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Zebra Tail Amplification: Accelerated Detection of Apoptotic Blunt-Ended DNA Breaks by In Situ Ligation

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Abstract

In situ ligation (ISL) is a simple and specific technique for apoptosis labeling in tissue sections. In its most economical version ISL uses ordinary PCR-labeled DNA fragments as probes. In tissue sections these makeshift probes are ligated to apoptotic DNA breaks by T4 DNA ligase. The approach can selectively label 5'PO₄ DNA breaks with blunt ends, and is the histological equivalent of electrophoretic apoptotic ladder detection. The main drawback of this technique is its low speed, as it requires 18 h-incubation for efficient labeling. Here, we describe an easy modification of ISL which reduces the incubation time to 1 h and converts ISL into a rapid detection method taking ~3 h overall. Signal enhancement is achieved by a new type of isothermal amplification reaction which generates “zebra tails”—long and labeled extensions of the probes attached to DNA breaks.

Keywords

In situ ligation; Zebra tail amplification; Rapid apoptosis detection; Isothermal amplification; Blunt-ended DNA breaks labeling; Signal enhancement

1 Introduction

The majority of DNA fragments produced in apoptotic DNA cleavage are blunt-ended and have 3'OH/5'PO₄ functional groups at the ends [1–3]. The techniques that simultaneously detect several of these DNA cleavage features can label apoptotic cells with increased specificity. In situ ligation (ISL) is one of those techniques [4]. This approach detects two markers of apoptotic DNA fragmentation: blunt DNA ends and terminal 5' phosphates in DNA breaks in fixed apoptotic cells. The reaction is carried out by adding blunt-ended 5'OH DNA probes and T4DNA ligase directly to tissue sections [5] (Fig. 1). The ISL labeling occurs only when both markers of apoptotic DNA cleavage are simultaneously present. As a result, the assay is more specific for apoptosis than TUNEL which labels a single marker of broken DNA—free 3'OH groups [4, 6–8]. Free 3'OH groups in DNA represent a much more generic type of DNA damage and can be produced as artifacts of tissue section processing, cutting, or heating. In addition, 3'OH termini in DNA are generated in necrosis, nonlethal DNA damage, and DNA repair. All of these can produce TUNEL staining not related to apoptosis. This shortcoming of the TUNEL assay is discussed in a number of cautionary notes [6, 9–17]. In spite of this limitation TUNEL is far more popular than the more specific ISL technique. One of the reasons is the time-consuming nature of ISL, requiring 18 h-long incubations, much more protracted than the ~3 h-long TUNEL [18].

Accelerating ISL detection would make it significantly more attractive for routine apoptosis labeling in the tissue section format. However, the considerable length of incubation in ISL is essential for the ligation of a sufficient number of probes to DNA breaks in tissue sections. The probe attachment to DNA ends that are hidden inside tissue section is slower than DNA ligations occurring entirely in solution. This is probably due to the lower accessibility of the cellular ligation targets which are partially concealed in the cross-linked matrix of the section. The ISL labeling reaction is not accelerated by commercially available ultrafast ligation mixes exploiting the volume exclusion effect of PEG, because similar or even higher PEG concentrations are already used in ISL protocols [5, 19, 20]. The same is true for boosting the T4 DNA ligase concentration, as the standard ISL protocol already uses the highly concentrated enzyme [5, 19]. Besides, following this approach even further can make the assay prohibitively expensive.

The other way to make this assay faster is by a major increase of the probe signal. Interestingly, such an approach was employed in the biochemical counterpart of ISL [2] which appeared soon after we developed the in situ ligation method [6]. Then a paper was published in 1997 describing a PCR-based technique, which also used ligase, this time for biochemical detection of double-strand breaks in DNA of apoptotic cells [2]. In this study, nucleosomal ladders were revealed by a ligation-mediated polymerase chain reaction (LMPCR), which amplified DNA fragments with blunt, 5' phosphorylated ends. LMPCR combined detection and PCR-based amplification steps into a single procedure and was therefore fast and sensitive.

However, the straightforward adaptation of PCR-based amplification to the ISL technique proved to be difficult. Therefore, we developed an alternative amplification technique, which specifically enhances ISL signal. This new amplification procedure eliminates the need for the time-consuming incubations with ligase because sufficient ISL signal is achieved after 1 h incubation.

The ISL signal is enhanced with the help of the new isothermal cyclic elongation reaction that makes the already ligated probes much longer by generating their protracted labeled extensions—“zebra tails.” These are produced by repetitive ligations of the new probes to the continually reactivated ends of the growing “seed” probes. The term “zebra tail” originates from the graphical representation of this perpetual lengthening when each new ligation event is marked with a black stripe. The ongoing probe extension produces the familiar “zebra” pattern.

