Instructions for Use

For detection of apoptotic cells.

This product is for research use only and is not intended for diagnostic use.
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1. **BACKGROUND**

*In situ* Apoptosis Detection Kit (ab206386) allows 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 Transf erase (TdT) binds to exposed 3’-OH ends of DNA fragments, which are 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 brown substrate at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells.

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. This apoptosis detection assay exploits the fact that, when the apoptotic endonucleases produce the classical DNA ladder, they generate free 3’-OH groups at the ends of these DNA fragments. These free 3’-OH groups are end-labelled by the *in situ* Apoptosis Detection Kit, allowing for the detection of apoptotic cells using a molecular biology-based end labelling technique.
## 2. ASSAY SUMMARY

<table>
<thead>
<tr>
<th>Activity</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehydrate samples</td>
<td>25</td>
</tr>
<tr>
<td>Permeabilize samples using Proteinase K</td>
<td>23</td>
</tr>
<tr>
<td>Inactivate endogenous peroxidases with 3% H₂O₂</td>
<td>5</td>
</tr>
<tr>
<td>Label with TdT Enzyme</td>
<td>125</td>
</tr>
<tr>
<td>Block samples with Blocking Buffer</td>
<td>10</td>
</tr>
<tr>
<td>Incubate with Conjugate</td>
<td>35</td>
</tr>
<tr>
<td>Prepare and incubate with DAB Solution</td>
<td>20</td>
</tr>
<tr>
<td>Counterstain with Methyl Green Counterstain</td>
<td>1-3</td>
</tr>
<tr>
<td>Dehydrate mount with coverslip</td>
<td>15</td>
</tr>
</tbody>
</table>

Note that this assay summary represents the use of ab206386 with paraffin-embedded tissue sections. A modified protocol should be followed for end-labelling of tissue cryosections and cell preparations fixed on slides (see Pages 10-16 for further details).
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature.

5. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
## 6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount (30 test size)</th>
<th>Amount (60 test size)</th>
<th>Storage Condition (Before preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase K</td>
<td>50 µL</td>
<td>100 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>TdT Equilibration Buffer</td>
<td>4 mL</td>
<td>8 mL</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>TdT Labeling Reaction Mix</td>
<td>1 x 1.3 mL</td>
<td>2 x 1.3 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>TdT Enzyme</td>
<td>40 µL</td>
<td>70 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Stop Buffer</td>
<td>4 mL</td>
<td>8 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>12 mL</td>
<td>24 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>25X Conjugate</td>
<td>150 µL</td>
<td>300 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>DAB Solution 1</td>
<td>150 µL</td>
<td>300 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>DAB Solution 2</td>
<td>4 mL</td>
<td>8 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Methyl Green Counterstain</td>
<td>1 x 3.5 mL</td>
<td>2 x 3.5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Xylene
- Ethanol, 100, 90, 80 and 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 (dH₂O)
- Coplin jars, glass or plastic with slide holders
- Humidified chamber (see Technical Hints section)
- Glass or plastic coverslips
- Mounting media (ab64230)
- Microscope
- 1-20 μL, 20-200 μL, and 200-1000 μL precision pipettes
- Sterile DNase/RNase free disposable pipette tips
- Microcentrifuge tubes
- Absorbent wipes
- Cold block or ice bath
- PAP pen (ab2601)
8. **TECHNICAL HINTS**

- Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Read through the protocol before running the assay as modified sample preparation protocols are required for suspension cell lines, fixed cell preparations and tissue cryosections.

- Please refer to Section 11 for suitable positive and negative controls.

- Incubation times for Proteinase K, DNase I and labeling may need to be optimized for your cell type and slide preparation using this protocol as a starting point.

- Keep enzymes, heat labile components and samples on ice during the assay. The TdT Enzyme should be placed in a -20°C storage device during use.

- Make sure all buffers and solutions are kept on ice before starting the experiment.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross-contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- A humidified chamber should be used where indicated to prevent reagent loss through evaporation. To construct a simple humidified chamber, place a moist paper towel at the bottom of a plastic box with a lid (ensure that the paper towel is not too wet). Either place slides carefully directly onto the moist surface or onto a support made by placing plastic pipettes onto the moist surface. Place lid on box.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening to avoid reagent loss in tube caps.

