Agrisera

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Product no AS20 4459

DNA Fragmentation Detection Kit (60 slides)

Background

This DNA Fragmentation Detection Kit utilizes the fact that Terminal deoxynucleotidyl Transferase (TdT) binds to exposed 3'-OH ends of DNA fragments. During apoptosis, such DNA fragments are generated, which catalyzes the addition of biotin-labeled deoxynucleotides. A streptavidin-horseradish peroxidase (HRP) conjugate is used to detect biotinylated nucleotides, and diaminobenzidine (DAB) reacts with the HRP-labeled sample, generating a brown, insoluble substrate at the sites where DNA fragmentation has taken place. Counterstaining with Methyl Green allows the morphological evaluation and characterization of normal and apoptotic cells. The DNA Fragmentation Detection Kit can be used for the identification apoptotic nuclei in paraffin-embedded tissue sections, frozen tissue sections, or in preparations of single cell suspensions fixed on slides.

DNA Fragmentation Detection Kit reagents

Proteinase K, a pH-stabilized Solution	100 µl
TdT Equilibration Buffer	8 ml
TdT Labeling Reaction Mix (mix of labeled and unlabeled nucleotides)	2 x 1.3 ml
TdT Enzyme (Terminal Deoxynucleotidyl Transferase)	70 µl
Stop Buffer	8 ml
Block Buffer	24 ml
Streptavidin-HRP Conjugate (25x concentrate)	300 µl
DAB Concentrate	300 µl
DAB Reaction Buffer	8 ml
Methyl Green Counterstain	2 x 3.5 ml

This kit contains reagents for 60 tests.

Storage

Store kit at -20°C. For long-term storage, it is recommended to keep the TdT Enzyme, TdT Labeling Reaction Mix and Streptavidin-HRP Conjugate (25x concentrate) frozen in aliquots. Use only the amount needed of these components for each test, while leaving the remaining aliquots at -20°C. For short-term storage (up to 2 weeks), the kit components can be kept at 4-8°C.

This kit should not be used beyond the expiration date.

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Material not provided in the kit but required to conduct the test

Xylene **|** Ethanol (70%, 80%, 90%, 100%) **|** Methanol **|** Hydrogen peroxide (30%) **|** Tris-buffered saline (1x TBS, 20 mM Tris pH 7.6, 140 mM NaCl) **|** DNase I (optional, positive control) **|** Deionized or distilled water **|** Coplin jars with slide holders **|** Humidifier chamber **|** Coverslips **|** Mounting media **|** Microscope **|** Micropipette (1-20 µl, 20-200 µl, 200-1000 µl) **|** Sterile DNase/RNase free disposable pipette tips **|** Microcentrifuge tubes **|** Absorbent wipes **|** Cold block or ice bath **|** Pap pen

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Important notes

The kit should not be used beyond the expiration date on the kit label. Reagents from different lots should not be mixed.

The TdT Enzyme contains glycerol and will not freeze solid at -20°C. To preserve the enzyme activity, keep the TdT Enzyme at -20°C until immediately before using it in preparing the labeling reaction mixture. Use a microcentrifuge to pulse-spin the TdT Enzyme tube prior to opening it. Keep the TdT Enzyme at -20°C for use, employing a cold block or ice bath. 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 components of the DNA Fragmentation Detection Kit, except the Stop Buffer, Block Buffer and Methyl Green Counterstain should be kept on ice or in a cold block during usage. After use, the components should be returned to -20°C for long-term storage, or 4-8°C for short-term storage (up to 2 weeks).

Briefly pulse-spin all solutions before use, to avoid reagent loss in tube caps.

Use a humidifier chamber for incubation and coverslips, when indicated, to ensure even reaction mixture distribution, and to avoid reagent loss from evaporation.

The supplied protocol should be used as a guideline. Empirical determination of the incubation time for Proteinase K, DNase I and labeling may be needed for the specific cell type and slide preparation in question.

Safety measures

Cacodylic acid, which is toxic and carcinogenic, is a component of the DNA Fragmentation Detection Kit TdT Equilibration Buffer and TdT Labeling Reaction Mix. Avoid contact with the eyes and skin. Do not ingest. The DNA Fragmentation Detection Kit Diaminobenzidine (DAB) solution contains potential carcinogens. Please use suitable protective gear for your eyes, hands, face, and clothes while handling these items.

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Protocol

Please read the whole protocol carefully before proceeding with your experiment.

The following protocol is for paraffin-embedded sections. Separate protocols for the end-labeling of tissue cryosections and cell preparations fixed on slides is provided at the end of this section.

1. **Rehydration of tissue**

Note: DO NOT let specimen dry out between steps. Cover or immerse specimen in TBS (1x) to keep hydrated, if necessary.