Remarkably, the “zebra tail” signal amplification is initiated by introducing a very minimal change to the standard ISL protocol. The amplification cycles start after the addition of a restriction enzyme to the labeling mix. The added restrictase is required to be a blunt-end cutter and to have a single cleavage site in the PCR fragment used as an ISL probe. The principles of the zebra tail amplification (ZTA) technique are illustrated in Fig. 2.

In sum, the amplification reaction constantly regenerates the ligatable blunt end of the growing probe enabling its continuous elongation. Every cleavage-ligation cycle adds either the full- or the partial-length of the initial labeled DNA fragment (*see Note 1*). When each

new ligation event is marked with a black stripe, the multiple cycles of cleavage-ligation produce a long “zebra tail” of labeled DNA (Fig. 2) (*see Note 2*).

Because “zebra tails” are the direct extensions of the reacted probes, they do not disperse or diffuse and indicate the probe’s location with high accuracy. This property can be useful in providing better resolution during in situ detection.

The presented protocol is applicable for specific non- fluorescent detection of apoptotic cells in tissue sections. The procedure is eightfold faster compared to the original ISL and overall takes ~3 h to complete.

In addition to the time economy, the increased signal intensity can be helpful for better visualization of morphological changes in individual apoptotic cells when strong and selective labeling of the apoptotic nuclear chromatin is needed (*see Note 3*). The assay can also be applied for specific identification of minute apoptotic bodies in preparations of microvesicles, exosomes, and other extracellular vesicles [21].

2 Materials

1. The 5–6 μm -thick sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks onto charged and precleaned slides.
2. Xylene.
3. 70, 80 and 96% Ethanol.
4. T4 DNA ligase 5 U/ μL (Roche Molecular Biochemicals, Indianapolis, IN) (*see Note 4*).
5. 10 $\times$ reaction buffer for T4 DNA ligase: 660 mM-Tris-HCl, 50 mM MgCl_2 , 10 mM dithioerythritol, 10 mM ATP, pH 7.5 (20 $^\circ\text{C}$) (Roche Molecular Biochemicals, Indianapolis, IN).
6. 30% (w/v) solution of PEG-8000 (Sigma, St. Louis, MO) in bidistilled water (*see Note 5*).
7. Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) 20 mg/mL stock solution in distilled water. Store at $-20\text{ }^\circ\text{C}$. In the reaction use 50 $\mu\text{g/mL}$ solution in PBS, prepared from the stock. Do not reuse (*see Note 6*).
8. PCR-derived digoxigenin-labeled DNA fragment obtained using Pfu polymerase (*see Note 7*).

The sequence of the fragment is not important. However, it should contain a unique restriction site. We used the same 226 bp long PCR-labeled fragment as before in regular ISL [6, 19]. It contained a single Sma I restriction site (*see Note 8*).
9. Sma I restriction endonuclease 20 U/ μL (New England Biolabs, Indianapolis, IN). This enzyme is used only with the PCR fragment described above. If

another fragment is used, check its restriction map, and select the appropriate blunt end-producing restrictase (*see Note 9*).

10. Phosphate-buffered saline (PBS), pH 7.4.
11. Washing buffer: 100 mM Tris-HCl, pH 7.5; 100 mM NaCl.
12. Blocking solution: 10% sheep serum in 100 mM Tris-HCl, pH 7.5; 100 mM NaCl.
13. 1-Step™ NBT/BCIP (Pierce)—ready-to-use substrate for alkaline phosphatase that yields a very intense purple signal. If background caused by endogenous phosphatases is a problem, then 1-Step NBT/BCIP plus Suppressor can be used. This substrate formulation additionally contains 1 mM levamisole to inhibit endogenous phosphatase activity.
14. Anti-digoxigenin antibody—alkaline-phosphatase conjugate (Roche Molecular Biochemicals, Indianapolis, IN). Fab fragments from an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase for the detection of digoxigenin-labeled compounds.
15. 22 × 22 mm or 22 × 40 mm glass or plastic coverslips. Plastic coverslips are preferable as they are easier to remove from the section.
16. Aqueous slide mounting media such as Aqua-Mount (Thermo Fisher Scientific, Waltham, MA) or ImmunoHistoMount (Santa Cruz Biotechnology, Santa Cruz, CA).
17. Microscope with appropriate filters and objectives.