9.1 **Proteinase K:**

Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Return to -20°C immediately after use.

9.2 **TdT Equilibration Buffer:**

Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Return to -20°C immediately after use.

9.3 **TdT Labelling Reaction Mix:**

Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Special care should be taken to keep cold. Return to -20°C immediately after use.

9.4 **TdT Enzyme:**

Ready to use as supplied. Contains glycerol and will not freeze solid at -20°C. Do not remove from -20°C freezer until immediately before use. Place in a -20°C storage device for use, special care should be taken to keep cold. Return to -20°C immediately after use.

9.5 **Stop Buffer:**

Ready to use as supplied. Thaw component 30 minutes prior to use. Return to -20°C after use.

9.6 **Blocking Buffer:**

Ready to use as supplied. Thaw component 30 minutes prior to use. Return to -20°C after use.

9.7 **25X Conjugate:**

Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Special care should be taken to keep cold. Return to -20°C immediately after use.
ASSAY PREPARATION

9.8 **DAB Solution 1:**
Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Return to -20°C immediately after use.

9.9 **DAB Solution 2:**
Ready to use as supplied. 30 minutes prior to use, thaw component and keep on ice. Return to -20°C immediately after use.

9.10 **Methyl Green Counterstain:**
Ready to use as supplied. 30 minutes prior to use, thaw component. Return to -20°C after use.
10. ASSAY PROCEDURE and DETECTION

Paraffin embedded tissue sections

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

10.1. Rehydration:

10.1.1 Immerse slides in xylene for 5 minutes at room temperature. Repeat (total two 5 minute incubations). Xylene should be changed frequently.

10.1.2 Immerse slides in 100% ethanol for 5 minutes at room temperature. Repeat (total two 5 minute incubations).

10.1.3 Immerse slides in 90% ethanol for 3 minutes at room temperature.

10.1.4 Immerse slides in 80% ethanol for 3 minutes at room temperature.

10.1.5 Immerse slides in 70% ethanol for 3 minutes at room temperature.

10.1.6 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, ab2601).

10.2. Permeabilization of specimen:

10.2.1 Dilute Proteinase K 1:100 in dH₂O (mix 1 μL of Proteinase K plus 99 μL dH₂O per specimen).

10.2.2 Cover the entire specimen with 100 μL of Proteinase K solution prepared above and incubate at room temperature for 20 minutes.

10.2.3 Rinse slide with 1X TBS for 5 minutes.

10.2.4 Gently tap off excess liquid and carefully dry the glass slide around the specimen using an adsorbent wipe. Care should be taken to not touch the specimen.
10.3. **Quenching: inactivation of endogenous peroxidases:**

10.3.1 Dilute 30% H$_2$O$_2$ 1:10 in methanol (mix 10 µL 30% H$_2$O$_2$ with 90 µL methanol per specimen).

10.3.2 Cover the entire specimen with 100 µL of 3% H$_2$O$_2$. Incubate at room temperature for 5 minutes.

10.3.3 Rinse slide with 1X TBS for 5 minutes.

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

10.4. **Equilibration:**

10.4.1 Cover the entire specimen with 100 µL of TdT Equilibration Buffer provided. Incubate at room temperature for 30 minutes. During the last five minutes of this incubation prepare the Labeling Reaction Mixture.

10.5. **Labeling Reaction:**

10.5.1 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 for the number of samples/ slides to be labeled. For each sample to be labeled, add 1 µL TdT Enzyme to 39 µL TdT Labeling Reaction Mix in a clean microfuge tube, mix gently and keep on ice or a cold block until use.

10.5.2 Carefully blot the TdT Equilibration Buffer from the specimen, taking care not to touch the Specimen.

10.5.3 Immediately apply 40 µL of TdT Labeling Reaction Mix (prepared above) onto each specimen and cover the specimen with a coverslip to assure even distribution of the reaction mixture and prevent loss due to evaporation during incubation.

