. 2611-500)
- Pepsin (Sigma Cat. No. P6887). Prepare a 0.5% pepsin solution on the same day it will be used in 1X PBS and adjust to pH 1.5 with 1 N HCl.
- Reagent-grade water (distilled and deionized)

EQUPMENT

- BD VACUTAINER™ red-top blood collection tubes (BD Cat. No. 6430)
- 1-mL disposable tuberculin plastic syringes (BD Cat. No. 5602)
- 12 x 75mm Falcon polystyrene test tubes (BD Cat. No. 2058) or equivalent
- Transfer pipettes, nonsterile (BD Cat. No. 7524) or equivalent
- 5-mL serological pipette, nonsterile (BD Cat. No. 7529) or equivalent
- Vortex mixer
- Centrifuge with swinging-bucket rotor and 12 x 75mm tube carriers
- Micropipettors with tips, variable to 1000 mL (Pipetman or equivalent)
- Vacuum aspirator with trap (low vacuum)
- Hemacytometer and light microscope
- Microtome to cut 50-µm sections
- 4.5-inch flat-tipped forceps (VWR Cat. No. 25681-269) or equivalent
- Water bath at 37°C
- 2 x 2-inch sterile cotton gauze
- 12 x 75-mm polystyrene test tube with 35-µm cell strainer cap, nonsterile (BD Cat. No. 2235) or 35-µm nylon mesh (Tetko Cat. No. 3-35/22) or equivalent.
- 14.6-cm disposable Pasteur pipettes and 1-mL pipette bulb
- Plastic funnel (Scientific Products Cat. No. F7450-1) or equivalent
- Powderless latex gloves
- Chemical fume hood

PROCEDURE

Tissue Sampling

Please read through this entire procedure before processing specimens.

1. Cut 50-µm sections from each formalin-fixed paraffin-embedded specimen. Start with at least two sections per embedded block. Increase the number of sections per specimen if a greater number of cells is desired. Cut and stain a 5-µm section before and after each sampling from the block to identify the relative positions and proportions of the normal and malignant tissues.

2. Place the sections from each block in a 10-mL red-top VACUTAINER tube. Label tubes with case number and block identification.

Deparaffinization

CAUTION: Perform the following deparaffinization steps under a chemical fume hood.

1. Begin to deparaffinize or dewax sections by adding at least 2 to 3 mL of AmeriClear to the BD VACUTAINER™ tube(s). There should be enough reagent to completely cover the paraffin sections during the incubation step.

2. Vortex the samples vigorously. Approximate time for incubation with AmeriClear is 10 to15 minutes with a maximum time of 3 hours. Before pipetting off the reagent, carefully pull the section(s) up onto the side of the tube with the pipette, keeping the tissue intact. Aspirate the remaining reagent.

3. Add an additional 2 to 3 mL of AmeriClear, vortex briefly, and incubate for 10 minutes. Make sure the tissue sections are covered by the reagent. Process as above. Before proceeding to the hydration steps, check each tube to see if the sections are transparent, indicating the paraffin is completely dissolved

Rehydration

The following steps are critical for proper hydration of the tissue and if not performed correctly may result in an increase in debris during sample preparation.

NOTE: While performing the first rehydration step, if the alcohol appears cloudy or if visible wax precipitates are present, remove the alcohol and perform an additional deparaffinization step by incubating in AmeriClear for 30 minutes to remove any residual paraffin. Then continue with the rehydration steps.

1. Rehydrate sections by sequential washes using 3 mL of each of the following ethanol solutions: 100%, 95%, 70%, and 50%. Vortex the specimen after the addition of each new ethanol solution and incubate for a minimum of 10 minutes at room temperature (20° to 25°C). Aspirate each solution before adding the next, keeping the tissue sections intact by pulling them up onto the side of the tube before pipetting.

   NOTE: If the tissue is brittle or very small, it may be difficult to pull the tissue up onto the wall of the tube. Careful attention must be paid to prevent the loss of too many cells.

