Title FragEL™ DNA Fragmentation Detection Kit, Colorimetric - TdT Enzyme | QIA33

- **Hae Jin Kee, et al. (2006) Inhibition of Histone Deacetylation Blocks Cardiac Hypertrophy Induced by Angiotensin II Infusion and Aortic Banding. *Circulation* 113, 51-59.**

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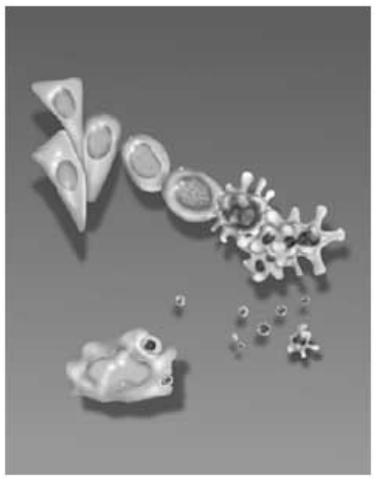
Revision	**09-Febuary-2009 RFH**
Synonyms	**TUNEL Assay**
Form	**50 Tests**
Format	**Microscopy**
Detection method	**Colorimetric**
Species	**a broad range of species**
Storage	**Upon arrival store the entire contents of the kit at -20°C in a non-frostfree freezer. Following initial thaw store the Stop 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.**
Intended use	**The Calbiochem® 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.** **Figure 1: Characteristics** 

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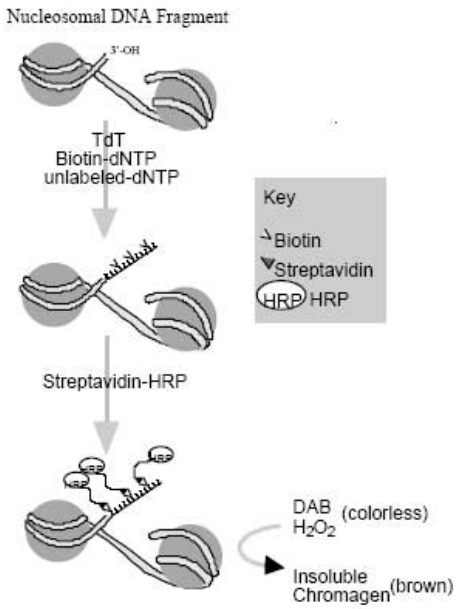
Principles of the assay	**The Calbiochem® DNA Fragmentation Detection Kits allow the recognition of apoptotic nuclei in paraffin-embedded tissue sections, tissue cryosections, or in cell preparations fixed on slides by Fragment End Labeling (FragEL™) of DNA. In this assay terminal deoxynucleotidyl transferase (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.** **Figure 2: Principle of the Assay** 
Materials provided	**The TdT-FragEL™ kit supplies sufficient reagents to stain 50 specimens.** **• PROTEINASE K (Kit Component No. JA1477-.24ML): 2 mg/ml Proteinase K in 10 mM Tris, pH 8** **• 5X TdT EQUILIBRATION BUFFER (Kit Component No. JA1558-1.75ML): 1 M Sodium Cacodylate, 0.15 M Tris, 1.5 mg/ml BSA, 3.75 mM $CoCl_2$, pH 6.6** **• TdT LABELING REACTION MIX (Kit Component No. 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 (Kit Component No. JA1560-.155ML): Terminal Deoxynucleotidyl Transferase** **• STOP BUFFER (Kit Component No. JA1479-6ML): 0.5 M EDTA, pH 8** **• BLOCKING BUFFER (Kit Component No. JA1480-12ML): 4% BSA in PBS** **• 50X CONJUGATE (Kit Component No. JA1481-.125ML): Peroxidase streptavidin conjugate 50-fold concentrated solution** **• DAB TABLETS (Kit Component No. JA1485-6EA): 3,3'Diaminobenzidine (0.7 mg/tablet)** **• H_2O_2/UREA TABLETS (Kit Component No. JA1486-6EA): H_2O_2/urea (1.6 mg/tablet)** **• METHYL GREEN COUNTERSTAIN (Kit Component No. JA1482-6ML): 0.3% Methyl green** **• CONTROL SLIDES (Kit Component No. JA1609-1EA): Two similar slides. A mixture of HL60 cells incubated with 0.5 µg/ml actinomycin D for 19 h to induce apoptosis and uninduced HL-60 cells. Supplied as formaldehyde-fixed cytospin® preparations.** **Note: Reagent volumes are stated on the particular reagent vial or bottle.**
Materials Required but not provided	**• 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 $MgSO_4$ in 1X TBS (optional, for use in generating positive control)** **• DNaseI (optional, for use in generating positive control)** **• distilled water** **• 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** **• ice**

