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Quick Detection of DNase II-Type Breaks in Formalin-Fixed Tissue Sections

Candace L. Minchew Vladimir V. Didenko

Abstract

Blunt-ended DNase II-type breaks with 5' hydroxyls are generated in phagocytic cells of any lineage during digestion of the engulfed DNA. These breaks indicate the ongoing active phagocytic reaction. They are produced by the acid deoxyribonuclease—DNase II which is the primary endonuclease responsible for DNA degradation after its engulfment.

Here, we present an express approach that detects blunt-ended 5' OH DNA breaks in fixed tissue sections. The technique is simple to perform and takes only 60 min to complete. It can be useful in studies of the clearance of dying cells in oncological, inflammatory, and autoimmune disorders.

Keywords

Phagocytic digestion of DNA; Labeling of phagocytosis; Phagolysosomes; Clearance of cell death; Express detection of DNase II cleavage; 5' OH DNA probes

1 Introduction

DNA breaks bearing 3' phosphates and 5' hydroxyls occur in phagolysosomes of those phagocytic cells that engulfed nuclear material from dying cells. They are produced by DNase II—a key endonuclease in the phagocytic degradation of DNA from apoptotic nuclei [1]. The amino acid sequence of this enzyme is highly conserved and close homologs of mammalian DNase II are present in invertebrates, such as worms and flies, which indicates its importance. The enzyme is located in lysosomes and is active in acidic conditions when it hydrolyzes the phosphodiester bonds in DNA [1].

Two different isoforms of lysosomal nucleases have been identified so far—DNase II α and DNase II β [2-4]. The primary lysosomal enzyme is DNase II α , often referred to as DNase II. This enzyme is expressed in all animal tissues. In contrast, DNase II β has a limited tissue distribution that varies between species. In mice it degrades nuclear DNA during lens cell differentiation [5] and is specifically expressed in liver [5], yet in human tissues it is absent from the liver but is highly expressed in the salivary gland [4].

Overall, the ubiquitous expression pattern of DNase II (DNase II α) and its presence in all tissues confirms that it is the principal lysosomal DNA digestion enzyme, whereas DNase II β performs specialized functions in selected tissues in different mammalian species and may also function as a secreted enzyme [1, 4].

Both isoforms of lysosomal DNase II produce DNA breaks that contain 3' PO₄/5' OH at the ends. DNA breaks having this configuration are often referred to as DNase II-type breaks [6–8]. This sort of DNA cleavage differs from DNA cuts produced by DNase I, DNase I-like nucleases, and caspase-activated deoxyribo-nuclease (CAD), which all create the reversed 3' OH/5' PO₄ end-group pattern. Such DNase I-type breaks are produced in apoptotic execution and are used as specific markers of apoptotic cells [9–11]. In the same way, the DNase II-type breaks serve as characteristic markers of DNase II activity and indicate the digestion of engulfed DNA [7]. Specific detection of these breaks in cells in fixed tissue sections indicates the ongoing active phagocytic reaction and labels phagocytes of any lineage participating in active clearance of dead cells [12].

Here, we present an express version of the technique which labels DNase II-type DNA breaks. The protocol takes only 60 min to complete. The increased speed of the assay is enabled by two factors: the quicker processing of sections, and the use of the ultrafast labeling enzyme—vaccinia topoisomerase (*see Note 1*).

The described approach selectively labels blunt-ended 5' OH DNA breaks in formaldehyde-fixed, paraffin-embedded tissues. The detected type of DNA breaks localize in phagolysosomes of phagocytizing cells and are produced by DNase II. The brightly labeled phagolysosomes filled with semi-digested DNA can be easily identified in the cytoplasm of phagocytic cells under fluorescence microscope observation (Fig. 1).

The technique does not label single-stranded DNA breaks or DNase I-type cleavage, such as the caspase-initiated apoptotic DNA fragmentation producing DNA breaks with 5' PO₄ instead of 5' OH. Instead, the labeling indicates the active phagocytic clearance of dying (either apoptotic or necrotic) cells. The assay is simple, economic, and fast. It can be easily mastered by a researcher new to the field of in situ labeling (*see Note 2*).