2. Complete the rehydration by adding 3 mL of reagent-grade water. Gently aspirate and replace with fresh water to eliminate any residual alcohol. Leave the tissue in reagent-grade water overnight at room temperature (20° to 25°C).

Dissociation

1. Gently aspirate the water and resuspend in 2 mL of 1X PBS while preparing the pepsin (10 minutes).

2. Gently aspirate the 1X PBS and add a minimum of 2 mL of freshly prepared 0.5% pepsin solution to each tube. Vortex on medium speed for approximately 30 seconds to ensure that the tissue is resuspended.

3. Incubate tube for 1 hour in a 37°C water bath. Shake or gently vortex the tube every 10 minutes and check the turbidity of the supernatant. With some tissues, a longer incubation period may be required to increase the recovery of cells. Some tissues, like spleen and lymph nodes, may require only 20 minutes before the supernatant turns turbid. This will also depend on the activity of the enzyme.

   NOTE: Remove Solutions A and B from the BD CycleTEST™ PLUS kit and leave them at room temperature for at least 10 minutes before use.

Staining

1. Filter cell suspensions through the plastic funnel lined with an opened layer of 2 x 2-inch cotton gauze into a 12 x 75mm test tube. Rinse the sample tube once with 1 to 2 mL of 1X PBS and pour through the gauze. Squeeze the gauze with flat-tipped forceps to increase cell yield. (BD Falcon™ 35-µm cell strainer cap tube or 35-µm nylon mesh can be substituted for the gauze.)

2. Centrifuge the cell suspensions at 400 x g for 5 minutes at room temperature (20° to 25°C). Carefully remove the supernatant.

3. Gently resuspend the cell pellet in 1 mL of 1X PBS and count the cells in a hemacytometer, using a light microscope. Adjust the cell concentration to a maximum of 5.0×10^5 cells/mL.

4. Centrifuge at 400 x g for 5 minutes at room temperature (20° to 25°C). Carefully decant all the supernatant.

5. Add 250 µL of Solution A (trypsin buffer) to the tube and gently mix. Do not vortex.

6. Allow the trypsin to react for 10 minutes at room temperature (20° to 25°C). Do not remove Solution A.

7. Add 200 µL of Solution B (trypsin inhibitor and RNase in a buffer) and gently mix. Do not vortex.

8. Incubate with the trypsin inhibitor and RNase for 10 minutes at room temperature (20° to 25°C).

9. Add 200 µL of cold (2° to 8°C) Solution C (propidium iodide stain solution). Gently mix as above and incubate for 10 minutes in the dark on ice or in the refrigerator (2° to 8°C).

10. Filter the sample through a BD Falcon 35-µm cell strainer cap tube or through 35µm nylon mesh into a 12 x 75-mm tube.

11. Keep the samples in an ice bath (2° to 8°C) and protected from light until flow cytometric analysis.

12. Run the sample on the cytometer within 3 hours after addition of Solution C.

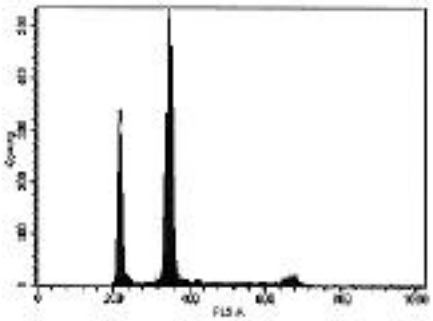

Figure 1. Histogram of stained DNA nuclei from paraffin-embedded tissue.

WARNING

Spermine tetrahydrochloride is irritating to the skin and mucus membranes. Propidium iodide is a suspected mutagen. Dimethyl sulfoxide (DMSO) is a possible teratogen.

LIMITATIONS

Fresh tissue is preferable to paraffin-embedded material for most purposes. This technique for extracting and staining nuclei from paraffin-embedded tissue is intended for use only in retrospective studies.

REFERENCES

1. Hedley DW. Flow cytometry using paraffin-embedded tissue: Five years on. *Cytometry*. 1989;10:229-241.

2. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem*. 1983;31:1333-1335.

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