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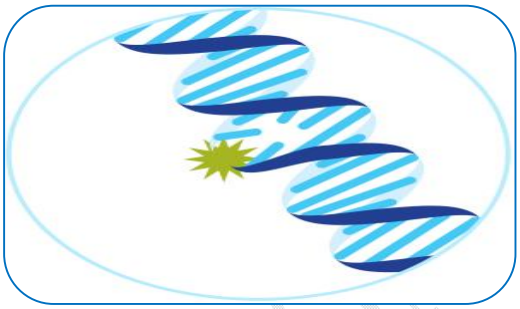
TUNEL ASSAY: AN EFFECTIVE CONVENTIONAL WAY TO EVALUATE DNA DAMAGE



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ABSTRACT:

Apoptosis, or programmed cell death, plays an important role in normal development and homeostasis of adult tissues. Apoptosis has also been linked to many disease states, including cancer. One of the biochemical hallmarks of apoptosis is the generation of free 3'-hydroxyl termini on DNA via cleavage of chromatin into single and multiple oligonuleosome-length fragments. The TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay exploits this biochemical hallmark by labeling the exposed termini of DNA, thereby enabling visualization of nuclei containing fragmented DNA. This article outlines a conventional method for in situ TUNEL staining of cultured cells and tissue sections which is highly effective in analysing PCD in broad range of test sections.

KEYWORDS: *Apoptosis, Programmed Cell Death, Biochemical, DNA fragmentation, Tunel Assay.*

1. INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a physiological form of cell death that plays a critical role in the development and maintenance of multicellular organisms. Apoptosis is mainly characterized based on morphological and biochemical criteria of the test cell (Taylor et al., 2008). Morphological characteristics include cell shrinkage, cytoplasmic condensation, chromatin segregation and condensation, membrane blebbing, and the formation of membrane-bound apoptotic bodies, whereas the biochemical criteria of apoptosis is internucleosomal DNA cleavage into oligonucleosome-length fragments. Informative data related to cell death and various other cellular parameters, including cell cycle and cell phenotype can be determined by biochemical methods such as terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling of DNA fragments (TUNEL).

Histological sections prepared in the conventional method includes pretreatment with protease, nick end labelling with biotinylated poly dU, introduced by terminal deoxy-transferase, and staining by avidin-conjugated peroxidase. In the conventional method, the reaction is specific because only nuclei located at positions where PCD (Programmed Cell Death) has occurred or is expected are stained (Srinivasan et al., 2002). The initial screening for PCD includes: small and large intestine, epidermis, lymphoid tissues, ovary, and other organs. The process for PCD is found to be initiated at the nuclear peripheral region. It is considered to be of relatively short duration (1-3 h from initiation to cell death) The PCD pattern appears in test tissues in clusters. The extent of apoptosis or tissue-PCD observed by this method is relatively more compared to detection by nuclear morphology which in turn paves the way for broader and better variety of studies.

2. MATERIAL AND METHODS

The conventional method for study of Tunel assay is done in two broad steps including preparation of Control (s) and Buffer solutions (Gavrieli Y, et al 1992).

Preparation of control (s)

Two control and two test sections per slide is recommended during preparation for Tunel assay . The control (s) may be as follows

1. **Positive Control(s): Positive control may be prepared by the following methods**
- The test sections should be incubated with DNase I (3000U/ml in 50 mM Tris-HCl, pH 7.5, 1mg/ml BSA) for 10 minutes at 15-25 ºC in order to induce DNA strand breaks, prior to labeling procedure.
- Known positive control (from Tunel assay Kits available) may be prepared or used as an alternative.

2. **Negative Control:** The test sections are incubated with label solution (in the absence of terminal transferase) only.