10.5.4 Place slides in a humidified chamber and incubate at room temperature (at least 22ºC) for 1.5 hours. **NOTE:** If room temperature is below 22°C the use of a 37ºC incubator is recommended.
10.6. **Termination of labeling reaction:**

10.6.1 Inspect the Stop Buffer. If a precipitate is present, warm the Stop Buffer to 37ºC for five minutes or until precipitate is no longer evident.

10.6.2 Remove coverslip* and rinse slide with 1X TBS for 5 minutes.

Cover slip is best removed by sub-merging 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 recommended but a plastic cover slip may be used.

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

10.6.4 Rinse slide with 1X TBS for 5 minutes.

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

10.7. **Blocking**

10.7.1 Cover the entire specimen with 100 μL of Blocking Buffer. Incubate at room temperature for 10 minutes. During the last 5 minutes of blocking, prepare the Conjugate solution.

10.8. **Detection**

10.8.1 Dilute the 25X Conjugate 1:25 in Blocking Buffer (mix 4 μL 25X Conjugate with 96 μL Blocking 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.

10.8.2 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.

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

10.8.4 Rinse slides with 1X TBS for 5 minutes.
10.9. **Development**

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

10.9.2 Prepare working DAB solution by adding 4 µL DAB Solution 1 to 116 µl DAB Solution 2 (1:30 dilution). Prepare only enough working DAB solution for specimens to be processed. Do not store diluted DAB solution. Prepare fresh on each occasion.

10.9.3 Cover the entire specimen with 100 µL of DAB solution prepared in section 10.9.2 above. Incubate at room temperature for 15 minutes.

10.9.4 Rinse slides gently with dH₂O.

10.10. **Counterstain and Storage**

10.10.1 Immediately cover the entire specimen with 100 µL of Methyl Green Counterstain solution provided.

10.10.2 Incubate at room temperature for 1-3 minutes.

10.10.3 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.

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

10.10.5 Blot slides briefly on an absorbent towel.

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

10.10.7 Dip slides 2-4 times into 100% xylene.

10.10.8 Wipe excess xylene from back of slide and around specimen.

10.10.9 Mount a glass coverslip using mounting media (such as ab64230) over the specimen.
Tissue cryosections

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

To avoid loss of tissue from glass slides during washing steps, gently dip the slides 2-3 times in a beaker of 1X TBS instead of rinsing with a wash bottle.

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

10.11. Tissue fixation and hydration

10.11.1 Immerse slides in 4% formaldehyde (prepared in 1X PBS) for 15 minutes at room temperature.

10.11.2 Gently drain off excess liquid and carefully dry the glass slide around the specimen.

10.11.3 Immerse slides in 1X TBS for 15 minutes at room temperature.

10.11.4 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, ab2601) or a hydrophobic slide marker.

10.12. Permeabilization of specimen

10.12.1 Dilute Proteinase K 1:100 in dH2O (mix 1 μL of Proteinase K plus 99 μL dH2O per specimen).

10.12.2 Cover the entire specimen with 100 μL of Proteinase K solution prepared above and incubate at room temperature for exactly 10 minutes. Do not incubate for longer than 10 minutes.

10.12.3 Rinse slide with 1X TBS for 5 minutes.

10.12.4 Gently tap off excess liquid and carefully dry the glass slide around the specimen using an adsorbent wipe. 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 Section 10.3 (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.

Cell suspensions or fixed cells
Cells grown in suspension (suspension cultures etc.) can be fixed and attached to slides. The protocol differs from that used for paraffin-embedded tissue sections as the deparaffinization step is replaced with a rehydration step and permeabilization with Proteinase K is performed only for 5 minutes.

10.13. Fixing cell preparations

10.13.1 Pelleted cells by gentle centrifugation for 5 minutes at 4°C. Wash cells twice with cold (4°C) PBS.

10.13.2 Re-suspend cells in 4% formaldehyde (in PBS) at a cell density of 1x10^6/mL and incubate at room temperature for 10 minutes.

