Published in final edited form as:

Methods Mol Biol. 2011 ; 682: 37–48. doi:10.1007/978-1-60327-409-8_4.

IN SITU LABELING OF DNA BREAKS AND APOPTOSIS BY T7 DNA POLYMERASE

Vladimir V. Didenko

Baylor College of Medicine, and Michael E. DeBakey VA Medical Center, Houston TX 77030

Abstract

The native T7 DNA polymerase is a fast and highly processive enzyme which can be used for *in situ* detection of apoptosis and various types of DNA breaks. The technique is quick and simple, and was shown to label earlier stages of apoptosis compared to the terminal transferase technique. The *in situ* labeling applications of T7 DNA polymerase are presented and summarized from the DNA damage detection standpoint. The detailed protocols are provided together with the discussion of their advantages and limitations.

Keywords

DNA breaks; apoptosis detection; T7 DNA polymerase; DNA damage; in situ labeling

1. INTRODUCTION

The technique using native T7 polymerase to identify apoptotic cells is one of several similar methods for detection of DNA breaks *in situ*. The other enzymes employed in this type of labeling include terminal deoxyribonucleotidyl transferase (TdT) and DNA polymerases, such as *E. coli* DNA polymerase I (Pol I) and Klenow fragment of DNA polymerase I.

Technically all these approaches are comparable and, when applied to fresh-frozen or fixed cells and tissue sections, use 3' termini at a variety of DNA breaks as priming points and synthesize new nucleic acid strands incorporating fluorescent or conventional tags. The methods subdivide into three categories based on the mechanism of labeling:

1. TdT-mediated dUTP nick-end labeling (TUNEL) (1,2), also sometimes called *in situ* nick-end labeling (ISNEL) (3), is based on the activity of TdT which, unlike Pol I or Klenow fragment, is template-independent and catalyses long single-stranded DNA tails at any free 3'OH DNA ends.
2. *In situ* nick translation (ISNT) uses Pol I and the nuclease DNase I to label actively transcribing DNA regions; if DNase I is omitted, the Pol I labels the pre-existing single-stranded DNA breaks (4, 5).
3. *In situ* (DNA) end labeling (ISEL) employs Klenow fragment or T7 DNA polymerase to label various protruding 5' DNA ends via fill-in reaction (3, 4, 6–7).

Although the major application of these polymerization-based methods is in apoptosis detection; they have also been successfully used to identify the specifics of DNA damage *in situ* (4, 8, 9).

The rationale for employing *in situ* labeling of DNA breaks for apoptosis detection is the assumption that DNA fragmentation is a characteristic apoptotic marker. In spite of the ever increasing number of exceptions (10–13), orderly and extensive DNA fragmentation is considered a hallmark feature of apoptosis (1, 14). When combined with morphological verification and immunohistochemistry, it can be used as a sensitive and specific indicator of this process.

T7 DNA polymerase-based *in situ* labeling is faster, cheaper and simpler than most other *in situ* approaches. The added advantage is in its ability to identify apoptotic cells at an earlier stage compared to TUNEL, the other popular technique (3).

Native T7 DNA polymerase is a heterodimeric 92 kDa enzyme. It was originally isolated from *E. coli* infected by T7 bacteriophage. It is composed of a polymerase proper- the T7 gene 5 protein, encoded by T7 bacteriophage, and a “booster” protein - thioredoxin, encoded by the *E. coli* bacterium. Both parts of the complex are essential for the efficient polymerization reaction. Without thioredoxin, the gene 5 protein is a nonprocessive DNA polymerase. It cannot continuously incorporate nucleotides without dissociating from the primer template. Thioredoxin significantly increases its affinity for the template, so that the DNA synthesis becomes processive for thousands of nucleotides (15, 16). By contrast, the Klenow fragment incorporates about 15 bases before dissociating, T4 DNA polymerase - about 10 bases, T5 DNA polymerase - about 180 bases (17, 18). In fact T7 DNA polymerase is one of the most processive thermolabile DNA polymerases, with higher processivity than Pol I, Klenow fragment or T4 DNA polymerase (15, 16). Importantly, T7 DNA polymerase efficiently incorporates not only normal dNTPs, but also biotinylated nucleotides (3).

