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Product no AS20 4458

DNA Fragmentation Detection Kit (30 slides)

Background

Cell death occurs by two major mechanisms, necrosis and apoptosis. Apoptosis is also known as programmed cell death or ankoikis (a form of apoptosis which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix). Apoptosis leads to the elimination of cells without releasing harmful substances into the surrounding area. Too little or too much apoptosis plays a role in a great many diseases.

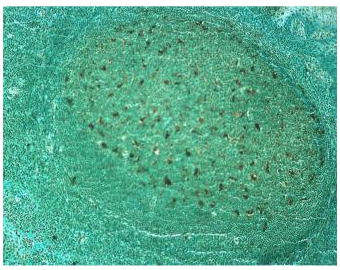

Figure 1. Positive result in DNA Fragmentation Detection Kit (X2044K) using paraffin fixed human tonsil tissue, 10 µm sections (1000X).

When apoptosis functions inappropriately, cells that should be eliminated survive and potentially become immortal, as in cancer or leukemia. When apoptosis works overly well, too many cells may 'die' and the result may be grave tissue damage. This is the case in stroke and neurodegenerative disorders such as Alzheimer, Huntington and Parkinson diseases. The term 'apoptosis' refers only to the structural changes a cell goes through during the process of programmed cell death and not to the process itself. Classical necrotic cell death occurs due to noxious injury or trauma to the cell while apoptosis is an energy dependent mechanism that takes place during normal cell development. 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. Apoptosis is the result of a cascade of molecular and biochemical events involving endogenous endonucleases that cleave DNA into the prototypical 'ladder of DNA fragments' that may be visualized 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.

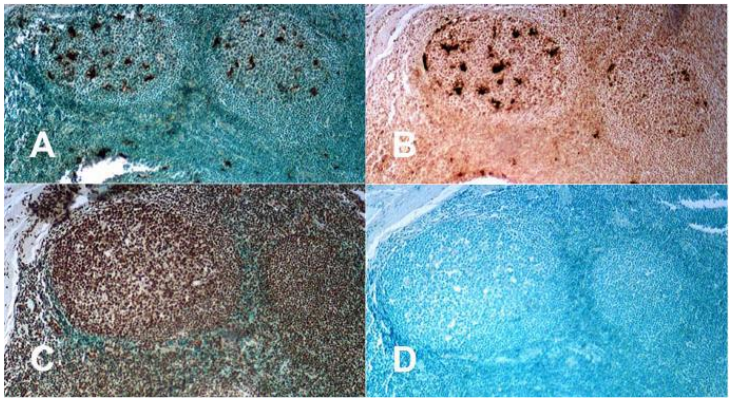

Figure 2. DNA Fragmentation Detection Kit (X2044K) using paraffin fixed human tonsil tissue, 10 µm sections (1000X). [A] Section processed and counter-stained with methyl green according to the DNA Fragmentation Detection Kit manual. [B] Counter-stain step was eliminated to more clearly illustrate the level of positive staining in the germinal centers of tonsil tissue. [C] Section treated with DNase I in order to generate a positive control slide. Note all nuclei stain positive. The use of DNase I generates free 3'-OH groups on cellular DNA, these free 3'-OH groups are then labeled with biotin-nucleotide by the TdT in the DNA Fragmentation Detection Kit (X2044K). [D] Negative control, where the TdT enzyme step was eliminated, thereby generating a negative slide.

Agrisera's DNA Fragmentation Detection Kit exploits the fact that 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 free 3'-OH groups are end-labeled by the DNA Fragmentation Detection Kit allowing for the detection of apoptotic cells using a molecular biology-based, end-labeling technique.

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Principle of the assay

Agrisera's DNA Fragmentation Detection Kit allows for the recognition of apoptotic nuclei in paraffin-embedded tissue sections, frozen tissue sections, or in preparations of single cell suspensions fixed on slides. 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 deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacts with the HRP labeled sample to generate an insoluble colored (brown) substrate at the site of DNA fragmentation. Counterstaining with Methyl Green aids in the morphological evaluation and characterization of normal and apoptotic cells.

Provided Material

The DNA Fragmentation Detection kit supplies sufficient reagents to stain 30 specimens of approx. 2.5 cm^2 size.