Immerse and incubate the slides in the following solvents and order at room temperature:
2 x 5 min in Xylene *(Note: Xylene should be changed frequently)*,
2 x 5 min in 100% ethanol,
1 x 3 min in 90% ethanol,
1 x 3 min in 80% ethanol,
1 x 3 min in 70% ethanol.
Rinse the slides for 5 min in TBS (1x). Carefully dry the glass around the specimen.
Note: Encircling the specimen using a waxed pen or Pap Pen may be helpful in containing the small reaction volumes around the specimen.

2. **Permeabilization of specimen**

Mix 1 µl of Proteinase K with 99 µl of deionized water (1:100 dilution). Cover the specimen completely with 100 µl diluted Proteinase K solution. Incubate for 20 min at room temperature. Rinse the slide for 5 min with TBS (1x). Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen.*

3. **Quenching (inactivating endogenous peroxidases)**

Mix 10 µl 30% hydrogen peroxide with 90 µl methanol (1:10 dilution). Cover the specimen completely with 100 µl of 3% hydrogen peroxide. Incubate for 5 min at room temperature. Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen.*

4. **Equilibration**

Cover the specimen completely with 100 µl of TdT Equilibration Buffer. Incubate for 30 min at room temperature. *Note: Prepare the Labeling Reaction Mixture during the last 5 min of this incubation.*

5. **Labeling reaction**

Prepare the Labeling Reaction Mixture by using a microcentrifuge to pulse-spin the TdT Enzyme tube. For each sample to be labeled, gently mix 1 µl TdT Enzyme with 39 µl TdT Labeling Reaction Mixture in a clean microfuge tube. Keep on ice or cold block until use. Blot the TdT Equilibrium Buffer from the specimen carefully. *Note: Care should be taken to not touch the specimen*. Immediately apply 40 µl of TdT Labeling Reaction Mixture onto each specimen. To ensure the even distribution of reaction mixture and prevent loss due to evaporation, cover the specimen with a coverslip during incubation. Place the slides in a humidifier chamber and incubate at a minimum of 22°C for 1.5 h.

6. **Termination of labeling reaction**

Prepare the Stop Buffer: if there is precipitation, heat the Stop Buffer for 5 min at 37°C. Remove the coverslip from the slide and rinse for 5 min with TBS (1x). *Note: Submerging the slide in TBS solution is a good way to remove the cover slip.* Cover the specimen completely with 100 µl of Stop Buffer. Incubate for 5 min at room temperature. Rinse the slide again with TBS (1x) for 5 min. Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen.

7. **Blocking**
   Cover the specimen completely with 100 µl Block Buffer. Incubate for 10 min at room temperature. *Note: Prepare the Streptavidin-HRP Conjugate solution during the last 5 min of this incubation.*

8. **Detection**
   Mix 4 µl of Streptavidin-HRP Conjugate (25x) with 96 µl Block Buffer (1:25 dilution) per specimen to be processed. Keep on ice or a cold block until ready to use. Blot the Block Buffer from the specimen carefully. *Note: Care should be taken to not touch the specimen*. Immediately apply 100 µl of diluted Streptavidin-HRP Conjugate (1x) to the specimen. Place the slides in a humidifier chamber and incubate at room temperature for 30 min. Rinse the slides for 5 min with TBS (1x).

9. **Development**
   Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen.*
   Prepare the working DAB solution: add 4 µl of DAB Concentrate to 116 µl DAB Reaction Buffer (1:30 ratio) per specimen to be processed. *Note: Prepare the DAB solution fresh. DO NOT store.* Cover the specimen completely with 100 µl DAB solution and incubate for 15 min at room temperature. Gently rinse the slides with deionized water.

10. **Counterstaining and storage**
    Cover the specimen completely with 100 µl of Methyl Green Counterstain solution, immediately following the previous step. Incubate for 1-3 min at room temperature. Draw off most of the counterstain by pressing the edge of the slide against an absorbent towel. Place the slide in a complin jar slide holder. Dip the slide in 100% ethanol 2-4 times, and subsequently blot the slide on an adsorbent towel. Repeat the previous dipping and blotting step with fresh ethanol. Dip slides in Xylene 2-4 times, and subsequently wipe the excess Xylene from the back of the slide and around the specimen. Mount a glass coverslip over the specimen using mounting media.

Bellow follow separate protocols for the end labeling of tissue cryosections and cell preparations fixed on slides.

Using the DNA Fragmentation Detection Kit with tissue cryosections
This protocol is similar to that for paraffin-embedded tissue, with the EXCEPTIONS that the paraffinization step is replaced by a hydration step, and that the Proteinase K permeabilization is only performed for 10 min.

Note: DO NOT let specimen dry out between steps. Cover or immerse specimen in TBS (1x) to keep hydrated, if necessary.
Note: It is recommended to dip the slides gently 2-3 times in a beaker of TBS (1x) instead of rinsing with a wash bottle, to avoid loss of tissue from the glass slide.