The optimal polymerization templates for T7 DNA polymerase are 5' overhangs and single-stranded gaps in double stranded DNA with available 3'OH DNA ends. The enzyme does not possess the 5'→3' exonuclease activity, and consequently cannot catalyze strand displacement synthesis at nicks (phosphodiester breaks) in duplex DNA (19). Although in solution, single-stranded DNA can serve as a polymerization template-primer for T7 DNA polymerase. This is not likely to happen in fixed DNA in tissue sections, because it occurs when ssDNA loops back upon itself and hybridizes to the same strand. Any unpaired nucleotides at the 3' terminus are then removed by the 3'→5' exonuclease activity until a base-paired terminus is reached and is used to prime the new strand synthesis (15, 19).

A significant feature of native T7 DNA polymerase, important for its labeling applications, is its strong 3'→5' exonuclease (proofreading) activity. The activity degrades a DNA strand in the direction opposite to the direction of polymerization. The hydrolysis reaction initiates from terminal 3' OH ends in both single- and double-stranded DNA. The double-strand exonuclease activity of T7 DNA polymerase is about 6 times stronger than its single-strand activity (20). However in the presence of four dNTPs, the 3'→5' exonuclease activity of T7 DNA polymerase is suppressed. dNTPs at a 30μM concentration completely inhibit 3'→5' exonuclease activity for either single- or double-stranded DNA (21). This probably reflects the inaccessibility of the 3' termini to the exonuclease because of the ongoing polymerization reaction at the termini (21, 22).

In the absence of nucleoside triphosphates, the enzyme initiates DNA hydrolysis at nicks and 3'OH termini in DNA (15). Starting from the available 3'OH ends, it will reduce 3' overhangs and blunt-ended DNA breaks into long 5'overhangs. It will expand nicks into

gaps and will enlarge the single-stranded gaps in the partially double-stranded DNA. Excessive incubation can result in complete DNA degradation.

The high processivity, strong 3'→5' exonuclease activity and the ability to tolerate various tagged nucleoside triphosphates are the properties most important for the application of T7 DNA polymerase for *in situ* labeling of DNA damage. For additional key biochemical properties and constants of this enzyme *see* Note 1.

What molecular targets are labeled by T7 DNA polymerase and justify its application for apoptosis detection?

Studies of apoptotic DNA fragmentation showed that the DNA breaks in it were either blunt-ended or possessed short, single nucleotide overhangs at 3' or 5' ends (23–26). The cutting properties of the major executioner apoptotic nucleases were found responsible for this effect, so that DNase I type enzymes produced blunt-ends or single-base 3' overhangs (23, 24), whereas caspase-3-activated deoxyribonuclease (CAD) generated mainly single-base 5' overhangs (25). From this standpoint, apoptosis labeling techniques which concentrate on the detection of these specific types of DNA brakes would be more specific. This was confirmed with the introduction of *in situ* ligation, which is highly specific for apoptosis and uses T4 DNA ligase to selectively label blunt ends or single-base 3' overhangs (23, 27, 28). However T7 DNA polymerase preferentially fills in 5' overhangs and single-stranded gaps in double-stranded DNA, so that neither blunt ends nor 3' overhangs are normally labeled. T7 DNA polymerase also cannot label nicks (single phosphodiester bond interruptions) in a DNA duplex because it is unable to displace the 5'-terminal strand at the nick.