1	Proteinase K, a pH-stabilized Solution	50 µl
2	TdT Equilibration Buffer	4 ml
3	TdT Labeling Reaction Mix[1]	1.3 ml
4	TdT Enzyme[2]	40 µl
5	Stop Buffer	4 ml
6	Block Buffer	12 ml
7	25x Streptavidin-HRP Conjugate	150 µl
8	DAB Concentrate	150 µl
9	DAB Reaction Buffer	4 ml
10	Methyl Green Counterstain	3.5 ml

[1]An optimized mix of labeled and unlabeled nucleotides
[2]Terminal Deoxynucleotidyl Transferase.

Storage

The DNA Fragmentation Detection Kit components are shipped on cold pack. Upon receipt, store kit at -20°C in a non-frost-free freezer. For long-term storage, it is recommended that you aliquot and freeze the TdT Enzyme (Component 4), TdT Labeling Reaction Mix (Component 3), and 25x Streptavidin-HRP Conjugate (Component 7) at -20°C. Thirty (30) minutes prior to use of each component, thaw component and keep on cold block or on ice. Return the components to -20°C for long-term storage or 4-8°C for short-term storage (up to 2 weeks) immediately after use. Special care should be taken to keep TdT Enzyme (Component 4), TdT Labeling Reaction Mix (Component 3) and 25x Streptavidin-HRP Conjugate (Component 7) cold by pulling out the number of aliquots needed for the test, keeping them on ice, and leaving the remaining aliquots at -20°C.

Not provided in the kit but required

Xylene **|** Ethanol, 100%, 90%, 80%, 70% **|** Methanol **|** 30% hydrogen peroxide **|** Tris-buffered saline (1x TBS, 20 mM Tris pH 7.6, 140 mM NaCl) **|** DNase I (optional, for use in generating positive control) **|** Distilled de-ionized water **|** Coplin jars, glass or plastic with slide holders **|** Humidified chamber **|** Glass or plastic coverslips **|** Mounting media (such as Permount®) **|** Microscope **|** 1-20 µl, 20-200 µl, and 200-1000 µl precision pipettors **|** Sterile DNase/RNase free disposable pipette tips **|** Microcentrifuge tubes **|** Absorbent wipes (such as Kimwipes) **|** Cold block or ice bath **|** Pap pen

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Important notes

READ ALL INSTRUCTIONS COMPLETELY BEFORE PERFORMING ASSAY.

• The TdT Enzyme contains glycerol and will not freeze solid at -20°C. To preserve the activity of this enzyme, do not remove it from the -20°C freezer storage until immediately before use in preparing the labeling reaction mixture. Pulse-spin the TdT Enzyme tube in a microcentrifuge prior to opening. Place the TdT Enzyme in a -20°C storage device (cold block or ice bath) for use. To preserve enzyme activity, return immediately to -20°C for long-term storage or 4-8°C for short-term storage (up to 2 weeks) after use. Components containing glycerol should not be stored at -80°C.

• All other DNA Fragmentation Detection Kit components, with the exception of Stop Buffer (Component 5), Block Buffer (Component 6), and Methyl Green Counterstain (Component 10), should be kept on ice or in a cold block during usage, and then promptly returned to -20°C for long-term storage or 4-8°C for short-term storage (up to 2 weeks) after use.

• To avoid reagent loss in tube caps, briefly pulse spin all solutions before removing caps and before use.

• Diaminobenzidine (DAB) solution contains potential carcinogens.

• Cacodylic acid is a component of the TdT Equilibration Buffer and TdT Labeling Reaction Mix. Cacodylic acid is toxic and carcinogenic. Avoid contact with eyes and skin. Do not ingest.

• Gloves, lab coat, and protective eyewear should be worn. Refer to your institution's health and safety guidelines for appropriate procedures.

• Separate protocols have been provided for the end labeling of paraffin-embedded tissue sections, tissue cryosections, and cell preparations fixed on slides. See 'Application Notes' at the end of this manual for tips on performing these procedures.

• 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.

• A humidified chamber should be used for all steps indicated to prevent reagent loss from evaporation.

