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ProductInformation

DNA STAINING REAGENTS KIT

STOCK NO. PI-STAIN

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Intended Use

The primary application of the DNA Staining Reagents Kit is the staining of cell nuclei isolated from fresh tissue or peripheral blood. Nuclei are stained with propidium iodide (PI) and analyzed by flow cytometry. Comparison of DNA analysis results from normal cells to tumor cells may be used to assess the cell cycle phase distribution. DNA staining of tumors is a useful tool in a variety of clinical research applications. The DNA Staining Reagent Kit allows measurement of DNA content which permits monitoring of cell activity over time. Quantitation of DNA may aid in the determination of cell ploidy status and S-phase modeling.

Background and Principle

Cell preparations are digested with trypsin and then further processed with RNase A to digest RNA. The remaining isolated nuclei are stained using propidium iodide solution. Analysis of the prepared samples is performed by flow cytometry. Analysis of the proportion of cells undergoing G_O/G_1, S and G_2 phases reveals information on the proliferative state of the cell sample. These results can also be used to analyze abnormal cell cycle distribution or DNA aneuploidy.[1,2]

Reagents and Materials Provided

1. **Solution A** **S-1407**
   Trypsin solution used for enzymatic digestion of tissue and cell membranes.

2. **Solution B** **S-1532**
   Trypsin inhibitor and RNase A solution used to stop trypsin activity and digest RNA.

3. **Solution C** **S-1657**
   Propidium Iodide solution (at least 125 µg/ml in final solution) used to stain DNA.

4. **Buffer Solution** **S-1282**
   Sodium Citrate buffer used for preparation and/or freezing of cell suspensions.

5. **Instructions for Use**

Reagents and Materials that may be Required, but are not Provided

1. Hemocytometer and microscope
2. 12 x 75 mm polypropylene tubes and caps (Sigma Product No. T-1911)
3. Ice bath
4. Aluminum foil
5. Vortex
6. Pipets, 2 ml capacity, disposable (Sigma Product No. P-3547)
7. Micropipettor and disposable tips; 200 µl capacity
8. Sheath Fluid (Using PBS instead of sheath fluid may result in different performance).
9. HISTOPAQUE® (Sigma Stock No. 1077-1)

Reagents and Materials that may be Required, but are not Provided (cont.)

10. Ethanol
11. Xylene
12. 15 ml conical tube (Sigma Product No. C-8046)
13. Pepsin (Sigma Product No. P-7012)

Flow Cytometry

The DNA Staining Reagent Kit is to be used for sample preparation and staining for the measurement

of total nuclear DNA using flow cytometry. The flow cytometer must be equipped with an argon laser with an optimal emission of 488 nm. Primary emission of propidium iodide is between 580 and 650 nm. For instruments lacking fixed filter configuration, a red or orange band pass emission filter is recommended. The flow cytometer must be equipped with the appropriate software to perform histogram analysis and calculate G_O/G_1, S and G_2/M phases. An electronic doublet discriminator is necessary for optimal performance.
Prior to analysis, linearity must be demonstrated in the flow cytometer to insure proper machine function. Sample flow rate must be low. High flow rate may cause falsely elevated G_O/G_1, S and G_2 /M percentages.

Procedure

Specimen Preparation

I. Blood

1. Separate mononuclear cells using HISTOPAQUE®.
2. Place white blood cells in clean 17 x 100 mm tube.
3. Resuspend cells in 5 ml citrate buffer solution.
4. Centrifuge for 5 minutes (300 x g) at room temperature.
5. Aspirate supernatant and resuspend pellet in 1.5 ml of citrate buffer solution.
6. Centrifuge for 5 minutes (300 x g) at room temperature.
7. Aspirate supernatant and resuspend pellet in 500 µl of citrate buffer solution.
8. Using citrate buffer solution, adjust the cell concentration to 2.5×10^6 cells/ml.

II. Cultured Cells

1. Place tissue culture suspension into a 17 x 100 mm tube allowing enough suspension to provide 2.5×10^6 cells/sample.
2. Centrifuge for 5 minutes (300 x g) at room temperature.
3. Aspirate media and resuspend pellet in 5 ml of citrate buffer solution.
4. Centrifuge for 5 minutes (300 x g) at room temperature.
5. Aspirate supernatant and resuspend pellet in 1.5 ml of citrate buffer solution.
6. Centrifuge tube for 5 minutes (300 x g) at room temperature.
7. Aspirate supernatant and resuspend pellet in 500 µl of citrate buffer solution.
8. Using citrate buffer solution, adjust the cell concentration to 2.5×10^6 cells/ml.

III. Fresh solid tumor

1. Using a scalpel, scrape cells from approximately 1 cm of the tissue to be tested.
2. Place the cellular material into a 17 x 75 mm tube containing 50% ethanol. The cells are now ethanol fixed.
3. Place a portion of the fixed cells into a 17 x 75 mm tube.
4. Centrifuge for 5 minutes (300 x g) at room temperature.
5. Aspirate the ethanol and resuspend pellet in 5 ml of citrate buffer solution.
6. Centrifuge tube for 5 minutes (300 x g) at room temperature.
7. Aspirate supernatant and resuspend pellet in 1.5 ml of citrate buffer solution.
8. Centrifuge tube for 5 minutes (300 x g) at room temperature.
9. Aspirate supernatant and resuspend pellet in 500 µl of citrate buffer solution.
10. Using citrate buffer solution, adjust the cell concentration to 2.5×10^6 cells/ml.

