

User Manual

FragEL DNA Fragmentation Detection Kit, Colorimetric - TdT Enzyme

QIA33

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for human or animal consumption.

Product Overview

Intended Use

The TdT-FragEL DNA Fragmentation Detection Kit is a non-isotopic system for the labeling of DNA breaks in apoptotic nuclei in paraffin-embedded tissue sections, tissue cryosections, and in cell preparations fixed on slides.

Background

Cell death has been shown to occur by two major mechanisms, necrosis, and apoptosis. Classical necrotic cell death occurs due to noxious injury or trauma while apoptosis takes place during normal cell development, regulating cellular differentiation and number.

While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane. A cascade of molecular and biochemical events has been identified including activation of an endogenous endonuclease that cleaves DNA into oligonucleosomes detectable as a ladder of DNA fragments in agarose gels. Observation of oligonucleosomal DNA fragments by DNA laddering has long been the most acceptable and only available assay for the detection of apoptosis. However, this

methodology has numerous disadvantages such as a lack of sensitivity and specificity; lengthy preparation time, requiring a high level of expertise; and inability to discern which cells in a population are undergoing apoptosis. Apoptotic endonucleases not only affect cellular DNA by producing the classical DNA ladder but also generate free 3'-OH groups at the ends of these DNA fragments. These groups are end-labeled by FragEL DNA Fragmentation Detection Kits allowing for the detection of apoptotic cells using a molecular biology-based, end-labeling, histochemical or cytochemical technique. DNA modification is not the only indicator of apoptotic cell death. One can histologically recognize apoptotic cells in a population by identifying the well-characterized morphological changes. FragEL DNA Fragmentation Detection Kits allow one to simultaneously evaluate both of these apoptotic indices. DNA strand breakage is identified within morphologically intact cell specimens allowing for a more concise differentiation between the normal and apoptotic cell. The versatility of these non-isotopic kits permits the identification of cellular apoptosis in fresh cultured cells, paraffin-embedded tissues, and tissue cryosections.

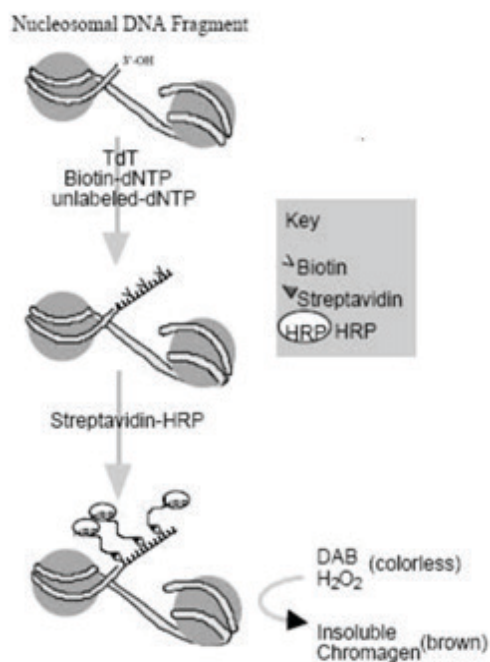
Principles of the Assay

The DNA Fragmentation Detection Kits allow the recognition of apoptotic nuclei in paraffin-embedded tissue sections, tissue cryosections, or in cell preparations fixed on slides by Fragment End Labeling (FragEL) of DNA. In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells.

Characteristics



Principle of the Assay



Materials Provided

The TdT-FragEL Kit supplies sufficient reagents to stain 50 specimens.

Note: Reagent volumes are stated on the particular reagent vial or bottle.

