ApoBrdU DNA Fragmentation Assay Kit

For the labeling of DNA breaks to detect apoptotic cells by immunohistochemistry.

For research use only - not intended for diagnostic use.

Materials Supplied

Item	Quantity	Storage Condition
Control Slides-pos/neg*	2 each	-20°C
Blocking Buffer	22 mL	-20°C
H2O2/Urea Tablets	6 each	-20°C
Proteinase K	0.11 mL	-20°C
DAB Tablets	6 each	-20°C
TdT Enzymes	0.041 mL	-20°C
Br-dUTP	0.44 mL	-20°C
200X Conjugate	0.035 mL	-20°C
5X Reaction Buffer*	1.75 mL	+4°C
Anti-BrdU-Biotin Antibody	0.275 mL	+4°C
Methyl Green	6 mL	RT

*The control slides contain a mixture of apoptotic and non-apoptotic cells, allowing visualization of both positive & negative labeling within the same microscope field

**After initial defrosting, the 5X Reaction Buffer and Anti-BrdU-Biotin Antibody should be stored at 4°C, and Methyl Green should be stored at room temperature. Do Not Refreeze.

Assay protocols

Staining of Paraffin Embedded Tissue (PET) PET-A.

Deparaffinization & Rehydration

1. Immerse slides in xylene for 5 minutes at room temperature. Repeat using fresh xylene for second 5 minutes incubation.
2. Immerse slides in 100% ethanol for 5 minutes at room temperature. Repeat using fresh 100% ethanol for second 5 minutes.

3. Immerse slides in 90% ethanol for 3 min, then 80% ethanol for 3 min, and then 70% ethanol for 3 minutes at room temperature.
4. Immerse slides briefly into 1X PBS and carefully dry the glass slide around the specimen. If processing the kit's control slides simultaneously with unknown samples, please refer to the CFS protocol (CSF-A).

   Notes: At this point it may be helpful to encircle the specimen using a waxed pen or a hydrophobic marker.

   Do not let tissue specimen dry out at any step! If necessary, cover or immerse specimen in 1X PBS to keep hydrated

   PET-B. Permeabilization, Inactivation of Endogenous Peroxidase, & Equlibration
5. Dilute only enough Proteinase K (pink cap) needed 1:100 in 10 mM Tris pH 8. Cover the entire specimen with 100 µl proteinase K. Incubate at room temperature for 20 minutes. DO NOT OVER INCUBATE.
6. Rinse slide with 1X PBS. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
7. Dilute 30% H_2O_2 1:10 in methanol. Cover the entire specimen with 100 µl of 3% H_2O_2. Incubate at room temperature for 5 minutes. DO NOT OVER INCUBATE.
8. Rinse slide with 1X PBS. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
9. Dilute only enough 5X Reaction Buffer (green cap) as needed 1:5 with dH2O. Cover the entire specimen with 100 µl of the 1X Reaction Buffer. Incubate at room temperature for 10 to 30 minutes while preparing the labeling reaction mixture below.
10. Prepare the Complete Labeling Reaction Mixture as follows (Note: Mix only enough DNA Labeling Solution to complete the number of assays prepared per session. The DNA Labeling Solution is active for approximately 24 hours.)

DNA Labelling Solution	1 Assay	5 Assays	10 Assays
5X Reaction Buffer	10 µl	50 µl	100 µl
TdT Enzyme	0.75 µl	3.75 µl	7.5 µl
Br-dUTP	8 µl	40 µl	80 µl
Distilled H20	32.25 µl	161.25 µl	322.5 µl
Total Volume	51 µl	255 µl	510 µl

11. Carefully blot the 1X Reaction Buffer from the specimen, taking care not to touch the specimen. Immediately apply 50 µl of Complete Labeling Reaction Mixture (prepared above) onto each specimen except for the control slides which require only 25 µl each (Note: The use of a cover slip at this point assures even distribution of the reaction mixture and prevents evaporation during incubation).
12. Cover the specimen with a piece of Parafilm cut slightly larger than the specimen (HINT: Folding up one corner of the Parafilm cover slip will aid in its application and removal). Place slides in a humid chamber and incubate at 37°C for 1 to 1.5 hours.

NOTE: The DNA End Labeling Reaction can also be carried out at 22-24°C overnight for the control slides. For samples other than the control slides provided in the kit, incubation times at 37°C may need to be adjusted to longer or shorter periods depending on the characteristics of the tissue you used.

13. Remove Parafilm cover slip and rinse slide with PBS. Gently tap off excess liquid and carefully dry the glass around the specimen.
14. Cover the entire specimen with 100 µl of Blocking Buffer (white cap). Incubate at room temperature for 10 minutes. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen.
15. Immediately cover specimen with 100 µl of Antibody Solution (prepared as described below)

Antibody Solution	1 Assay	5 Assays	10 Assays
Anti-BrdU-Biotin Antibody	5 µl	25 µl	50 µl
Blocking Buffer	95 µl	475 µl	950 µl
Total Volume	100 µl	500 µl	1000 µl

16. Incubate with the Antibody Solution in the dark for 1-1.5 hours at room temperature (Hint: Cover slides with aluminum foil).
17. Rinse slide in PBS. Gently tap off excess liquid and carefully dry the glass around the specimen. Cover the entire specimen with 100 µl of Blocking Buffer (white cap).
18. Dilute only enough of the 200X Conjugate (black cap) needed 1:200 in Blocking Buffer (white cap). Prepared as described below.