3 Method

1. Place the sections in a slide rack and dewax in xylene for 5 min, transfer to a fresh xylene bath two more times for an additional 2 min each. For each transfer, dip cassette up and down three times.
2. Rehydrate by passing through graded ethanol concentrations: 96% Ethanol—2 × 2 min; 80% Ethanol—2 × 2 min; 70% Ethanol—2 min; water—2 × 2 min. For each transfer, dip cassette up and down three times.
3. Digest section with Proteinase K. Use 100 µL of a 50 µg/mL solution per section. Incubate for 10 min at room temperature in a humidified chamber (*see Note 10*).
4. Rinse in distilled water for 3 × 2 min. For each transfer, dip cassette up and down three times.
5. Apply 100 µL per section of the pre-incubation solution. Incubate for 5 min at room temperature (23 °C). The pre-incubation solution consists of a 1× T4 DNA ligase reaction buffer supplemented with PEG-8000 to the final concentration of 15%. It contains 66 mM-Tris HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithioerythritol, 1 mM ATP, and 15% polyethylene glycol (*see Note 11*).

6. Aspirate the pre-incubation solution and apply the full ligase reaction mix with the probe (750 ng/ μ L stock) and T4 DNA ligase (*see Note 12*).
In situ ligation labeling solution (20 μ L per section):
Prepare on ice in this order:
4 μ L—bidistilled water.
10 μ L—PEG8000 (30% stock solution).
2 μ L—10 $\times$ buffer for T4 DNA ligase.
1 μ L—PCR labeled probe (0.5 μ g/ μ L).
2 μ L—T4 DNA ligase (5 U/ μ L) (*see Note 4*).
1 μ L—Sma I restrictase (20 U/ μ L) (*see Note 9*).
The total volume of the labeling solution can be scaled up to accommodate the bigger sections.
Incubate for 1 h. at room temperature (23 $^{\circ}$ C) (*see Note 13*) in a humidified chamber with a plastic coverslip.
The total volume of the labeling solution can be scaled up to accommodate the bigger sections.
7. Remove coverslips by gently immersing the slides vertically in a Coplin jar containing water at room temperature. Then wash sections 3 $\times$ 5 min in distilled water.
8. Apply 100 μ L per section of the blocking solution containing 10% sheep serum. Incubate for 15 min at room temperature (23 $^{\circ}$ C) in a humidified chamber.
9. Aspirate the pre-incubation solution and apply 50 μ L per section of solution of anti-digoxigenin antibody—alkaline-phosphatase conjugate diluted 1:100 in the blocking solution. Incubate for 10 min at room temperature (23 $^{\circ}$ C) in a humidified chamber.
10. Wash 2 $\times$ 10 min in 100 mM Tris-HCl, pH 7.5; 100 mM NaCl.
11. Apply 1-StepTM NBT/BCIP substrate solution for alkaline phosphatase. Keep in the dark. Monitor color development. The color usually develops in 5–20 min.
12. Stop reaction by rinsing sections in water. Add water-soluble mounting media such as Aqua-Mount or ImmunoHistoMount and coverslip.

4 Notes

1. The elongation reaction is a single-strand ligation occurring between 3'OH and 5'PO₄ termini, whereas 3'OH and 5'OH termini cannot be linked by T4DNA ligase. Therefore, each fully formed double-strand “zebra tail” contains the regularly positioned nicks in one opposing strand.

2. Some free-floating “zebra tails” are also created in solution, where restriction and amplification can occur prior to probe ligation to DNA breaks in the section. This does not change the results of the labeling reaction, because the longer probes created in this case have the same configuration of ends as the original fragment. When ligated to breaks in the section, they detect the same type of DNA damage and produce enhanced signal.
3. Both ISL and its ZTA enhancement presented here can selectively detect blunt-ended and 5′ phosphorylated DNA breaks. This is convenient for apoptosis labeling because the majority of DNA breaks in apoptosis are blunt-ended with the 3′OH/5′PO₄ at the ends [1–3]. However, smaller numbers of these same breaks are produced in other important situations, such as V(D)J recombination [22], as a result of action of ionizing radiation, various chemicals, free radicals, and biological processes [23, 24].
4. In our experiments the highly concentrated (5 U/μL) (Roche Molecular Biochemicals, Indianapolis, IN) ligase preparation repeatedly gives the best signal.
5. DNA ligation is strongly stimulated by 15% PEG-8000 in the reaction solution due to the volume exclusion effect [20]. However, sometimes PEG presence can cause nonspecific background staining. In such cases reducing PEG concentration can significantly reduce or eliminate the problem.
6. In high concentrations (1 mg/mL and higher) proteinase K is a very stable enzyme and can be stored for extended time. However, it gradually loses activity when stored in aqueous solutions at low concentrations (~10 μg/mL) [25].
7. Pfu is a proofreading polymerase and exclusively produces blunt-ended DNA fragments in PCR. The non-proofreading polymerases, such as Taq, produce PCR fragments, which contain single base extensions at their 3′ ends. This prevents ZTA from progression because the fragments with single base overhangs do not ligate to the blunt ends of the growing “zebra tails.”