Kit Components

- Proteinase K (JA1477-.24ML)
2 mg/mL Proteinase K in 10 mM Tris, pH 8
- 5X TdT Equilibration Buffer (JA1558-1.75ML)
1 M Sodium Cacodylate, 0.15 M Tris, 1.5 mg/mL BSA, 3.75 mM CoCl₂, pH 6.6
- TdT Labeling Reaction Mix (JA1559-1ML)
3 vials containing a mixture of labeled and unlabeled deoxynucleotides at a ratio optimized for DNA Fragment End Labeling with TdT
- TdT Enzyme (JA1560-.155ML)
Terminal Deoxynucleotidyl Transferase
- Stop Buffer (JA1479-6ML)
0.5 M EDTA, pH 8
- Blocking Buffer (JA1480-12ML)
4% BSA in PBS
- 50X Conjugate (JA1481-.125ML)
Peroxidase streptavidin conjugate 50-fold concentrated solution
- DAB Tablets (JA1485-6EA)
3,3'-Diaminobenzidine (0.7 mg/tablet)
- H₂O₂ /Urea Tablets (JA1486-6EA)
H₂O₂ /Urea (1.6 mg/tablet)
- Methyl Green Counterstain (JA1482-6ML)
0.3% Methyl green
- Control Slides (JA1609-1EA)
Two similar slides. A mixture of HL60 cells incubated with 0.5 µg/mL actinomycin D for 19 hours to induce apoptosis and uninduced HL-60 cells. Supplied as formaldehyde fixed Cytospin® preparations.

Materials Required (Not supplied)

Reagents

- Xylene
- Ethanol, 100, 90, 80, 70%
- Methanol
- 30% Hydrogen peroxide
- Tris-buffered saline (1X TBS, 20 mM Tris pH 7.6, 140 mM NaCl)
- 10 mM Tris pH 8
- 1 mM MgSO₄ in 1X TBS (optional, for use in generating positive control)
- DNaseI (optional, for use in generating positive control)
- Distilled water
- Ice

Equipment

- Coplin jars, glass or plastic with slide holders
- Wash bottle or beaker for rinsing slides
- Humidified chamber
- Glass coverslips
- Mounting media (such as Permount®)
- Microscope
- 2-20 µL, 20-200 µL, and 200-1000 µL precision pipettors with disposable tips
- Microcentrifuge tubes
- Parafilm®
- Absorbent wipes

Warnings and Precautions

For optimal results, read these instructions before using this kit.

- The TdT Enzyme will not solidify at -20 °C. To preserve the activity of this enzyme, do not remove it from the freezer until immediately before use in preparing the labeling reaction mixture. Pulse-spin the TdT enzyme tube in a microcentrifuge prior to opening. At this point either pipet the enzyme directly from the freezer or momentarily place it in a -20 °C storage device designed for bench top use. Do not store at -80 °C.

- All other FragEL kit components, with the exception of Stop Buffer, Blocking Buffer, and Methyl Green Counterstain, should be kept on ice during usage, and then promptly returned to -20 °C.
- To avoid reagent loss in tube caps, briefly pulse spin all thawed solutions before removing caps.
- Diaminobenzidine (DAB) solution contains potential carcinogens.
- Cacodylic acid is a component of the 5X TdT Equilibration Buffer. Cacodylic acid is toxic and carcinogenic. Avoid contact with eyes and skin. Do not ingest.
- Gloves, lab coat, and protective eyewear should be worn.
- A separate protocol has been provided for the end labeling of paraffin-embedded tissue sections, tissue cryosections, and cell preparations fixed on slides. See the section appropriate to your sample. HL60 control slides should be labeled using the fixed cells protocol.
- HL60 control slides contain a mixture of apoptotic (positive) and normal (negative) cells. In determining the amount of labeling reaction mixture to prepare, each control slide counts as one sample.
- Incubation time for Proteinase K, DNase I, and labeling may need to be empirically determined for your particular cell type and slide preparation. Use this protocol as a guideline.
- The use of coverslips is recommended during the labeling step to assure even distribution of the reaction mixture and to prevent loss due to evaporation during incubation. To make a coverslip, cut a piece of Parafilm® just slightly larger than the specimen. Fold up one corner of the Parafilm® to aid in its application and removal.
- To construct a humidified chamber, wet several paper towels with water and place them along the bottom of a glass or plastic container with sides. Place two pipets parallel to each other on top of the towels. Keeping slides level, lay them face up across the pipets. To avoid unwanted drainage of reagents from slides make sure that the towels do not contact the slides in any way. Cover the container with a lid or plastic wrap to provide a humidified environment.