Blunt ends and 3' overhangs become the targets of polymerase-based labeling due to the utilization of replacement synthesis. The replacement synthesis procedure was initially developed for *in vitro* applications of T4 DNA polymerase and employed the strong 3'→5' exonuclease activity of that enzyme (29). The approach was adapted for T7 DNA polymerase because it possesses an equally strong 3'→5' exonuclease activity. To make use of replacement synthesis, the labeling procedure includes the step where, prior to the labeling reaction, the tissue sections are pre-incubated with T7 DNA polymerase without

¹Here is a brief synopsis of the properties of native (unmodified) T7 DNA polymerase relevant to its usage for *in situ* labeling of DNA breaks and apoptosis.

Temperature sensitivity: Optimal temperature 37°C; increasing temperature to 42° C inhibits polymerase and exonuclease activities with half-time ~ 3min (in the absence of Mg²⁺) (21). 10 min at 75° C completely inactivates the enzyme (in the presence of Mg²⁺). Lowering temperature below 37°C reduces polymerization rates and processivity. One week at room temperature (23°C) results in 30% loss of enzymatic activity (15). Enzyme is stable when stored at -20°C.

pH sensitivity : Optimal pH 7.6–7.8 in potassium phosphate buffer. The enzyme is less active (retains ~40% activity) in Tris buffer in the same pH range (30). The enzyme is remarkably stable at alkaline pH values, whereas it is irreversibly inactivated at acidic pH (retains 83% activity after 2hrs at pH 12, while it is only 4% active after 2hrs at pH 2.2) (31).

Sensitivity to ions: Mg²⁺ or Mn²⁺ are absolutely required for polymerization reaction. The Mg²⁺ optimum is 10 mM at 150 μM of each of the four nucleotides (31). Optimal concentration of Mn²⁺ is 0.1mM (32). Substitution Mn²⁺ for Mg²⁺ changes some enzyme properties such as fidelity (15). KCl at 20 mM increases enzyme activity 20% followed by complete inhibition at 300 mM (31).

Effects of chemicals: Sensitive to sulfhydryl inactivation and in order to remain active requires 2-mercaptoethanol or dithiothreitol. When 2-mercaptoethanol is omitted from the incubation mixture the reaction proceeds at only half the optimal rate (30). EDTA chelates Mg²⁺ and is a strong polymerase inhibitor. 30μM dNTPs (but not rNTPs) inhibit 3'→5' single-strand and double-strand exonuclease activities (21).

Nucleotide incorporation: Shows the comparatively high K_m value (Michaelis constant) for an equimolar mixture of the four dNTPs = 80μM (31). The rate of synthesis is ~ 100 nucleotides per second and a single enzyme molecule is capable of synthesizing thousands of nucleotides from the same primer without dissociating (33).

Efficiently incorporates ddNTPs, dNTPαS, dITP, 7-deaza-dGTP, biotin-14-dATP (3, 15.). The efficiency of incorporation significantly increases when Mn²⁺ (2mM) is substituted for Mg²⁺ (5mM) (32). The native enzyme cannot incorporate 1,N⁶ etheno-2'-deoxyadenosine 5'-triphosphate unlike modified enzyme without exonuclease activity (33).

Native T7 DNA polymerase cannot catalyze strand-displacement synthesis and stalls when synthesizing DNA at a nick (33). The unmodified native enzyme also does not polymerize nucleotides through hairpin structures and terminates the extension within three to four nucleotides of the base of the hairpin (33).

adding dNTPs. This pretreatment significantly expands the spectrum of DNA breaks labeled by T7 DNA polymerase because its 3'→5' exonuclease activity converts blunt ended DNA breaks and 3' overhangs into the detectable 5' overhangs. It also expands nicks into single-stranded gaps and lengthens the pre-existing 5' overhangs and gaps in DNA making them more prominent. Although an overly long pre-incubation can extensively degrade DNA, short pre-incubation serves as a signal amplifier.