In PCR probe labeling we prefer using digoxigenin over biotin as it produces less background staining in our experiments. In general, Pfu efficiently incorporates digoxigenin- 11-dUTP or biotin -dUTP if the ratio of substituted-dUTP to dTTP in the labeling mix is 1:2. Therefore usually ~35% of a dTTP in the Pfu PCR reaction mix is substituted by its tagged analog [19]. This is expected to translate into the substitution of ~35% of thymidines in our 226-bp probe by the tagged nucleotides and would result in the insertion of 26 tags. The PCR labeling of in situ ligation probes and their application is described in detail elsewhere [19].
8. The complete sequence of the fragment is presented in [26]. However, this specific sequence has little importance for the labeling reaction. Almost any 100–500-bp long PCR-derived fragment can be used. This range of probe lengths was successfully employed by different laboratories for the original ISL procedure [6, 27–35], and can serve as a starting point for ZTA, which is an accelerated ISL staining. Much longer probes are impractical as they can break

during purification and storage, producing smaller fragments with nonspecific configurations of ends.

9. Different restrictases can be used to drive the reaction. Although we have successfully used frequent cutters, such as Alu I, and more rare cutters such as Sma I, but it is probably better to use rare cutters whenever possible, because the fewer restriction sites per genome potentially limit the likelihood of creating artifactual blunt-ended DNA breaks in the section. In any case the control sections are still needed. Such control sections with confirmed absence of ds DNA breaks should be treated with restriction enzyme alone and then tested by in situ ligation to exclude the possibility of the creation of additional DNA breaks. In our experience, short time (1 h) incubation with Sma I did not create DNA breaks in formalin-fixed human sections, most likely because DNA in the formalin-fixed tissues is cross-linked and is not readily available for cleavage by restrictases. Sma I is used at room temperature (25 °C is recommended by New England Biolabs). At 37 °C it has a half-life of only 15 min.
10. Proteinase K digestion time may need adjustment depending on the tissue type. Insufficient digestion may result in a weaker signal. Overdigestion results in signal disappearance and section disruption. Most often 10–15 min digestion is sufficient.
11. Pre-incubation with ligation buffer ensures even saturation of the section prior to the addition of the enzyme and the probe, and was shown to increase the ligation efficiency. Prepare pre-incubation solution by adding 10 µL of the 10× ligase buffer (Roche) to 40 µL of bidistilled water and 50 µL of 30% PEG-8000.
12. A mock reaction is recommended as a regular control in order to rule out nonspecific background staining. In the mock reaction an equal volume of 50% glycerol in water is substituted for T4 DNA ligase.
13. Lowering of the temperature to 16 °C reduces the signal; a temperature increase to 37 °C completely eliminates the signal.

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Fig. 1.

ISL probe and regular ISL labeling. **(a)** A regular ISL probe is a blunt-ended DNA fragment with 3'OH/5'OH at the ends. It can be tagged with either digoxigenin or fluorophores. The probe's actual sequence is unimportant for the regular ISL procedure. The probe can be used in the isothermal signal amplification procedure if it contains a single recognition site for a blunt-end restrictase (Sma I in this example). **(b)** In the regular ISL, probes are ligated to 5'PO₄ blunt-ended apoptotic DNA breaks in tissue sections. They cannot ligate to each other due to the absence of 5'PO₄ termini required by T4 DNA ligase. Therefore, the regular ISL reaction stops when all DNA probes are ligated to 5'PO₄ breaks in apoptotic DNA

Fig. 2.

Zebra tail amplification of ISL signal. **Step 1.** A restrictase present in the labeling mix cleaves the ligated ISL probes in the section and produces the new generation of blunt ends with 5'PO₄. These serve as selective targets for the second round of probe ligation. **Step 2.** Any ISL probe in solution, regardless if it is cut or not (has 5'PO₄ or 5'OH at the ends), can now ligate to the cleaved probes in the section possessing 5'PO₄ at the ends. In some cases it can restore the original probe (when 3'OH/5'PO₄ end of the solute probe is ligated to 3'OH/5'PO₄ end of the probe in the section); this will be immediately re-cleaved and will not hamper the amplification reaction (not shown). In other cases, such as the reverse end ligation, (when 3'OH/5'OH end of the solute probe is ligated to 3'OH/5'PO₄ end of the probe in the section) the restriction site is not regenerated, so the extended probe cannot be re-cut. This permanently elongates the “seeded” probe and exposes the new 5'PO₄ at its growing end, thus enabling another round of extension. The newly-generated 5'PO₄ at blunt ends are responsible for the growth of the labeled DNA strand and the ISL signal amplification. “Zebra tail” image is formed when each new ligation event on the growing DNA strand is marked with a black stripe