- Cells grown in suspension can be fixed and attached to slides as follows. Cells are pelleted by gentle centrifugation for 5 min at 4 °C. Cells are then resuspended in 4% formaldehyde (in 1X PBS) at a cell density of 1×10^6 /mL and incubated at room temperature for 10 min. Cells are pelleted by gentle centrifugation for 5 min at room temperature and resuspended, at the same concentration, in 80% ethanol. Store fixed cells at 4 °C. Fixed cells (100-300 μ L) can be immobilized onto glass slides by directly placing the cell suspension onto the slide or by using a Cytospin® centrifuge. Precoating slides with poly-L-lysine may enhance cell adherence. Store Cytospin® samples at -20 °C.

Storage and Stability

Upon arrival store the entire contents of the kit at -20 °C in a non-frost free freezer. Following initial thaw store the Stop Buffer and Methyl Green Counterstain at room temperature and the Blocking Buffer at 4 °C. Refreezing of these components, however, does not appear to affect their performance.

Protocol

Preparation

Generation of Negative Control Samples

A negative control of your specific sample can be generated by substituting dH₂O for the TdT in the reaction mixture or by keeping the specimen in 1X reaction buffer (with a cover slip) during the labeling step. Perform all other steps as described. This controls for endogenous peroxidases and non-specific conjugate binding. A non-apoptotic control is also critical since cells and tissue begin to undergo apoptosis from the very beginning of the excision, fixation, and processing steps. A delay in fixation or routine mechanical manipulation may result in the unwanted breakage of DNA that could be misinterpreted as apoptosis.

Generation of Positive Control Samples

A positive control of your specific tissue sample can be generated by covering the entire specimen with 1 μ g/ μ L DNase I in 1X TBS/1 mM MgSO₄ following proteinase K treatment. Incubate at room temperature for 20 min. Perform all other steps as described. This will fragment DNA in normal cells. Cytoplasmic as well as nuclear DAB staining of DNase treated cells may be observed.

Detailed Protocol

TdT-FragEL Of Paraffin Embedded Tissue

Deparaffinization and Rehydration

1. Immerse slides in xylene for 5 min at room temperature. Repeat using fresh xylene for second 5 min incubation.
2. Immerse slides in 100% ethanol for 5 min at room temperature. Repeat using fresh 100% ethanol for second 5 min incubation.
3. Immerse slides in 90% ethanol for 3 min at room temperature.
4. Immerse slides in 80% ethanol for 3 min at room temperature.
5. Immerse slides in 70% ethanol for 3 min at room temperature.
6. Rinse slides briefly with 1X TBS and carefully dry the glass slide around the specimen.

Note: To help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen or a hydrophobic slide marker.

DO NOT LET THE SPECIMEN DRY OUT DURING OR BETWEEN ANY STEP! If necessary, cover or immerse the specimen in 1X TBS to keep hydrated.

Permeabilization of Specimen

1. Dilute 2 mg/mL Proteinase K 1:100 in 10 mM Tris pH 8 (mix 1 μ L of 2 mg/mL Proteinase K plus 99 μ L 10 mM Tris per specimen).
2. Cover the entire specimen with 100 μ L of 20 μ g/mL Proteinase K. Incubate at room temperature for 20 min. DO NOT OVERINCUBATE.
3. Rinse slide with 1X TBS.
4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Generation of a Positive Control (optional)

1. Cover the entire specimen with 1 μ g/ μ L DNase I in 1X TBS/1 mM MgSO₄. Incubate at room temperature for 20 min.
2. Rinse slide with 1X TBS.
3. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Note: For generation of a negative control, see [DNA Ladder TdT-FragEL Staining on page 8](#)).

Inactivation of Endogenous Peroxidases

1. Dilute 30% H₂O₂ 1:10 in methanol (mix 10 µL 30% H₂O₂ with 90 µL methanol per specimen).
2. Cover the entire specimen with 100 µL of 3% H₂O₂. Incubate at room temperature for 5 min. DO NOT OVERINCUBATE.
3. Rinse slide with 1X TBS.
4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Equilibration and Labeling Reaction

1. Dilute 5X TdT Equilibration Buffer 1:5 with dH₂O (mix 20 µL 5X Buffer with 80 µL dH₂O per specimen).
2. Cover the entire specimen with 100 µL of 1X TdT Equilibration Buffer. Incubate at room temperature for 10 to 30 min while preparing the labeling reaction mixture.