The assay utilizes the unique enzymatic properties of vaccinia DNA topoisomerase I (TOPO), a virus-encoded eukaryotic type IB topoisomerase. When applied to tissue sections this enzyme specifically attaches the preactivated blunt-ended hairpin-shaped oligoprobes to the 5' OH ends of blunt-ended DNA breaks. This type of ligation is not possible for DNA ligases which all require 5' PO₄ at DNA ends. However, vaccinia topoisomerase I used in this assay joins DNA molecules employing a different mechanism.

In nature this topoisomerase untwists cellular DNA to release its torsional stress. At first the enzyme binds to the specific recognition sequence and makes a single-strand cut at its end thus freeing an opposite 5' OH DNA terminus. The DNA molecule then rotates around the remaining strand and releases the stress. Next, TOPO re-ligates the DNA strand back to the momentarily released 5' OH DNA terminus. The assay uses this re-ligation activity of the topoisomerase to detect DNase II breaks with 5' OH ends. Its labeling principle is presented in Fig. 2 (*see Note 1*).

This chapter presents a step-by-step express labeling protocol which requires 1 h to complete. The protocol deals with the rapid preparation and usage of fluorescent TOPO probes in fixed tissue sections to label the phagocytic clearance of dying cells.

2 Materials

1. 5-6 μm -thick sections cut from paraformaldehyde-fixed, paraffin- embedded tissue blocks. Sections of different thicknesses (3–50 μm -thick) can potentially be used but might require different time of dewaxing and/or Proteinase K treatment (**step #3**). Use charged and precleared slides that retain sections well.

For the positive control experiments, as the source of DNase II type DNA breaks, we recommend using sections of dexamethasone- treated rat thymus (*see Note 3*).
2. Xylene.
3. 70, 80, and 96% Ethanol.
4. Oligo 1. Double-hairpin vaccinia topoisomerase I cleavable oligonucleotide. The oligonucleotide is labeled with a single fluorescein.

5'-AAGGGACCTGCGFCAGGTCCCTTAACGCATATGCGTT-3'

F – FITC-dT

PAGE or HPLC purification is recommended. Dilute with bidistilled water to 100 pmol/ μL stock concentration. Store at -20°C protected from light.
5. Vaccinia DNA topoisomerase 1–6 pmol/ μL stock (*see Note 4*).
6. 50 mM Tris-HCl, PH 7.4.
7. Proteinase K (Roche Diagnostics Corporation, Inianapolis, IN) 20 mg/mL stock in distilled water. Store at -20°C . In the reaction use 50 $\mu\text{g}/\text{mL}$ solution in PBS, prepared from the stock. Do not reuse (*see Note 5*).
8. Vectashield with DAPI (Vector Laboratories, Burlingame, CA).
9. Phosphate-buffered saline (1 $\times$ PBS): dissolve 9 g NaCl, 2.76 g $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 5.56 g $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$ in 800 mL of distilled water. Adjust to pH 7.4 with NaOH, and fill to 1 L with distilled water.
10. Fluorescent microscope with appropriate filters and objectives.

3 Method

3.1 Labeling 5' OH Blunt-Ended DNA Breaks in Tissue Sections

1. Place the sections in a slide rack and dewax in xylene for 5 min, ransfer to a fresh xylene bath two more times for an additional 2 min each. For each transfer, dip cassette up and down three times (*see Note 6*).
2. Rehydrate by passing through graded ethanol concentrations: 96% Ethanol—2 $\times$ 2 min; 80% Ethanol—2 $\times$ 2 min; 70% Ethanol—2 min; water—2 $\times$ 2 min. For each transfer, dip cassette up and down three times (*see Note 6*).

3. Digest section with Proteinase K. Use 100 μ L of a 50 μ g/mL solution per section. Incubate for 10 min at room temperature (23 °C) in a humidified chamber (*see Note 7*).
4. Rinse in distilled water for 3×2 min. For each transfer, dip cassette up and down three times (*see Note 6*).
5. While sections are rinsing, combine 100 pmoles of Probe 1 and 100 pmoles (3.3 μ g) of TOPO in a solution of 50 mM Tris-HCL, pH 7.4 (*see Note 8*). Use 100 μ L of this reaction solution per section.
6. Aspirate water from sections and apply the labeling mix containing the oligoprobes and TOPO enzyme.
7. Incubate for 15 min at room temperature (23 °C) in a humidified chamber, protected from light.
8. Wash sections 3×2 min in distilled water. For each transfer, dip cassette up and down three times (*see Note 6*).
9. Cover sections with an antifading solution (Vectashield with DAPI), coverslip and analyze the signal using a fluorescent microscope. Double-strand DNA breaks with 5' OH will fluoresce green.