Two modifications of the *in situ* labeling technique using T7 DNA polymerase are described in this chapter. Both presented protocols efficiently label apoptotic cells and proceed through similar technical steps. The first technique was initially introduced by Wood et al (6, 7). It employs non-fluorescent detection and labels apoptosis in unfixed, fresh-frozen sections and in cultured cells. The second protocol was developed by Tanaka and co-authors (3). It uses fluorescence labeling and is applicable to paraffin-embedded sections. This technique can also be performed in a double-staining format with immunohistochemistry for additional verification of cell death by apoptosis-related antibodies. The formalin-fixed paraffin-embedded sections generally provide significantly better morphology compared to frozen sections and permit individual morphological evaluations of labeled cells.

The T7 DNA polymerase-based technique was shown to label cells in an earlier stage of apoptosis compared to TUNEL (3). In experiments, the TdT-based and T7 polymerase-based labeling of apoptotic rat brain neurons were compared with immunohistochemical detection of active caspase-3 in these cells. The earlier stage was indicated because the T7 DNA polymerase labeled more neurons with cytoplasmic localization of active caspase-3 compared with the TdT technique (40% vs. 13%). The T7-labeled cells also had morphological signs of earlier apoptosis, such as significantly larger nuclei than those labeled by TdT ($17.0 \pm 2.3\mu\text{m}^2$ vs. $9.1 \pm 0.8\mu\text{m}^2$) (3).

The reasons for this preference towards earlier apoptosis detection are not completely clear. The explanation provided in the original paper is not convincing. It stated that TdT preferentially labels single-stranded DNA, whereas T7 DNA polymerase exclusively labels DNA duplexes, which supposedly decrease as apoptosis progresses (3). A more likely explanation could be in the ability of T7 DNA polymerase to expand the few nicks or single-stranded DNA breaks produced in earlier apoptosis into wide single-stranded gaps which are efficiently labeled by the enzyme.

Below we describe two protocols using T7DNA polymerase for apoptosis detection in frozen cells and tissue sections and in formalin-fixed tissues. The descriptions are aimed at the beginner in this field without previous experience with the method.

2. MATERIALS

1. 20 μm -thick cryostat-cut tissue sections from fresh frozen tissue blocks, unfixed cultured cells, or 5–6 μm -thick sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks.
2. Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, Pa) for cryostat sections and unfixed cells.
3. ProbeOn™ Plus charged and precleaned slides (Fisher Scientific, Pittsburgh, Pa) for the sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks. Other slide brands can also be used if they retain tissue well.
4. 10× Phosphate-buffered saline (PBS). For 1 Liter: dissolve 2.0 g KH_2PO_4 , 11.5g Na_2HPO_4 , 80g NaCl, 2g KCl in 800 mL distilled water, mix and adjust pH to 7.4 with HCl. Add water to bring volume to 1L.

5. 4% formaldehyde. Dilute 37% formaldehyde (Sigma, St. Louis, MO) in PBS 1:9. Store at 4°C, protect from light. Avoid using after three weeks of storage.
6. 70%, 80%, 95% and 100% ethanol.
7. Chloroform (Sigma, St. Louis, MO).
8. Unmodified T7 DNA polymerase 10 U/μL (Epicentre Biotechnologies Inc., WI) (*see* Note 2).
9. 10× T7 Reaction Buffer (Epicentre Biotechnologies Inc., WI) – the reaction buffer for T7 DNA polymerase: 330 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 100 mM magnesium sulfate, 5 mM dithiothreitol.
10. 0.5 mM EDTA, pH 8.0, in PBS.
11. Nucleotides: 200 mM dGTP, dTTP, dCTP, 20 mM dATP and 40 mM biotin-14-dATP (Sigma, St. Louis, MO).
12. Streptavidin-Cy2 conjugate or streptavidin-fluorescein conjugate or streptavidin–Texas Red conjugate (Molecular Probes, Eugene, OR).
13. 0.1% BSA (USB Corporation, Cleveland, OH).
14. Biotin-blocking reagents, such as those provided in the Cell and Tissue Staining kit (R&D Systems, Minneapolis, MN)
15. Streptavidin-peroxidase conjugates (Rockland Immunochemicals, Inc., Gilbertsville, PA).
16. ImmPACT™ DAB Peroxidase Substrate (Vector Laboratories, Burlingame, CA). DAB is a potential carcinogen.
17. Permount mounting media (Fisher Scientific, Pittsburgh, Pa).
18. Vectashield (Vector Laboratories, Burlingame, CA) antifading-counterstaining solution with DAPI (1μg/ml). Store at 4°C in the dark. DAPI is a potential carcinogen.
19. Glass or plastic coverslips.
20. Fluorescent or conventional microscope with appropriate filters and objectives.