Note: Alternatively slides can be immersed in a coplin jar containing enough 1X TdT buffer to cover the specimen. Do NOT allow the specimens to dry out.

3. Prepare the working TdT Labeling Reaction Mixture as follows:

Lightly vortex contents of the TdT Labeling Reaction Mix tube pulse-spin the TdT enzyme tube in a microcentrifuge prior to opening for each sample to be labeled, transfer to a clean microfuge tube ON ICE and mix gently:

- 57.0 µL TdT Labeling Reaction Mix
- 3.0 µL TdT Enzyme

4. Carefully blot the 1X TdT Equilibration Buffer from the specimen, taking care not to touch the specimen.
5. Immediately apply 60 µL of TdT Labeling Reaction Mixture (prepared above) onto each specimen.
6. Cover the specimen with a piece of Parafilm® cut slightly larger than the specimen.

Hint: Folding up one corner of the Parafilm® coverslip will aid in its application and removal.

Note: The use of a coverslip assures even distribution of the reaction mixture and prevents loss due to evaporation during incubation.

7. Place slides in a humidified chamber and incubate at 37 °C for 1.5 hours.

Termination of Labeling Reaction

1. If a precipitate is present, prewarm the Stop Buffer to 37 °C for five min.
2. Remove Parafilm®, coverslip and rinse slide with 1X TBS.
3. Cover the entire specimen with 100 µL of Stop Solution. Incubate at room temperature for 5 min.
4. Rinse slide with 1X TBS.
5. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Detection

1. Cover the entire specimen with 100 µL of Blocking Buffer. Incubate at room temperature for 10 min.
2. Dilute the 50X Conjugate 1:50 in Blocking Buffer (mix 2 µL 50X Conjugate with 98 µL Blocking Buffer per specimen).
3. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen. Immediately apply 100 µL of diluted 1X conjugate to the specimen.
4. Place slides in a humidified chamber and incubate at room temperature for 30 min.
5. Five min before concluding incubation prepare DAB solution by dissolving one tablet of DAB and one tablet of H₂O₂/Urea in 15 mL of tap/faucet H₂O. This yields enough DAB solution for at least 10 specimens.
Note: Tap/faucet H₂O may contain metal ions that enhance the DAB reaction.
6. Rinse slides with 1X TBS.
7. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
8. Cover the entire specimen with 100 µL of DAB solution. Incubate at room temperature for 10-15 min.
9. Rinse slides with dH₂O.

Counterstain

1. Immediately cover the entire specimen with 100 μ L of Methyl Green counterstain solution.
2. Incubate at room temperature for 3 min.
3. Press an edge of the slide against an absorbent towel to draw off most of the counterstain and place in a coplin jar slide holder.
4. Dip slides 2-4 times into 100% ethanol.
5. Blot slides briefly on an absorbent towel.
6. Repeat step 4 using fresh 100% ethanol. Blot slides briefly on an absorbent towel.
7. Dip slides 2-4 times into xylene.
8. Wipe excess xylene from back of slide and around specimen.
9. Mount a glass coverslip using a mounting media such as Permount® over the specimen.

TdT-FragEL of Tissue Cryosections

This protocol is similar to FragEL of paraffin-embedded tissue sections EXCEPT that the deparaffinization step is replaced with a short hydration step and permeabilization with Proteinase K is performed for only 10 min. Fixation of cryopreserved tissue is required prior to performing the assay.

To avoid loss of tissue from glass slides during washing steps, it is recommended that slides be dipped 2-3 times into a beaker of 1X TBS rather than rinsed with a wash bottle.

DO NOT LET THE TISSUE DRY OUT BETWEEN OR DURING ANY STEP! If necessary cover or immerse the slides in 1X TBS to keep hydrated.

Tissue Fixation and Hydration

1. Immerse slides in 4% formaldehyde (in 1X PBS) for 15 min at room temperature.
2. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
3. Immerse slides in 1X TBS for 15 min at room temperature.
4. Carefully dry the glass slide around the specimen.