Procedure Outline

Paraffin-Embedded Sections

Activity	**Time (min)**
Rehydrate samples	29
Rinse with 1x TBS	5
Permeabilize samples using Component 1	20
Rinse with 1x TBS	5
Inactivate endogenous peroxidases. Incubate with 3% H_2O_2	5
Rinse with 1x TBS	5
Equilibrate samples with Component 2	30
Add Component 4 to Component 3 and incubate	90
Rinse with 1x TBS	5
Terminate reaction with Component 5	5
Rinse with 1x TBS	5
Block samples with Component 6	10
Dilute Component 7 to 1x and add to each slide	30
Rinse with 1x TBS	5
Prepare DAB Solution (Components 8 & 9) and add to all slides	15
Rinse with dH_2O	5
Counterstain with Component 10	1-3
Dehydrate with 100% ethanol - 100% Xylene and mount with coverslip	15

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Detailed Assay Protocol

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.

1. Rehydration of tissue

1.1 Immerse slides in Xylene for 5 minutes at room temperature. Repeat (total two 5-minute incubations).
NOTE: Xylene should be changed frequently.
1.2. Immerse slides in 100% ethanol for 5 minutes at room temperature. Repeat (total two 5-minute incubations).
1.3. Immerse slides in 90% ethanol for 3 minutes at room temperature.
1.4. Immerse slides in 80% ethanol for 3 minutes at room temperature.
1.5. Immerse slides in 70% ethanol for 3 minutes at room temperature.
1.6. Rinse slides briefly with 1x TBS for 5 minutes and 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 (Pap Pen).

2. Permeabilization of Specimen

2.1. Dilute Proteinase K (Component 1) 1:100 in dH_2O (mix 1 µl of Component 1, Proteinase K, plus 99 µl dH_2O per specimen).
2.2. Cover the entire specimen with 100 µl of Proteinase K solution prepared above and incubate at room temperature for 20 minutes.
2.3. Rinse slide with 1x TBS for 5 minutes.
2.4. Gently tap off excess liquid and carefully dry the glass slide around the specimen using a Kimwipe or other adsorbent material. Care should be taken to not touch the specimen.

3. Quenching: Inactivation of Endogenous Peroxidases

3.1. Dilute 30% H_2O_2 1:10 in methanol (mix 10 µl 30% H2O2 with 90 µl methanol per specimen).
3.2. Cover the entire specimen with 100 µl of 3% H_2O_2. Incubate at room temperature for 5 minutes.
3.3. Rinse slide with 1x TBS for 5 minutes.
3.4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

4. Equilibration

4.1. Cover the entire specimen with 100 µl of TdT Equilibration Buffer provided (Component 2). Incubate at room temperature for 30 minutes. During the last five minutes of this incubation prepare the Labeling Reaction Mixture.

5. Labeling Reaction

5.1. Prepare the working TdT Labeling Reaction Mixture as follows: Pulse-spin the TdT Enzyme tube in a microcentrifuge prior to opening. Prepare only enough TdT Labeling Reaction Mix only for the number of samples/slides to be labeled. For each sample to be labeled add 1 µl TdT Enzyme (Component 4) to 39.0 µl TdT Labeling Reaction Mix (Component 3) in a clean microfuge tube, mix gently and keep on ice or a cold block until use.
5.2. Carefully blot the TdT Equilibration Buffer from the specimen, taking care not to touch the Specimen.
5.3. Immediately apply 40 µl of TdT Labeling Reaction Mixture (prepared above) onto each specimen and cover the specimen with a coverslip to assures even distribution of the reaction mixture and prevent loss due to evaporation during incubation.
5.4. Place slides in a humidified chamber and incubate at room temperature (at least 22°C) for 1.5 hours.
NOTE: If room temperature is below 22°C the use of a 37°C incubator is recommended.

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6. Termination of Labeling Reaction

6.1. Locate the Stop Buffer (Component 5). If a precipitate is present, warm the Stop Buffer to 37°C for five minutes or until precipitate is no longer evident.

6.2. Remove coverslip* and rinse slide with 1x TBS for 5 minutes.

*HELPFUL HINT: Cover slip is best removed by submerging the slide in TBS solution in a Coplin jar or beaker and allowing cover slip to gently slide off specimen. A glass cover slip is best, but plastic cover slip may be used.