3.METHOD

3.1. Protocol 1. Fresh-frozen cryostat sections or unfixed cells

- 1 Air-dry unfixed cryostat sections, and then place them for 5 min in PBS. For cells, air-dry 100 μL of cells (approx. 1×10^6 cells/mL) on a glass microscope slide, then wash gently in PBS.
- 2 Place sections or cells in 4% formaldehyde for 10 min, then wash 2×5 min in PBS.
- 3 Dehydrate sections or cells through serial ethanol washes for 2 min in each 70%, 80%, 95% and 100% ethanol.
- 4 Place in chloroform for 2 min.

²Unmodified i.e. native T7 DNA polymerase is used in this labeling technique. In modified T7 DNA polymerase, such as Sequenase™ from USB Corporation, the 3'→5' exonuclease activity of the original enzyme has been removed. Although such an enzyme will still label 5' overhangs, its utility for apoptosis detection *in situ* has not been tested.

- 5 Rehydrate by passing through graded ethanol concentrations: 95% Ethanol –2 min; 70% Ethanol – 2min; PBS – 3×5 min. (*see* Note 3)
- 6 Apply T7 Reaction Buffer. Incubate for 10 min at room temperature (23° C) (*see* Note 4).
- 7 Replace buffer with 0.1 U/μL unmodified T7 DNA Polymerase in T7 Reaction Buffer; Incubate for 10 min at room temperature (23° C).
- 8 Replace with 0.1 U/μl unmodified T7 DNA Polymerase, 0.2 mM each dCTP, dGTP, dTTP, 0.02mM dATP, 0.04 mM biotin-14-dATP in T7 Reaction Buffer; Incubate for 10 min at room temperature (23° C) (*see* Note 5).
- 9 Aspirate the solution and stop the reaction by adding 0.5 mM EDTA, pH 8.0, in PBS.
- 10 Wash sections or cells in 3 changes of PBS.
- 11 For color development Incubate slide in 0.1% BSA for 30 min, then rinse twice in PBS.
- 12 Cover specimen with 10 μg/ml streptavidin-peroxidase in PBS for 30 min in the dark.
- 13 Wash specimen in 3 changes of PBS.
- 14 Incubate specimens with ImmPACT™ peroxidase DAB substrate solution for 5–15 min at room temperature. Wear gloves, DAB is a suspected carcinogen.
- 15 Stop the reaction by washing in 3 changes of distilled water (*see* Note 6).
- 17 For viewing, the specimen is dehydrated for 10 min each in 70%, 95% then 100% ethanol, and clarified in xylene for 10 min. Mount a coverslip using Permount. A positive apoptosis reaction is indicated by a dark purple color (*see* Note 7).

3.2. Protocol 2. Formaldehyde-fixed, paraffin-embedded tissues

1. Place the sections 5–6μm-thick sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks onto ProbeOn™ Plus charged and precleaned slides (Fisher Scientific, Pittsburgh, Pa). Other slide brands can also be used if they retain tissue well.
2. Dewax the sections in xylene for 15 min, transfer to a fresh xylene bath for additional 5 minutes.
3. Rehydrate by passing through graded ethanol concentrations: 95% Ethanol – 2×5min; 80% Ethanol – 5min; water – 2×5 min (*see* Note 8).