To help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen or a hydrophobic slide marker.

Permeabilization of Specimen

1. Dilute 2 mg/mL Proteinase K 1:100 in 10 mM Tris pH 8 (mix 1 μ L of 2 mg/mL Proteinase K plus 99 μ L 10 mM Tris per specimen).
2. Cover the entire specimen with 50-100 μ L of 20 μ g/mL Proteinase K. Incubate at room temperature for 10 min. DO NOT OVERINCUBATE.
3. Dip slide 2-3 times into a beaker of 1X TBS.
4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
5. All remaining steps are identical to those steps outlined for TdT-FragEL of paraffin-embedded tissue sections:
 - Inactivation of Endogenous Peroxidase
 - Equilibration and Labeling Reaction
 - Termination of Labeling Reaction
 - Detection
 - Counterstain

Note: Remember to wash slides by dipping into a beaker of 1X TBS rather than using a wash bottle.

TdT-FragEL of Fixed Cell Preparations

- For fixing cells, see directions at top of page 6.
- This protocol is similar to FragEL of paraffin-embedded tissue sections EXCEPT that the deparaffinization step is replaced with a short rehydration step and permeabilization with proteinase K is performed for only 5 min.
- To avoid loss of cells from glass slides during washing steps, it is recommended that slides be dipped 2-3 times into a beaker of 1X TBS rather than rinsed with a wash bottle.
- Reagent volumes may be decreased to account for the lower surface area that usually accompanies cells fixed on slides.
- DO NOT LET THE CELLS DRY OUT BETWEEN OR DURING ANY STEP! (if necessary cover or immerse the slide in 1X TBS to keep hydrated).

Rehydration

1. Immerse slides in 1X TBS for 15 min at room temperature.
2. Carefully dry the glass slide around the specimen.

Note: To help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen or a hydrophobic slide marker.

Permeabilization of Specimen

1. Dilute 2 mg/mL Proteinase K 1:100 in 10 mM Tris pH 8 (mix 1 μ L of 2 mg/mL Proteinase K plus 99 μ L 10 mM Tris per specimen).
2. Cover the entire specimen with 50-100 μ L of 20 μ g/mL Proteinase K. Incubate at room temperature for 5 min. DO NOT OVERINCUBATE.
3. Dip slide 2-3 times into a beaker of 1X TBS.
4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

All remaining steps are identical to those steps outlined for Klenow-FragEL of paraffin-embedded tissue sections (Page 6):

- [Inactivation of Endogenous Peroxidases](#)
- [Equilibration and Labeling Reaction](#)
- [Termination of Labeling Reaction](#)
- [Detection](#)
- [Counterstain](#)

Note: Remember to wash slides by dipping into a beaker of 1X TBS rather than using a wash bottle.

Product Performance

Assay Characteristics and Examples

A dark brown DAB signal indicates positive staining while shades of blue-green to greenish tan signifies a non-reactive cell. Since the 3'-OH ends of DNA fragments generated by apoptosis are concentrated within the nuclei and apoptotic bodies, morphology as well as DAB staining can be used to interpret FragEL results. Characteristic morphological changes during apoptosis have been well characterized (see next page) and should be used as verification of programmed cell death. Non-apoptotic cells do not incorporate significant amounts of labeled nucleotide since they lack an excess of 3'-OH ends.