10.13.3 Pellet cells by gentle centrifugation for 5 minutes at room temperature and re-suspended, at the same concentration, in 80% ethanol.

10.13.4 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 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.

**NOTE:** To avoid loss of cells from glass slides during washing steps, dip slides 2-3 times into a beaker of 1X TBS instead of rinsing with a wash bottle.
10.14. **Rehydration**

10.14.1 Immerse slides in 1X TBS for 15 minutes at room temperature.

10.14.2 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, ab2601).

10.15. **Permeabilization of specimen**

10.15.1 Dilute 2 mg/ml Proteinase K 1:100 in 10 mM Tris pH 8 (mix 1 μL of 2 mg/ml Proteinase K with 99 μL 10 mM Tris per specimen).

10.15.2 Cover the entire specimen with 50 -100 μL of 20 μg/ml Proteinase K. Incubate at room temperature for exactly 5 minutes. Do not incubate for longer than 5 minutes.

10.15.3 Dip slide 2-3 times into a beaker of 1X TBS.

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

10.15.5 Please follow protocol for paraffin embedded sections from section 10.2 (Quenching: inactivation of endogenous peroxidases).
11. ANALYSIS

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

To generate a positive control, treat one or more slides with 1 µg/µL DNase I in TBS/1 mM MgSO$_4$ for 20 minutes at room temperature (perform all other steps as described). Slides of 10 µm thickness are preferred. The DNase I treatment will fragment DNA in normal cells to generate free 3’OH groups identical to those generated during apoptosis.

To add a negative control, substitute the TdT Enzyme with dH$_2$O in the reaction mix or keep the specimen in reaction buffer (with cover slip to prevent drying out) during the labeling stage. Perform all other steps as described. This is a suitable control for endogenous peroxidases and non-specific conjugate binding or background in the assay. A non-apoptotic control is also a useful control. A delay in fixation or routine mechanical manipulation may result in unwanted DNA breakage that could be read as apoptosis.

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 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 labelled nucleotide since they lack free 3’-OH ends (indicative of apoptosis).

After performing the in situ apoptosis detection test, careful evaluation of the slides should be performed using a light microscope.
Figure 1. Using paraffin fixed human tonsil tissue, 10 μm sections (1000X).
Figure 2. Using paraffin fixed human tonsil tissue, 10 μm sections (1000X). A] Section processed and counter-stained with methyl green according to the manual. B] Counter-stain step was eliminated to more clearly illustrate the level of positive staining in the germinal centres 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 labelled with biotin-nucleotide by the TdT in the kit. D] Negative control, the TdT Enzyme step was eliminated thereby generating a negative slide.
13. **QUICK ASSAY PROCEDURE**

**NOTE:** This procedure, which starts at the permeabilization step for paraffin-embedded tissue sections, is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time. This procedure should be modified for frozen sections and fixed cells.

- Cover specimen with 100 µL Proteinase K solution for 20 minutes.
- Rinse with TBS for 5 minutes.
- Cover specimen with 100 µL of 3% H₂O₂ for 5 minutes.
- Rinse with TBS for 5 minutes.
- Cover specimen with 100 µL TdT Equilibration buffer for 30 minutes.
- Add 40 µL of TdT labeling Reaction Mix to each sample, place cover slip over the top and incubate at 37°C for 90 minutes.
- Remove coverslip and rinse with TBS for 5 minutes.
- Cover specimen with 100 µL Stop Buffer and incubate at room temperature for 5 minutes.
- Rinse with TBS for 5 minutes.
- Add 100 µL Blocking Buffer to the specimen and incubate at room temperature for 10 minutes.
- Carefully blot the Blocking Buffer from the specimen.
- Immediately apply 100 µL of 1X Conjugate to the specimen and incubate for 30 minutes.
- Rinse with TBS for 5 minutes.
- Cover specimen with 100 µL DAB solution and incubate for 15 minutes.
- Rinse with dH₂O.
- Cover specimen with 100 µL Methyl green for 3 minutes.
- Dehydrate slides by repeated immersion in 100% ethanol followed by xylene before adding a glass coverslip.
14. FAQ
15. NOTES
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