³Alternately, after fixing, the cells or sections can be permeabilized by incubating 5 min in 0.1% Triton X-100 followed by three washes in PBS. To suppress endogenous peroxidase activity, treat the sections with 0.1% hydrogen peroxide for 20 min. To block endogenous biotin, incubate with biotin-blocking reagents, such as those provided in the Cell and Tissue Staining kit. Wash 2X in PBS.

⁴Pre-incubation with T7 buffer ensures even saturation of the section prior to addition of the enzyme and dNTPs for uniform staining. Use 50–200 μL of solution, as needed to cover the section or cells.

⁵Direct fluorescent labeling is possible by using fluorescent dNTPs instead of biotin. To ensure the absence of nonspecific background staining due to interactions between fluorophores and charged groups in cellular proteins, the signal should be verified using control sections incubated with fluorescent dNTPs without the enzyme.

⁶At this stage, if required, the specimen may be counterstained by using Eosin, Methyl Green or Hematoxylin.

⁷A mock reaction is recommended as a regular control in order to rule out nonspecific background staining. In a mock reaction all labeling reaction components except the enzyme are present. A positive control with verified apoptosis is also recommended (such as dexamethazone-treated rat thymus, etc (23)).

4. Equilibrate in T7 buffer for 10 min at room temperature (23°C) (*see* Note 4).
5. Replace buffer with 1 U/μL unmodified T7 DNA Polymerase in T7 Reaction Buffer; Incubate for 10 min at room temperature (23° C). (*see* Note 9)
6. Apply the full reaction solution composed of 0.1 U/μL T7 DNA polymerase, 200 mM dGTP, dTTP, dCTP, 20 mM dATP and 40 mM biotin-14-dATP in T7 buffer. Incubate for 1 hr at 37°C in a humidified chamber. (*see* Note 10)
7. Aspirate the solution and stop the reaction by incubating 10 min in 50 mM EDTA, pH 8.0, in PBS at room temperature.
8. Wash sections 3×10 min in distilled water by gently immersing the slides in a coplin jar containing water at room temperature.
9. Dilute 2 μL of streptavidin-Cy2 conjugate in 1 mL of sodium bicarbonate buffer (*see* Note 11). Add 100 μL of this solution to the section. Incubate 45 min at room temperature in a covered humidified chamber (*see* Note 12).
10. Wash 3×10 min in distilled water.
11. Add Vectashield with DAPI and a coverslip (*see* Note 7).

Acknowledgments

This research was supported by grant R01 NS062842 from the National Institute of Neurological Disorders and Stroke, National Institutes of Health and by grants R21 NS064403 from the National Institute of Neurological Disorders and Stroke, National Institutes of Health through ARRA and R21 EB006301 National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health.

References

1. Walker, PR.; Carson, C.; Leblanc, J.; Sikorska, M. Labeling DNA damage with terminal transferase: applicability, specificity and limitations. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols*. 2001. p. 3-19.
2. Loo, DT. TUNEL Assay: An Overview of Techniques. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols*. 2001. p. 21-30.
3. Tanaka M, Momoi T, Marunouchi T. In situ detection of activated caspase-3 in apoptotic granule neurons in the developing cerebellum in slice cultures and in vivo. *Developmental Brain Research*. 2000; 121:223–28. [PubMed: 10876036]

⁸At this point, many protocols which use other DNA polymerases include a tissue permeabilization step accomplished by proteinase K treatment (4). This permits better access of polymerases to the sites of the breaks in formaldehyde-fixed sections. However the proteinase K step is absent in all T7 DNA polymerase protocols, developed for either frozen or formalin-fixed sections (3, 6, 7, 34). In frozen sections the proteinase K digestion can easily detach frozen sections from glass slides. Therefore cells and sections are in this case permeabilized by incubation for 5 min in 0.1% Triton X-100 followed by three washes in PBS. However in case of formalin-fixed sections, the absence of proteinase K treatment limits the amounts of detectable DNA breaks and could be one of the factors responsible for the detection of earlier apoptotic DNA breaks by this modification of the T7 DNA polymerase technique.