6.3. Cover the entire specimen with 100 µl of Stop Buffer. Incubate at room temperature for 5 minutes.

6.4. Rinse slide with 1x TBS for 5 minutes.

6.5. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

7. Blocking

7.1. Cover the entire specimen with 100 µl of Block Buffer (Component 6). Incubate at room temperature for 10 minutes. During the last 5 minutes of blocking prepare the Streptavidin-HRP Conjugate solution.

8. Detection

8.1. Dilute the 25x Streptavidin-HRP Conjugate (Component 7) 1:25 in Block Buffer (Component 6) by mixing 4 µl 25x Streptavidin-HRP Conjugate with 96 µl Block Buffer per specimen. Prepare only enough working solution for the number of slides/specimens being processed. Keep on ice or a cold block until ready to use.

8.2. Carefully blot the Block Buffer from the specimen, taking care not to touch the specimen. Immediately apply 100 µl of diluted 1x Streptavidin-HRP Conjugate to the specimen.

8.3. Place slides in a humidified chamber and incubate at room temperature for 30 minutes.

8.4. Rinse slides with 1x TBS for 5 minutes.

9. Development

9.1. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

9.2. Prepare working DAB solution by adding 4 µl DAB Concentrate (Component 8) to 116 µl DAB Reaction Buffer (Component 9) (1:30 ratio). Prepare only enough working DAB solution for specimens to be processed. Do not store diluted DAB solution. Prepare fresh on each occasion.

9.3. Cover the entire specimen with 100 µl of DAB solution prepared in section 9.2 above. Incubate at room temperature for 15 minutes.

9.4. Rinse slides gently with dH_2O.

10. Counterstain and Storage

10.1. Immediately cover the entire specimen with 100 µl of Methyl Green Counterstain solution provided (Component 10).

10.2. Incubate at room temperature for 1-3 minutes.

10.3. 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.

10.4. Dip slides 2-4 times into 100% ethanol.

10.5. Blot slides briefly on an absorbent towel.

10.6. Repeat step 4 using fresh 100% ethanol. Blot slides briefly on an absorbent towel.

10.7. Dip slides 2-4 times into 100% Xylene.

10.8. Wipe excess Xylene from back of slide and around specimen.

10.9. Mount a glass coverslip using a mounting media (such as Permount®) over the specimen.

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Application notes

A. How to make a humidified chamber.
A simple humidified chamber may be constructed using an empty plastic pipette box, Tupperware™ (or another plastic container with lid). Place a moist paper towel or Kimwipe™ on the bottom taking care not to over wet. Place slides carefully onto moist surface or construct a support scaffolding using plastic pipettes and place slides onto this support.

B. Using the DNA Fragmentation Detection Kit with Tissue Cryosections
This protocol is similar the protocol for 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 minutes. 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 gently 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 additional time is needed between steps to prepare reagents etc. cover or immerse the slides in 1X TBS to keep hydrated until use.

Tissue Fixation and Hydration
1. Immerse slides in 4% formaldehyde (prepared in 1x PBS) for 15 minutes at room temperature.
2. Gently drain off excess liquid and carefully dry the glass slide around the specimen.
3. Immerse slides in 1x TBS for 15 minutes 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 (Pap pen) or a hydrophobic slide marker.

Permeabilization of Specimen
1. Dilute Proteinase K (Component 1) 1:100 in dH_2O (mix 1 µl of Component 1, Proteinase K, plus 99 µl dH_2O per specimen).
2. Cover the entire specimen with 100 µl of Proteinase K solution prepared above and incubate at room temperature for 10 min. DO NOT OVERINCUBATE.
3. Rinse slide with 1x TBS for 5 minutes.
4. Gently tap off excess liquid and carefully dry the glass slide around the specimen using a Kimwipe or other adsorbent material. Care should be taken to not touch the specimen

All remaining steps are identical to those steps outlined for paraffin-embedded tissue sections. Proceed from *Quenching: Inactivation of Endogenous Peroxidases* and complete procedure.

Care should be taken during wash steps to avoid losing tissue sections. Washing by gentle emersion is recommended.

C. Using the DNA Fragmentation Detection Kit with Fixed Cell Preparations
Procedure for fixing cell suspensions or cell lines as preparation for performing the DNA Fragmentation Detection Kit procedure.