A. Tissue fixation and hydration
Immerse the slide in 4% formaldehyde, prepared in PBS (1x), for 15 min at room temperature. Drain off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. Immerse the slide in TBS (1x) for 15 min at room temperature. Carefully dry the glass slide in the areas around the specimen. *Note: Encircling the specimen using a waxed pen or Pap Pen, may be helpful in containing the small reaction volumes around the specimen.*

B. Permeabilization of specimen
Mix 1 µl of Proteinase K with 99 µl deionized water (1:100 dilution). Cover the specimen completely with 100 µl diluted Proteinase K solution. Incubate for 10 min at room temperature. DO NOT OVERINCUBATE. Rinse the slide with TBS (1x) for 5 min. Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen.*

All further steps are identical to those described in the protocol for paraffin-embedded sections. Continue from the step **Quenching (inactivating endogenous peroxidases)**.

Using the DNA Fragmentation Detection Kit with fixed cell preparation
This protocol is similar to that for paraffin-embedded tissue, with the EXCEPTIONS that the paraffinization step is replaced by a rehydration step, and that the Proteinase K permeabilization is only performed for 5 min. Included in this protocol is also the fixation of cell preparations.

Note: DO NOT let specimen dry out between steps. Cover or immerse specimen in TBS (1x) to keep hydrated, if necessary.
Note: It is recommended to dip the slides gently 2-3 times in a beaker of TBS (1x) instead of rinsing with a wash bottle, to avoid loss of tissue from the glass slide.

A. **Fixing cell preparations**
Pellet the cells by centrifuging them gently for 4 min at 4°C. Wash the cells twice with cold (4°C) PBS. Resuspend the cells at a cell density of $1x10^6$/ml in 4% formaldehyde, prepared in PBS. Incubate for 10 min at room temperature. Pellet the cells by centrifuging them gently for 5 min at room temperature. Resuspend the cells at a cell density of $1x10^6$/ml in 80% ethanol. Store fixed cells at 4°C. Immobilization of fixed cells (100-300 µl) can be done by directly placing the cell suspension onto the slide and letting air dry. *Note: Cell adherence may be enhanced by pre-coating the slide with poly-L-lysine. Cytospun samples should be stored at -20°C. Slides may be stored at -20°C for 6 months until used.*

B. Rehydration
Immerse slides in TBS (1x) for 15 min at room temperature. Carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen. Note: Encircling the specimen using a waxed pen or Pap Pen, may be helpful in containing the small reaction volumes around the specimen.*

C. Permeabilization of specimen
Mix 1 µl of Proteinase K with 99 µl deionized water (1:100 dilution). Cover the specimen completely with 50-100 µl diluted Proteinase K solution. Incubate for 5 min at room temperature. DO NOT OVERINCUBATE. Dip the slide 2-3 times into a beaker of TBS (1x). Tap off excess liquid gently, and carefully dry the glass slide in the areas around the specimen. *Note: Care should be taken to not touch the specimen.*

All further steps are identical to those described in the protocol for paraffin-embedded sections. Continue from the step **Quenching (inactivating endogenous peroxidases)**.

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Results

Positive staining in the DNA Fragmentation Detection Kit assay is indicated by a dark brown (DAB) signal. A nonreactive or negative cell is indicated by lighter shades of brown and/or shades of blue-green to greenish tan.

As verification of programmed cell death, the characteristic morphological changes during apoptosis should be used. As the 3'-OH ends of the DNA fragments that are generated during apoptosis are concentrated in the nuclei and apoptotic bodies, morphology should be used together with DAB staining to interpret the DNA Fragmentation Detection Kit results. Since non-apoptotic cells lack free 3'-OH ends, these do not incorporate significant amounts of biotin-labeled nucleotides.

The evaluation of the slides processed using the DNA Fragmentation Detection Kit test should be carefully evaluated using a light microscope.

Generating control samples

Negative control

A negative control may be used to control for endogenous peroxidases, non-specific conjugate-binding, or assay background. A duplicate slide eliminated of the TdT Enzyme is an appropriate negative control. To create such, follow the protocol as outlined, while substituting the TdT Enzyme with deionized water in the Label Reaction Mixture, or simply keep the specimen in reaction buffer (under a cover slip) during the labeling step. All other steps should be performed as described in the protocol. Beyond this, a non-apoptotic control may be useful as mechanical manipulation or delays in fixation may result in unwanted DNA breakage, which in turn could lead to apoptosis.

Positive control

DNase I treatment fragments DNA in normal cells, which generates free 3'OH groups identical to those created during apoptosis. To generate a positive sample this way, treat one of your slides with 1 µg/µl DNase I (in TBS/1 mM $MgSO_4$) for 20 min at room temperature, immediately following the Proteinase K treatment step according to the above protocol. All other steps should be performed as described.

Related products

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