4 Notes

1. TOPO can perform the specific ligation to 5' OH ends in tissue sections in 15 min [13]. This high speed of attachment is explained by the intrinsically fast ligation activity of the vaccinia topoisomerase enzyme. In the kinetic analysis of DNA strand cleavage and ligation reactions, this topoisomerase demonstrated ligation of 85% of oligoprobes within a 15 s interval [14].

The detailed analysis of various probe designs and this labeling approach that uses vaccinia topoisomerase are discussed elsewhere [7, 13].
2. The other assays commonly used for detection of fragmented DNA in fixed cells rely on labeling of 3' OH groups (TUNNEL assay) or 5' PO₄ groups (in situ ligation) and cannot label the same marker [7–9].
3. Apoptotic thymus contains both DNase I type and DNase II type DNA breaks [15] and is useful in control experiments. To make apoptotic thymus, subcutaneously inject Sprague-Dawley rats (150 g) with 6 mg/kg dexamethasone (Sigma) dissolved in 30% dimethyl sulfoxide in water. Animals should be sacrificed 24 h post injection. To fix the thymus, incubate it for 18 h in 4% paraformaldehyde. For paraffin embedding, pass the tissue through graded alcohols finishing with 100% ethanol, then place it overnight in chloroform and embed in paraffin.
4. Highly concentrated TOPO, which works well with the described assay, can be purchased from Millipore, sold as a part of the ApopTag® ISOL Dual Fluorescence Apoptosis Detection Kit. We have also used the highly

concentrated preparation of this enzyme obtained from Vivid Technologies (Houston, TX).

5. At concentrations higher than 1 mg/mL proteinase K is very stable and can be stored for years at -20°C . At low concentrations ($\sim 10\text{ }\mu\text{g/mL}$) it is less stable and its activity gradually decreases due to autolysis [16].
6. We consider the cassette dipping important for speeding up the processing of sections. The gentle and consistent dipping enhances the interaction between the section and the dewaxing (or washing) solution due to the increased convection.
7. The duration of Proteinase K digestion may need adjustment depending on the tissue type. Harder tissues might require longer digestion. Times of 10 min are usually used. The complete omission of the digestion step results in a weaker signal. On the other hand, overdigestion can result in signal disappearance and disruption of entire section.
8. In the initial experiments we used 215 pmoles ($7.1\text{ }\mu\text{g}$) of the enzyme per section in $25\text{ }\mu\text{L}$ of the reaction mix. However, the topoisomerase concentration can be significantly reduced without any loss of sensitivity. We later used four times less of the enzyme per section 53 pmol ($1.76\text{ }\mu\text{g}$ per section) with similar results. Reducing the amount of enzyme to 26 pmol (880 ng per section) resulted in a weaker signal and 266 fmol (8.8 ng per section) of enzyme produced no signal.

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Fig. 1. Phagolysosomes in macrophages clearing apoptotic cells. Phagolysosomes in the cytosol of macrophages, which engulfed apoptotic cells, are brightly labeled by the TOPO assay (FITC —green fluorescence). Nuclei of macrophages are visualized by DAPI (blue fluorescence). Rat thymus 24 h after injection of dexamethasone [7, 12]. Bar, 50 μ m

Fig. 2.

Assay for detection of DNase II-type breaks in tissue sections. *Left:* TOPO binds to the recognition sequence in the double-hairpin oligo and cleaves it activating left hairpin for ligation to 5' OH DNA termini. *Center:* Active Probe—fluorescent complex of oligo and TOPO covalently linked at the ligatable 3' end of the hairpin (*asterisk*). *Right:* Active probe specifically ligates to the 5' OH double strand DNase II-type break generated by DNase II in phagocytic cells digesting engulfed chromatin