Preparation of required reagents and solutions are as follows

1) **TdT Buffer Stock Solution** (pH 6.6)

To make the buffer:

125mM Tris-HCl (MW 157.6) ---------- 1.97 g
1M Sodium cacodylate, Trihydrate (MW 214.0) --------- 21.4 g
BSA (1.25mg/ml) ---------- 0.125 g
Distilled water ---------- 100 ml

(pH adjusted to 6.6 and solution stored at –20 ºC. till use)

2) **Cobalt Chloride Stock Solution**

To make the buffer:

25mM Cobalt chloride, Hexahydrate (MW 237.9) ---------- 0.6 g
Distilled water ---------- 100 ml

Prepare 25mM Cobalt Chloride in Distilled Water. Aliquot and store at –20 ºC.

3) **TdT Reaction Buffer**

To make the buffer:

TdT Buffer Stock Solution (as prepared previously) --40 ul
1mM Cobalt Chloride Stock Solution ---------- 8 ul
Distilled water ---------- 160 ul

TdT Reaction Buffer and store at –20 ºC

4) TdT Storage Buffer

To make the buffer (pH 7.2):

60mM K_2HPO_4 (MW174.18) ---------- 1.05 g
150mM KCl (MW 74.55) ---------- 1.12 g
Distilled water ---------- 50 ml

Dissolve in distilled water by constant stirring to mix well. Adjust pH 7.2 using concentrated HCl.

To the above solution add 50 ml of glycerin (100% glycerol), 0.5 ml of Triton X-100, and 8 ul of 2-Mercaptoethanol (99% Solution. MW 78.13) and mix well. Store at –20 ºC

5) Enzyme Reagent:

Terminal Transferase (TdT) (obtained from Roche Diagnostic) ------ 4 ul
TdT Storage Buffer (as previously prepared) ---------- 100 ul
(All solutions should be mixed properly and stored at –20 ºC till use)

6) Label Reagent:

Biotin-16-dUTP (obtainedfrom Roche Diagnostic) ---- 4 ul
TdT Reaction Buffer(as previously prepared) --------- 1 ml
(All solutions should be mixed properly and stored at –20 ºC till use)

7) TdT Reaction Mixture:

Enzyme Reagent ---------- 100 ul
Label Reagent ---------- 900 ul
(The solutions should be mixed properly just before use).
The remaining 100 ul of Label Solution can be used as negative control.

8) Stop Wash Buffer:

To make the buffer:

300mM NaCl (MW 58.44) ---------- 1.75 g
30mM Sodium citrate, Trihydrate (MW294.11) ---------- 0.88 g
Distilled water ---------- 100 ml

(All solutions should be mixed properly and stored at room temperature till use).

The following steps are performed for Tunel assay

1. **Deparaffinize** : Sections are deparaffinised in 2 changes of xylene for 5 minutes each, and hydrated with 2 changes of 100% ethanol for 3 minutes each, and in 95% ethanol for 1 minute.
2. The sections are then rinsed in distilled water.
3. **Pretreatment:** For pretreatment of test sections, proteinase K digestion method is preferred. In case of frozen sections or culture cells grown on slides,the sections should be incubated with 0.2% Triton X-100 in PBS-Tween for 30 minutes if required.
4. The sections are rinsed in 2 changes of PBS-Tween 20, 2 minutes each.
5. **Peroxidase Blocking**:In order to block endogenous peroxidase activity the test sections are incubated in 3% H_2O_2 in PBS for 10 minutes .
6. Rinse the test sections in PBS-Tween 20 for 3x2 min.
7. **Pre-incubation:** Incubate the test sections in TdT Reaction Buffer for 10 minutes.
8. **TdT Reaction**: Incubate the test sections in TdT Reaction Mixture for 1-2 hours at 37-40 °C in humidified chamber.
9. **Stop Reaction:** Rinse the test sections in stop wash buffer for 10 minutes to stop further reactions.
10. The above step should be followed by rinsing the section in PBS-Tween 20 for 3x2min.
11. **Detection:** Incubate the test sections with Streptavidin-HRP in PBS for 20 minutes at room temperature.
12. Rinse again in PBS-Tween 20 for 3x2min.
13. Examine sections under the microscope before starting the experiment to choose sections you want to use for TUNEL staining (better looking sections) and for control sections.
14. **Chromagen/Substrate:** Incubate the test sections with DAB (3,3′-Diaminobenzidine) for 1-2 minutes.