Fixing Cell Preparations
Cells grown in suspension (suspension cultures etc.) can be fixed and attached to slides.
1. Cells are pelleted by gentle centrifugation for 5 minutes at 4°C.
2. Wash cells 2x with cold (4°C) PBS.
3. Cells are then resuspended in 4% formaldehyde (in PBS) at a cell density of 1x106/ml and incubated at room temperature for 10 minutes.
4. Cells are pelleted by gentle centrifugation for 5 minutes at room temperature and resuspended, at the same concentration, in 80% ethanol.
5. Store fixed cells at 4°C.

6. Fixed cells (100-300 µl) can be immobilized onto glass slides by directly placing the cell suspension onto the slide and allowing to air dry. The use of a cytospin may also be used – follow manufacturer's recommendation for slide preparation.

NOTE: Pre-coating slides with poly-L-lysine may enhance cell adherence. Store cytospun samples at -20°C. Slides may be stored for up to 6 months at -20°C until used.

This protocol varies from the standard DNA Fragmentation Detection Kit procedure for paraffin-embedded tissue sections. Replace the deparaffinization step with a rehydration step. Permeabilization with Proteinase K is performed for only 5 minutes.

NOTE: 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.

NOTE: DO NOT LET THE CELLS DRY OUT BETWEEN OR DURING ANY STEP. If necessary, cover or immerse the slide in TBS to keep hydrated.

Rehydration
1. Immerse slides in 1x TBS for 15 minutes at room temperature.
2. 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 (Pap pen).

Permeabilization of specimen
1. Dilute Proteinase K (Component 1) 1:100 in dH_2O (mix 1 µl of Component 1, Proteinase K, plus 99 µl dH_2O per specimen).
2. Cover the entire specimen with 50-100 µl of Proteinase K solution.
3. Incubate at room temperature for 5 min. DO NOT OVERINCUBATE.
4. Dip slide 2-3 times into a beaker of 1x TBS.
5. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

All remaining steps are identical to those steps outlined for paraffin-embedded tissue sections. Proceed from *Quenching: Inactivation of Endogenous Peroxidases* and complete procedure.

Evaluation of Results

An apoptosis end point, indicative of positive staining in the DNA Fragmentation Detection Kit assay, is represented by a dark brown (DAB) signal. Lighter shades of brown and/or shades of blue-green to greenish tan indicate a nonreactive/negative cell.

Since 3'-OH ends of DNA fragments generated during apoptosis are concentrated within the nuclei and apoptotic bodies, morphology as well as DAB staining can and should be used to interpret DNA Fragmentation Detection Kit results. Characteristic morphological changes during apoptosis are well characterized and should be used as verification of programmed cell death. Non-apoptotic cells do not incorporate significant amounts of biotin labeled nucleotide since they lack free 3'-OH ends (indicative of apoptosis).
After performing the DNA Fragmentation Detection Kit test, careful evaluation of the slides should be performed using a light microscope.

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Generation of Control Samples

A. Generation of Negative Control

An appropriate negative control to employ is the elimination of the TdT enzyme from a duplicate slide. Simply perform the DNA Fragmentation Detection Kit test as outlined and substitute dH_2O for the TdT in the Labeling Reaction Mixture or keep the specimen in reaction buffer (with a cover slip to prevent drying out) during the labeling step. Perform all other steps as described in the manual. This is a suitable control for endogenous peroxidases and non-specific conjugate binding or background in the assay. A nonapoptotic control is also a useful control. A delay in fixation or routine mechanical manipulation may result in the unwanted breakage of DNA that could be read as apoptosis.

B. Generation of Positive Control

A positive control can be generated from your slides (or any commercially available slide preparation of any species or tissue - slides of 10 µm thickness are preferred).

A positive slide/sample can be generated by treating a slide(s) with 1 µg/µl DNase I in TBS/1 mM $MgSO_4$ for 20 minutes at room temperature immediately following the Proteinase K treatment step in the DNA Fragmentation Detection Kit manual.

Perform all other steps as described in the DNA Fragmentation Detection Kit manual.

The DNase I treatment will fragment DNA in normal cells, generating free 3'-OH groups identical to those generated during apoptosis.