ab206386 – TUNEL Assay Kit - HRP-DAB

For the detection of apoptotic cells.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab206386 (use www.abcam.cn/ab206386 for China, or www.abcam.co.jp/ab206386 for Japan)

Storage and Stability: Store kit at -20ºC in the dark immediately upon receipt. Aliquot components in working volumes before storing at the recommended temperature.

Materials Supplied:

Item	Amount (30 test size)	Amount (60 test size)	Storage Condition
Proteinase K	50 µL	100 µL	-20°C
TdT Equilibration Buffer	4 mL	8 mL	-20°C
TdT Labeling Reaction Mix	1 x 1.3 mL	2 x 1.3 mL	-20°C
TdT Enzyme	40 µL	70 µL	-20°C
Stop Buffer	4 mL	8 mL	-20°C
Blocking Buffer	12 mL	24 mL	-20°C
25X Conjugate	150 µL	300 µL	-20°C
DAB Solution 1	150 µL	300 µL	-20°C
DAB Solution 2	4 mL	8 mL	-20°C
Methyl Green Counterstain	1 x 3.5 mL	2 x 3.5 mL	-20°C

Materials Required, Not Supplied:

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

Ethanol, 100, 90, 80 and 70%
Humidified chamber (see Technical Hints)
Hydrophobic slide marker (such as PAP pen - ab2601)
Distilled de-ionized water (dH2O)
Coplin jars, glass or plastic with slide holders
Sterile DNase/RNase free disposable pipette tips
1-20 µL, 20-200 µL, and 200-1000 µL precision pipettes
DNase I (optional, for generating positive control)
Organic mounting media (such as ab188804 or ab104141)
Tris-buffered saline (1X TBS, 20 mM Tris pH 7.6, 140mM NaCl)
30% hydrogen peroxide
Absorbent wipes
Cold block or ice bath
Methanol
Xylene
Glass or plastic coverslips
Microscope
Microcentrifuge tubes

Technical Hints:

- To construct a humidified chamber, place a moist paper towel into a plastic box. Place slides onto the moist surface or elevated by using a support. Place lid on box.
- To avoid loss of sample from glass slides during washing steps, dip slides 2-3 times into 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.

Reagent Preparation:

- Components ready to use as supplied.
- Briefly centrifuge small vials at low speed prior to opening.
- Prepare only enough reagent for the number of samples/ slides required.

- Return components to -20°C immediately after use.
- TdT Enzyme contains glycerol and will not freeze solid at -20°C. Remove from -20°C freezer immediately before use. Place in a -20°C storage device for use.
- Thaw all other components 30 minutes prior to use and keep on ice.

Assay Procedure:

1. Paraffin embedded tissue sections

Rehydration

1.1. Immerse slides in xylene for 5 minutes at room temperature (RT). Repeat (total 2 x 5 minute incubations). Xylene should be changed frequently.
1.2. Immerse slides in 100% ethanol for 5 minutes at RT. Repeat (total 2 x 5 minute incubations).
1.3. Immerse slides in 90% ethanol for 3 minutes at RT.
1.4. Immerse slides in 80% ethanol for 3 minutes at RT.
1.5. Immerse slides in 70% ethanol for 3 minutes at RT.
1.6. Rinse slides briefly with 1X TBS for 5 minutes. Dry the slide around the specimen. To contain small reagent volumes, encircle the specimen using a hydrophobic slide marker.

Permeabilization of specimen:

1.7. Dilute Proteinase K 1/100 in dH2O (1 µL of Proteinase K + 99 µL dH2O per specimen).
1.8. Cover specimen with 100 µL of dilute Proteinase K solution. Incubate at RT for 20 min.
1.9. Rinse slide with 1X TBS for 5 minutes.
1.10. 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.

Quenching: inactivation of endogenous peroxidases:

1.11. Dilute 30% H2O2 1/10 in methanol (10 µL 30% H2O2 + 90 µL methanol per specimen).
1.12. Cover specimen with 100 µL of 3% H2O2. Incubate at RT for 5 min.
1.13. Rinse slide with 1X TBS for 5 minutes.
1.14. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Equilibration:

1.15. Cover specimen with 100 µL of TdT Equilibration Buffer. Incubate at RT for 30 minutes.

Labeling Reaction:

1.16. Pulse-spin the TdT Enzyme tube in a microcentrifuge prior to opening. 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.
1.17. Blot the TdT Equilibration Buffer from the specimen. Avoid touching the specimen.
1.18. Immediately apply 40 µL of TdT Labeling Reaction Mix onto each specimen. Cover the specimen with a coverslip to assure even distribution of the reaction mixture and prevent loss due to evaporation during incubation.
1.19. Place slides in a humidified chamber at RT for 1.5 hours. If RT is below 22ºC, the use of a 37°C incubator is recommended.