⁹Note that in case of formalin-fixed sections the amount of T7 DNA polymerase in the pre-incubation solution is 10 times higher compared to fresh-frozen sections.

¹⁰Note the higher temperature (37°C) and the longer time of the labeling reaction (1hr) in case of formalin-fixed sections compared to fresh-frozen sections and unfixed cells.

¹¹Other fluorescent conjugates can be used instead, such as streptavidin-fluorescein or streptavidin-Texas Red, etc. Some tissue sections have higher nonspecific background labeling with one particular type of fluorophore but not the other. In this case, using a different conjugate can significantly improve the signal/noise ratio.

¹²Non-fluorescent signal sometimes produces less background as compared with fluorescent labeling. It can be achieved by substituting streptavidin-peroxidase for streptavidin-fluorophores with subsequent color development by using ImmPACT™ peroxidase DAB substrate solution as described in protocol 1. In the color development step incubate tissue sections with the DAB substrate working solution at room temperature until suitable staining develops. The optimal development time is determined experimentally and generally is in the 2 to 15 minutes range.

4. Dierendonck, JH. DNA damage detection using DNA polymerase I or its Klenow fragment: applicability, specificity, limitations. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols*. 2001. p. 81-108.
5. Thiry, M. *In Situ* Nick Translation at the Electron Microscopic Level. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols*. 2001. p. 121-130.
6. Wood KA, Dipasquale B, Youle RJ. In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron*. 1993; 4:621-32. [PubMed: 8398151]
7. Wood KA, Youle RJ. The Role of Free Radicals and ~ 53 in Neuron Apoptosis in vivo. *J Neurosci*. 1995; 15:5851-57. [PubMed: 7643225]
8. Didenko VV, Ngo H, Baskin DS. Early necrotic DNA degradation: presence of blunt-ended DNA breaks, 3' and 5' overhangs in apoptosis, but only 5' overhangs in early necrosis. *Am J Pathol*. 2003; 162:1571-78. [PubMed: 12707041]
9. Otsuki, Y.; Ito, Y. Quantitative Differentiation of Both Free 3' OH and 5' OH DNA Ends Using Terminal Transferase-Based Labeling Combined with Transmission Electron Microscopy. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols*. 2001. p. 41-54.
10. De Felici M, Lobascio AM, Klinger FG. Cell death in fetal oocytes: many players for multiple pathways. *Autophagy*. 2008; 4:240-42. [PubMed: 18094606]
11. Hirata H, Hibasami H, Yoshida T, Morita A, Ohkaya S, Matsumoto M, Sasaki H, Uchida A. Differentiation and apoptosis without DNA fragmentation in cultured Schwann cells derived from wallerian-degenerated nerve. *Apoptosis*. 1998; 3:353-60. [PubMed: 14646482]
12. Métrailler-Ruchonnet I, Pagano A, Carnesecchi S, Ody C, Donati Y, Barazzzone Argiroffo C. Bcl-2 protects against hyperoxia-induced apoptosis through inhibition of the mitochondria-dependent pathway. *Free Radic Biol Med*. 2007; 42:1062-74. [PubMed: 17349933]
13. Cohen GM, X-M, Sun RT, Snowden D, Dinsdale, Skilleter DN. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem J*. 1992; 286:331-34. [PubMed: 1530564]
14. Zhang J, Xu M. Apoptotic DNA fragmentation and tissue homeostasis. *Trends Cell Biol*. 2002; 12:84-9. [PubMed: 11849972]
15. Eun, H-M. *Enzymology Primer for Recombinant DNA Technology*. Academic Press; San Diego CA: 1996. p. 377-407.
16. Tabor, S.; Struhl, K.; Scharf, SJ.; Gelfand, DH. DNA-dependent DNA polymerases. In: Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K., editors. *Current Protocols in Molecular Biology*. John Wiley & Sons; Hoboken, NJ: 1997. p. 3.5.1-3.5.15.
17. Das SK, Fujimura RK. Processiveness of DNA polymerases. A comparative study using a simple procedure. *J Biol Chem*. 1979; 254:1227-32. [PubMed: 368069]
18. Bambara RA, Uyemura D, Choi T. On the processive mechanism of *Escherichia coli* DNA polymerase I. *J Biol Chem*. 1978; 253:413-23. [PubMed: 338607]
19. Lehman, IR. T-Phage DNA Polymerases. In: Boyer, PD., editor. *The Enzymes*. Vol. 14. 1981. p. 64
20. Tabor S, Huber HE, Richardson CC. *Escherichia coli* thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. *JBC*. 1987; 262:16212-223.
21. Adler S, Modrich P. T7-induced DNA polymerase. Characterization of associated exonuclease activities and resolution into biologically active subunits. *JBC*. 1979; 254:11605-614.
22. Hori K, Mark DF, Richardson CC. Deoxyribonucleic acid polymerase of bacteriophage T7. Purification and properties of the phage-encoded subunit, the gene 5 protein. *JBC*. 1979; 254:11591-97.
23. Didenko VV, Hornsby PJ. Presence of double-strand breaks with single-base 3' overhangs in cells undergoing apoptosis but not necrosis. *J Cell Biol*. 1996; 135:1369-1376. [PubMed: 8947557]
24. Didenko VV, Tunstead JR, Hornsby PJ. Biotin-labeled hairpin oligonucleotides. Probes to detect double-strand breaks in DNA in apoptotic cells. *Am J Pathol*. 1998; 152:897-902. [PubMed: 9546350]