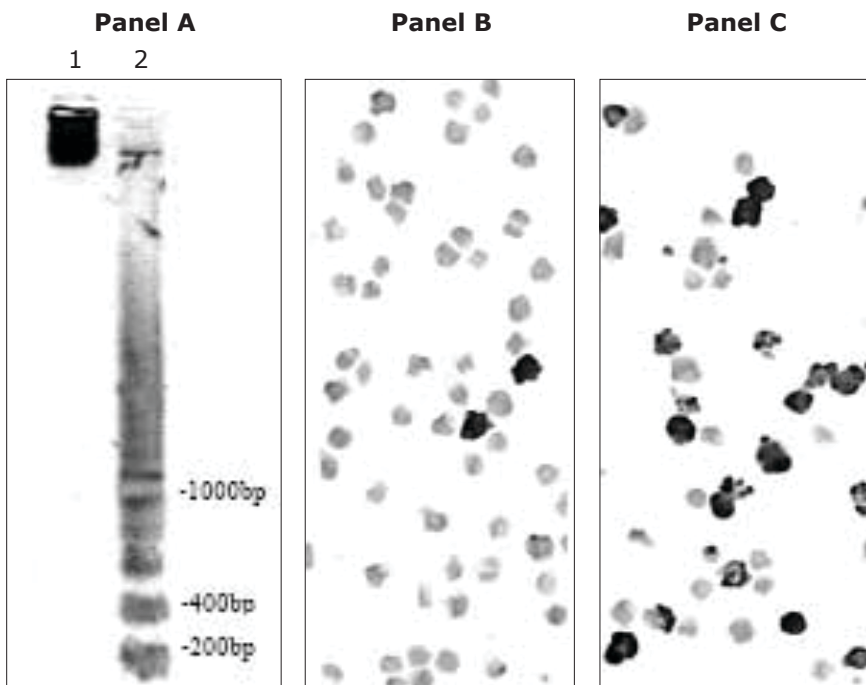
After labeling, carefully evaluate the control slides provided in the FragEL kit using a light microscope. The control slides contain a mixture of normal and apoptotic cells. Non-apoptotic cells should be predominantly rounded and appear counterstained with methyl green. Since a small number of cells naturally die during culturing or may be damaged during processing steps, 1-5% of a normal, healthy cells may stain positively. Actinomycin D (Act D) induces apoptosis by inhibiting RNA synthesis. Distinctive changes in cell shape take place within HL60 cells that have been treated with Act D. Nuclear chromatin condenses and aggregates to the inner surface of the nuclear membrane. The nuclear membrane convolutes followed by a budding-off process resulting in production of discrete apoptotic bodies. nuclear changes are accompanied by protrusion or blebbing of the cell membrane (see Panel B and C, [DNA Ladder TdT-FragEL Staining on page 8](#)) that should be readily observable in the dark brown apoptotic (positive) cells.

In tissue sections, convoluted budding and blebbing of the cell membrane is rarely seen. Many apoptotic nuclei are pyknotic and roughly rounded or oval in shape. Well preserved apoptotic bodies may be present. Since apoptosis is an asynchronous event, apoptotic cells within a tissue may be scattered throughout the cell population rather than located in groups of contiguous cells as observed with necrosis. Additionally, cytoplasmic or diffuse overall staining is often seen in necrotic cells due to loss of membrane integrity.

DNA Ladder TdT-FragEL Staining

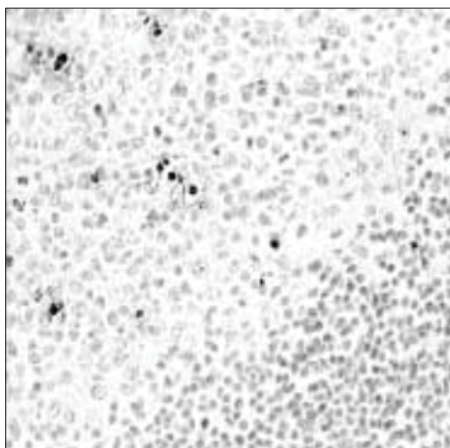
Agarose gel electrophoresis of DNA isolated from promyelocytic leukemia cells (HL60) induced to undergo apoptosis with 0.5 µg/mL actinomycin D (lane 2). DNA from control (untreated) HL60 cells is present in lane 1. Panels B and C: TdT-FragEL staining of HL60 cells induced to undergo apoptosis with 0.5 µg/mL actinomycin D (Panel C). Control (untreated) cells are shown in Panel B.

The following types of tissues were used to validate TdT-FragEL staining: human colon and breast carcinoma; prostate tumor; glioblastoma multiform; normal human skin, lymph node, tonsil, colon, lung, heart, ovary, uterus, testis, and kidney; rat uteri containing decidual tissue from day 8-11 of pseudopregnancy; and rat brain following acute subdural hematoma.