Termination of labeling reaction:

1.20. Warm the Stop Buffer to 37°C for five minutes to remove precipitate.
1.21. Remove coverslip by sub-merging the slide in TBS solution in a Coplin jar or beaker, allowing the cover slip to gently slide off specimen. A glass cover slip is recommended but a plastic cover slip may be used. Rinse slide with 1X TBS for 5 min.
1.22. Cover specimen with 100 µL of Stop Buffer. Incubate at RT for 5 min.
1.23. Rinse slide with 1X TBS for 5 minutes.
1.24. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

Blocking

1.25. Cover specimen with 100 µL of Blocking Buffer. Incubate at RT for 10 min.

Detection

1.26. Dilute the 25X Conjugate 1/25 in Blocking Buffer (4 µL 25X Conjugate + 96 µL Blocking Buffer per specimen). Keep on ice or a cold block until ready to use.
1.27. 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.
1.28. Place slides in a humidified chamber and incubate at RT for 30 min.
1.29. Rinse slides with 1X TBS for 5 minutes.

Development

1.30. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
1.31. Prepare working DAB solution by adding 4 µL DAB Solution 1 to 116 µL DAB Solution 2 (1/30 dilution). Do not store diluted DAB solution. Prepare fresh on each occasion.
1.32. Cover specimen with 100 µL of diluted DAB solution. Incubate at RT for 15 minutes.
1.33. Rinse slides gently with dH2O.

Counterstain and Storage

1.34. Immediately cover specimen with 100 µL of Methyl Green Counterstain solution.
1.35. Incubate at RT for 1-3 minutes.
1.36. 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.
1.37. Dip slides 2-4 times into 100% ethanol.
1.38. Blot slides briefly on an absorbent towel.
1.39. Repeat step 38 using fresh 100% ethanol. Blot slides briefly on an absorbent towel.
1.40. Dip slides 2-4 times into 100% xylene.
1.41. Wipe excess xylene from back of slide and around specimen.
1.42. Mount a glass coverslip using organic mounting media over the specimen.

2. Tissue cryosections

Tissue fixation and hydration

2.1. Immerse slides in 4% formaldehyde (prepared in 1X PBS) for 15 minutes at RT.
2.2. Gently drain off excess liquid and carefully dry the glass slide around the specimen.
2.3. Immerse slides in 1X TBS for 15 minutes at RT.
2.4. Dry the glass slide around the specimen. To contain small reaction volumes, encircle the specimen using a hydrophobic slide marker.

Permeabilization of specimen

2.5. Dilute Proteinase K 1/100 in dH2O (1 µL of Proteinase K + 99 µL dH2O per specimen).
2.6. Cover specimen with 100 µL of diluted Proteinase K solution and incubate at RT for exactly 10 minutes. Do not incubate for longer than 10 minutes.
2.7. Proceed by following protocol for paraffin embedded sections from Section 1.9.

3. Cell suspensions or fixed cells

Fixing cell preparations

3.1. Pellet cells by gentle centrifugation for 5 min at 4°C. Wash twice with cold (4°C) PBS.
3.2. Re-suspend cells in 4% formaldehyde (in PBS) at a cell density of $1x10^6$/mL and incubate at RT for 10 minutes.
3.3. Pellet cells by gentle centrifugation for 5 minutes at RT and re-suspended, at the same concentration, in 80% ethanol. Store fixed cells at 4°C.
3.4. Fixed cells (100-300 µL) can be immobilized by placing the cell suspension onto the glass slide and allowing to air dry. A cyto-spin may also be used. Pre-coating slides with poly-L-lysine may enhance cell adherence. Store cyto-spun samples at -20°C.

Rehydration

3.5. Immerse slides in 1X TBS for 15 minutes at RT.
3.6. Dry the glass slide around the specimen. To contain small reaction volumes, encircle the specimen using a hydrophobic slide marker.

Permeabilization of specimen

3.7. Dilute 2 mg/ml Proteinase K 1/100 in 10 mM Tris pH 8 (1 µL of 2 mg/ml Proteinase K + 99 µL 10 mM Tris per specimen).
3.8. Cover specimen with 50 -100 µL of 20 µg/mL Proteinase K. Incubate at RT for exactly 5 minutes. Do not incubate for longer than 5 minutes.
3.9. Dip slide 2-3 times into a beaker of 1X TBS.
3.10. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
3.11. Proceed by following protocol for paraffin embedded sections from section 1.11.

Analysis:

An apoptosis end point, indicative of positive staining, 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 RT immediately following Proteinase K treatment (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 dH2O 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 assay, evaluate the slides using a light microscope.

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)