25. Widlak P, Li P, Wang X, Garrard WT. Cleavage preferences of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates. *J Biol Chem.* 2000; 275:8226–8232. [PubMed: 10713148]
26. Staley K, Blaschke AJ, Chun J. Apoptotic DNA fragmentation is detected by a semiquantitative ligation-mediated PCR of blunt DNA ends. *Cell Death Diff.* 1997; 4:66–75.
27. Didenko, VV. Detection of specific double-strand DNA breaks and apoptosis in situ using T4 DNA ligase. In: Didenko, VV., editor. *In Situ Detection of DNA Damage: Methods and Protocols.* 2002. p. 143-151.
28. Al-Lamki RS, Skepper JN, Loke YW, King A, Burton GJ. Apoptosis in the early human placental bed and its discrimination from necrosis using the in-situ DNA ligation technique. *Hum Reprod.* 1998; 13:3511–9. [PubMed: 9886542]
29. Challberg MD, Englund PT. Specific labeling of 3' termini with T4 DNA polymerase. *Methods Enzymol.* 1980; 65:39–43. [PubMed: 6990190]
30. Grippo P, Richardson CC. Deoxyribonucleic acid polymerase of bacteriophage T7. *JBC.* 1971; 246:6867–73.
31. Nordstrom B, Randahl H, Slaby I, Holmgren A. Characterization of bacteriophage T7 DNA polymerase purified to homogeneity by antithioredoxin immunoabsorbent chromatography. *JBC.* 1981; 256:3112–17.
32. Tabor S, Richardson CC. Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and Escherichia coli DNA polymerase I. *PNAS.* 1989; 86:4076–80. [PubMed: 2657738]
33. Tabor S, Richardson CC. Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by in vitro mutagenesis. *JBC.* 1989; 264:6447–58.
34. Wood, KA. Unmodified T7 DNA Polymerase for the In Situ Detection of DNA Fragmentation Associated with Apoptosis. *Epicentre Forum.* 1994. p. 1(http://www.epibio.com/f1_2/f1_2t7.asp)