15. Rinse thoroughly in tap water.
16. **Counterstain** with Gill's hematoxylin for 30 seconds.
17. Rinse the section in running tap water for 5 minutes.
18. **Dehydrate**: Dehydrate the test section in 95% ethanol for 5min followed by 100% ethanol for 2x3min.
19. Cleanby keepingthe slidein xylene for 2x5min.
20. **Coverslip** with xylene based mounting medium.
21. The results are visualized with brown DAB under bright field microscope.

3. RESULTS AND DISCUSSION

Staining pattern or the nuclear image obtained is shown in Fig.1

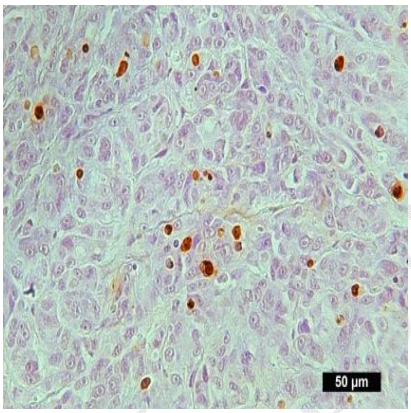

Fig 1. Detection of Apoptosis in mouse cell by TUNEL method

TUNEL-based assays were used to demonstrate the presence of apoptotic cells in tissue sections derived from target tissues of animal models of different diseases. Emphasis was placed on tissue preparation and fixation, as these are crucial to successful histological staining.

Though several factors effect the intensity of labeling of DNA breaks (Dierendonck*et al*, 2002)the most important one is the accessibility of the DNA ends to the specific labeling reagents. The accessibility of DNA ends is facilitated by treating the tissue sections with proteases like proteinase K. However,calibration of the reaction is essential because excess protein digestion may lead to DNA extraction (Migheli *et al*., 1994).

The protocol suggested here facilitates not only the reliable detection of TUNEL-positive cells but the immunodetection of different proteins in these cells and the surrounding tissues by DAB or fluorescence-based immunostaining. Although Tunel assay generates both false positive and negative results, optimization by manipulating the different factors influencing the assay and their standardization enable specific detection of relatively low, but significant number of DNA breaks caused by various sources including genotoxic agents.

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REFERENCES

1. Gavrieli Y, et al (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol.**119**:493-501.
2. Ansari, B, Coates, BD, Greenstein, BD, Hall, PA (1993) In situ end labeling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J Pathol***170**:1–8

3. Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level, *Nature reviews* **9**, 231–241.
4. Wyllie, AH, Kerr, JFR, Currie, AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol **68**:251–306
5. Willingham, M. C. (1999). Cytochemical methods for the detection of apoptosis, *J Histochem Cytochem* **47**, 1101–1110.
6. Allen, R. T., Hunter, W. J., III, and Agrawal, D. K. (1997). Morphological and biochemical characterization and analysis of apoptosis, *J Pharmacol Toxicol Meth* **37**, 215–228.
7. Darzynkiewicz *et al.* (2008) Analysis of apoptosis by cytometry using TUNEL assay. *Methods* **44** (3):250.
8. Barrett, K. L., Willingham, J. M., Garvin, A. J., and Willingham, M. C. (2001). Advances in cytochemical methods for detection of apoptosis, *J Histochem Cytochem* **49**, 821–832.
9. Srinivasan, M., Sedmak, D., and Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids, *Am J Pathol* **161**, 1961–1971.
10. Beckstead, J. H. (1994). A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues, *J Histochem Cytochem* **42**, 1127–1134.
11. Migheli, A, Atanasio, A, Schiffer, D (1995) Ultrastructural detection of DNA strand breaks in apoptotic neural cells by in situ end-labelling techniques. *J Pathol***176**:27–35.
12. Szabò, G, Boldog, F, Wikonkàl, N (1990) Dissassembly of chromatin into 50 kb units by detergent. *Biochem Biophys Res Commun* **169**:706–712.