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Precautions and recommendations	• 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 FragELTM 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 $1x10^6$/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 µl) can be immobilized onto glass slides by directly placing the cell suspension onto the slide or by using a Cytospin®. Precoating slides with poly-L-lysine may enhance cell adherence. Store cytospun samples at -20™C.
Preparation	1. Generation of Negative Control Samples A negative control of your specific sample can be generated by substituting dH_2O 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. 2. Generation of Positive Control Samples: A positive control of your specific tissue sample can be generated by covering the entire specimen with 1 µg/µl DNase I in 1X TBS/1 mM $MgSO_4$ 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	A. TdT-FragEL™ OF PARAFFIN EMBEDDED TISSUE A1. 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) A2. PERMEABILIZATION OF SPECIMEN 1. Dilute 2 mg/ml Proteinase K 1:100 in 10 mM Tris pH 8 (mix 1 µl of 2 mg/ml Proteinase K plus 99 µl 10 mM Tris per specimen).

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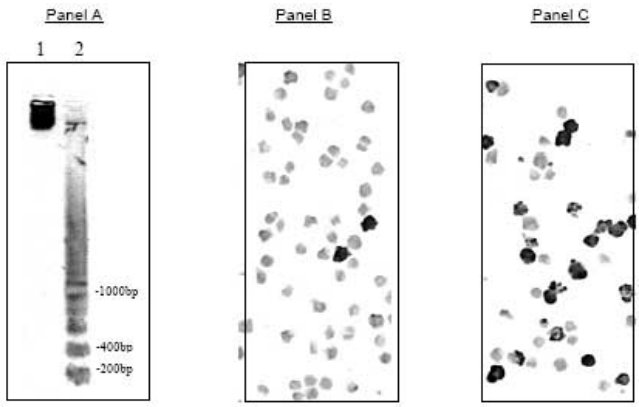
	4. Gently tap off excess liquid and carefully dry the glass slide around the specimen. **All remaining steps:** **• Inactivation of Endogenous Peroxidases** **• Equilibration and Labeling Reaction** **• Termination of Labeling Reaction** **• Detection** **• Counterstain** **are identical to those steps outlined for Klenow-FragEL™ of paraffin-embedded tissue sections (steps A4-A8)** ***Note: REMEMBER to wash slides by dipping into a beaker of 1X TBS rather than using a wash bottle.***
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 below) 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 below) 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.** **Figure 3: 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-FragELTM 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.** **Figure 4: DNA Ladder TdT-FragEL™ Staining**

SDS
Certificates of Analysis
References
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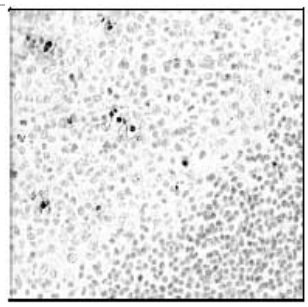
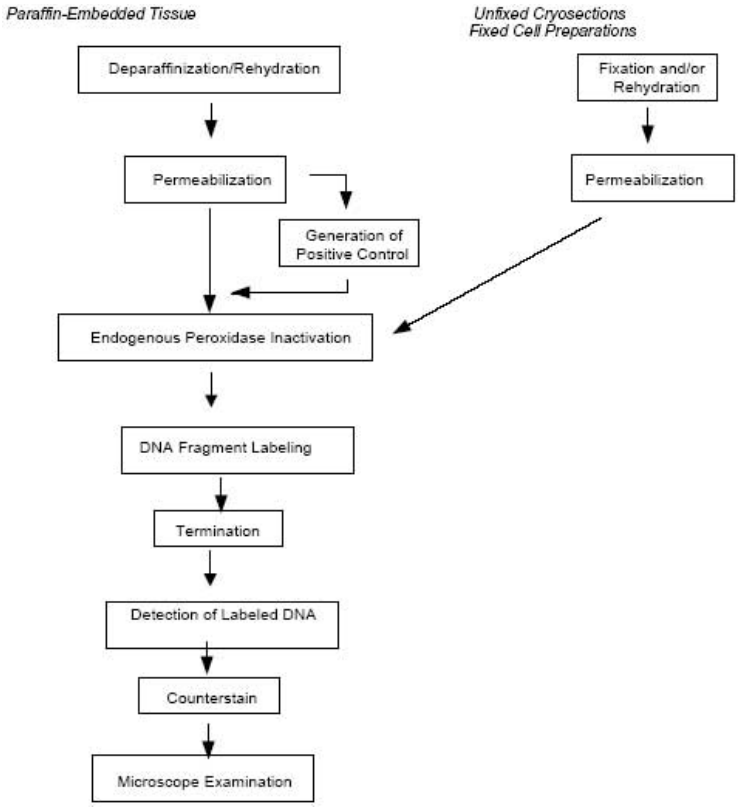
	 **Panel D: Tonsil stained with TdT-FragEL™ DNA Fragmentation Detection Kit. Apoptotic cells with markedly pyknotic nuclei are easily distinguishable from non-apoptotic cells. The tissue was fixed in 10% formalin and embedded in paraffin.**
Protocol Summary	**Figure 5: Protocol Summary** 
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