Conjugate Solution	1 Assay	5 Assays	10 Assays
200X Conjugate	0.5 µl	2.5 µl	5 µl
Blocking Buffer	100 µl	500 µl	1000µl

19. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen. Immediately apply 100 µl of diluted conjugate to the specimen. Incubate at room temperature for 30 minutes.
20. Five minutes before concluding incubation prepare DAB solution by dissolving one tablet of DAB (amber vial) and one tablet of H_2O_2/Urea (amber vial) in one ml of tap H_2O. This yields enough DAB solution for 10 specimens. (Note: Tap H_2O may contain metal ions that enhance the DAB reaction. DAB is highly carcinogenic and care should be taken when handling).
21. Rinse slides with 1X PBS. Gently tap off excess liquid and carefully dry the glass slide around the specimen. Cover the entire specimen with 100 µl of DAB solution. Incubate at room temperature for 15 minutes. Rinse slides with H_2O and blot. PET-D. Counterstain
22. Immediately cover the entire specimen with 100 µl of Methyl Green Counterstain (natural cap) solution. Incubate at room temperature for 3 minutes. Press edge of the slide against an absorbent towel to draw off most of the counterstain and place in a coplin jar slide holder.
23. Dip slides 2 times briefly into 100% ethanol. Blot slides briefly on an absorbent towel. Repeat step 22 using fresh 100% ethanol.

24. Blot slides briefly on an absorbent towel. Dip slides into xylene (or xylene substitute). Wipe excess xylene from back of slide and around specimen.10. Mount a glass cover slip using a mounting media such as per mount (r) over the specimen.
25. Mount a glass cover slip using a mounting media such as per mount (r) over the specimen

Staining of Cell Preparations Fixed on Slides (CFS)

The following protocol describes the method for measuring apoptosis in the positive and negative control slides that are provided in the APO-BRDUIHCTM kit. The same procedure should be employed for measuring apoptosis in your own slide specimens.

Important points to remember before starting this assay: The cells must be fixed prior to performing this assay. DO NOT LET THE CELLS DRY OUT BETWEEN OR DURING ANY STEPS! To avoid loss of cells from glass slides during washing steps, it is recommended that slides be dipped into a beaker of 1X PBS rather than rinsed with a wash bottle.

CFS-A. Cell Fixation, Rehydration, & Permeabilization

1. Pellet cells at 300xg for 5 minutes at 4°C. Remove media. Add enough 1-4% formaldehyde (in PBS pH 7.4) to the pelleted cells to create a cell density of 1x106 cells/ml and incubate 15 minutes at room temperature.
2. Centrifuge at 300xg for 5 minutes at room temperature and resuspend at the same density in 70% ethanol. An aliquot of fixed cells (100-300 µl) can then be adhered to glass slides by directly placing the suspension onto the slide or by using a Cytospin. Slides precoated with poly-L-lysine may enhance cell adherence.
3. Immerse slides in 1X PBS for 10 minutes at room temperature. Carefully dry the glass around the specimen. ** At this point it may be helpful to encircle the specimen using a waxed pen or a hydrophobic slide marker **
4. Dilute Proteinase K (pink cap) 1:100 in Tris pH 8. Cover the entire specimen with 50 - 100 µl of the diluted proteinase K. Incubate at room temperature for 5 minutes. DO NOT OVER INCUBATE.
5. Dip slide 2-3 times into a beaker of 1X PBS. Gently tap off excess liquid and carefully dry the glass slide around the specimen. All the remaining steps of staining cells fixed on slides are identical to those steps outlined in the previous section for staining of paraffin embedded tissue sections:

   Inactivation of endogenous peroxidases & equilibration (pet-b, from step 7)

   End labeling & detection (pet-c)

   Counterstain (pet-d)

Staining of Tissue Cryosections (TCS)

Important points to remember before starting this assay: Fixation of cryopreserved tissue is required prior to performing this assay. DO NOT LET THE TISSUE DRY OUT BETWEEN OR DURING ANY STEPS! if necessary cover or immerse the slide in 1X PBS to keep hydrated. To avoid loss of tissue from glass slides during washing steps, it is recommended that slides be dipped 2-3 times into a beaker of 1X PBS rather than rinsed with a wash bottle.

TCS-A. Tissue Fixation, Rehydration, & Permeabilization

1. Immerse slides in 4% formaldehyde (in PBS, pH 7.4) for 15 minutes at room temperature. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
2. Immerse slides in 1X PBS for 15 minutes at room temperature. Carefully dry the glass slide around the specimen. **At this point it may be helpful to encircle the specimen using a waxed pen or hydrophobic slide marker**
3. Dilute proteinase K (pink cap) 1:100 in 10 mM Tris, pH 8. Cover the entire specimen with 50-100 µl of the diluted proteinase K solution. Incubate at room temperature for 10 minutes. DO NOT OVER INCUBATE.
4. Dip slide 2 - 3 times into a beaker of 1X PBS. Gently tap off excess liquid and carefully dry the glass slide around the specimen. All the remaining steps of staining tissue cryosections on slides are identical to those steps outlined for staining of paraffin-embedded tissue sections:

1. **Inactivation of endogenous peroxidases & equilibration (pet-b, from step 7) End labeling & detection (pet-c) Counterstain (pet-d)**

For research use only! Not to be used in humans.

For more details, please visit http://www.apexbt.com/or contact our technical team.

APExBIO Technology
www.apexbt.com
7505 Fannin street, Suite 410, Houston, TX 77054.
Tel: +1-832-696-8203 | Fax: +1-832-641-3177 | Email: info@apexbt.com