IV. Paraffin-embedded tissue

1. Select a 50 micron section from an area of interest in a paraffin-embedded block.
2. Place 1-4 of these sections in a 16 x 125 mm glass tube.
3. Add 10 ml of xylene to the tube and allow to stand for 10 minutes.
4. Aspirate the supernatant.
5. Repeat steps 3 and 4.
6. Add 5 ml of 100% ethanol and allow to stand for 10 minutes.
7. Aspirate the supernatant.
8. Add 5 ml of 95% ethanol and allow to stand for 10 minutes.
9. Aspirate the supernatant.
10. Add 5 ml of 70% ethanol and allow to stand for 10 minutes.
11. Aspirate the supernatant.
12. Add 5 ml of 50% ethanol and allow to stand for 10 minutes.
13. Aspirate the supernatant.
14. Add 5 ml of dH_2O.
15. Vortex and aspirate the supernatant.
16. Repeat steps 14 and 15.
17. Using a watch glass, mince the tissue and add 2 drops of warmed 0.5% pepsin.
18. Using a 15 ml conical tube, resuspend the tissue with 2 ml of warmed 0.5% pepsin.
19. Incubate the tube in a 37°C waterbath for 30 minutes, vortexing at 5 minute intervals.
20. Centrifuge for 3 minutes (1000 x g).
21. Aspirate the supernatant.
22. Resuspend the pellet in 5 ml of citrate buffer solution.
23. Centrifuge tube for 5 minutes (300 x g) at room temperature.
24. Aspirate supernatant and resuspend pellet in 1.5 ml of citrate buffer solution.
25. Centrifuge tube for 5 minutes (300 x g) at room temperature.
26. Aspirate supernatant and resuspend pellet in 500 µl of citrate buffer solution.
27. Using citrate buffer solution, adjust the cell concentration to 2.5×10^6 cells/ml.

Staining Procedure

1. Place 200µl of the prepared cell suspension (2.5 x 10^6 cells/ml) into a 17 x 100 mm tube.
2. Add 1.8 ml of Solution A. Resuspend the cells by gentle inversion.
3. Incubate for 10 minutes at room temperature with constant mixing.
4. Add 1.5 ml of Solution B. Incubate for 10 minutes at room temperature with constant mixing.
5. Add 1.5 ml of cold (4°C) Solution C. Incubate for 10 minutes at room temperature protected from light with constant mixing.
   (Note: Volume may be decreased at this step by slow centrifugation and aspiration of supernatant.)
6. Store samples in an ice bath, protected from light, until analysis. Analysis should be performed within 3 hours of staining.
7. Analyze samples utilizing lab-specific protocols. Refer to instrument guidelines for proper operation. Optimal analysis requires low sample flow rate.

Controls

Control/standard cells are useful tools in DNA analysis. Cells of known DNA content may be used as a reference point for instrument setup and for determination of the DNA Index. They may also be used as a control to monitor staining conditions. Chicken red cell nuclei, trout red cell nuclei or human lymphocyte nuclei are commonly used controls/standards. Chicken red cell nuclei contain 33% of the DNA content of normal human nuclei. Trout erythrocyte nuclei contain approximately 80%. These cells may be stained at the same time as the test sample or stained separately.

Histogram Analysis

Analysis and proper interpretation of data obtained using the DNA Staining Reagent Kit depends on the user's application. The histograms below are typical of those produced by samples stained using the DNA Staining Reagent Kit. Several key phases of the cell cycle may be defined:

G_0/G_1 = Phase in which the cell is resting or preparing for mitosis.

S-Phase = Phase between G_0/G_1 and G_2/M when the cell is in the process of DNA synthesis.

G_2/M = Phase in which the DNA content of the cell is twice the amount of the DNA content of the cell in the resting phase.

DNA ploidy status of tumors may be expressed as the amount of DNA in the test sample compared to a known normal sample.[8]

$$\text{DNA index} = \frac{\text{mean or modal channel number of DNA aneuploid } G_0/G_1 \text{ peak}}{\text{mean or modal channel number of DNA diploid } G_0/G_1 \text{ peak}}$$

Applications Notes

1. Light microscopic examination of tumor cell suspensions is necessary to insure that tumor cells have been properly represented.
2. Malignancy must be evaluated using standard Hematoxylin & Eosin staining by a trained pathologist.
3. It is essential that the recommended cell concentration be used. Increasing the number of cells may produce poor results.
4. Propidium iodide uptake (fluorescence intensity) depends on histone content and chromatin structure density. Histone and other protein digestion by the trypsin in solution A is essential to furnish homogenous nuclei suspensions for analysis.
5. pH and ionic strength of the liquid solution[6] used directly effect the fluorescence of propidium iodide. Use of PBS instead of sheath fluid may result in different performance.
6. Reagent volumes and incubation times have been optimized in the development of this kit. Alteration of the recommended quantities from those specified may generate inaccurate results.
7. PI has a high affinity for plastic tubing. When samples are analyzed using flow cytometry, an equilibrium must be established within the instrument. Data should be acquired after a minimum of 30 seconds of aspiration.
8. G_0/G_1 peak CV should be ≤ 5 for fresh cells. High CV's are a result of poor technique during sample prep staining. Higher CV's are found with paraffin-embedded tissue.

Storage

Store at -20°C. Repeated freezing and thawing is **not** recommended. Storage in "frost-free" freezers is **not** recommended. After thawing, reagents stored at 0-5°C are stable for 4 weeks. Reagents may be briefly heated to 37°C in the process of thawing. Protect Solution C (PI) (Sigma Product No. S-1657) from light and keep at 0-5°C after thawing.

References

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