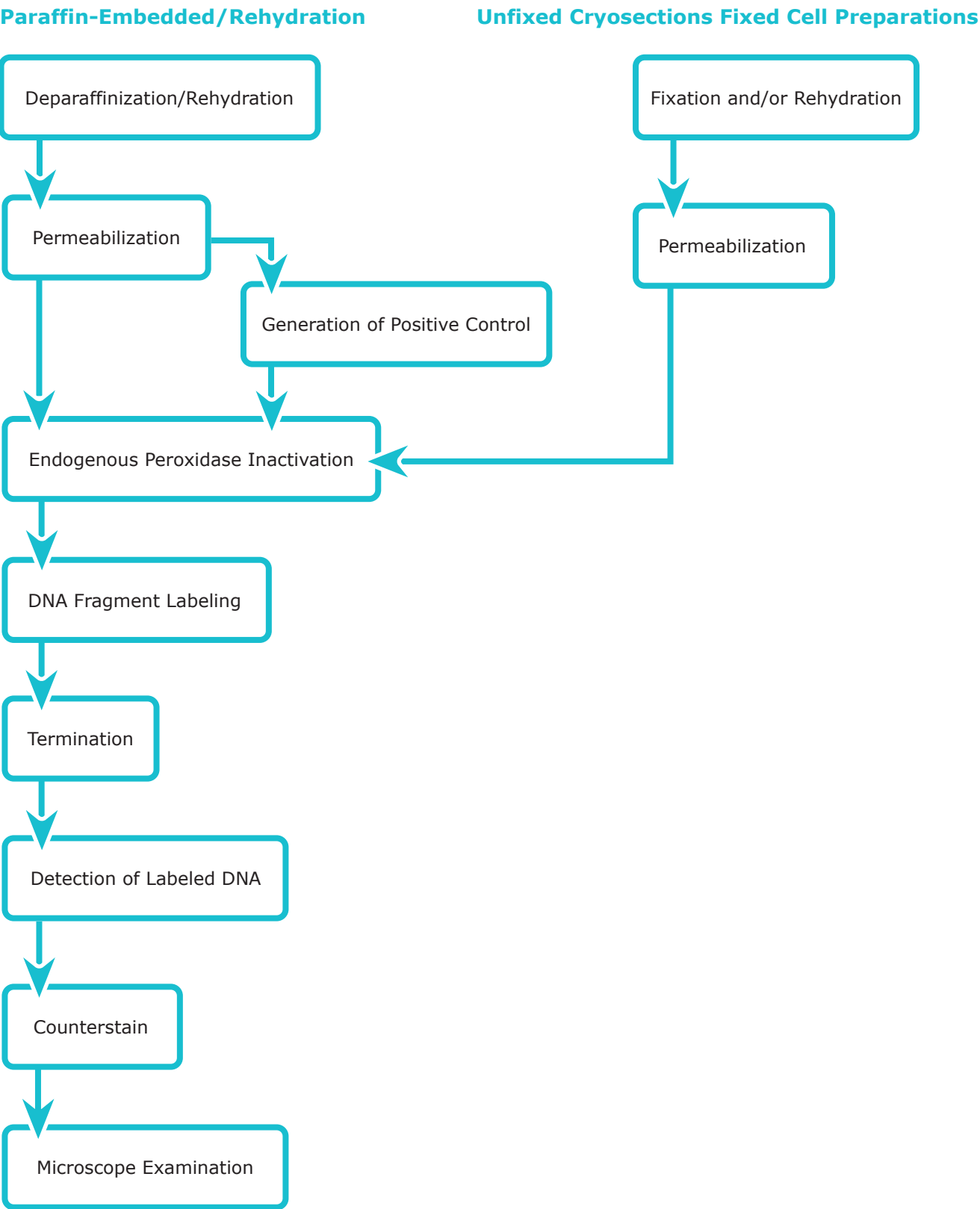


Tonsil stained with TdT-FragEL DNA Fragmentation Detection Kit

Apoptotic cells with markedly pyknotic nuclei are easily distinguishable from non-apoptotic cells. The tissue was fixed in 10% formalin and embedded in paraffin.



Protocol Summary



Notice

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