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IN SITU LIGATION SIMPLIFIED: USING PCR FRAGMENTS FOR DETECTION OF DOUBLE-STRAND DNA BREAKS IN TISSUE SECTIONS

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Abstract

The simplified *in situ* ligation procedure is described. All reagents for the assay can be easily obtained in any molecular or cell biology laboratory. The technique uses ligation of double-stranded, PCR-derived DNA fragments labeled with digoxigenin or fluorophores for highly selective detection of apoptotic cells in paraffin-embedded tissue sections. Two types of DNA fragments prepared by PCR are employed. The fragment synthesized by Taq polymerase contains single-base 3' overhangs, whereas the Pfu polymerase-made fragment is blunt ended. Both fragments can be used as specific, sensitive and cost-effective DNA damage probes. After ligation to apoptotic nuclei in tissue sections, they indicate the presence of double-strand DNA breaks with single-base 3' overhangs as well as blunt ends.

Keywords

blunt-ended DNA breaks; double-strand DNA breaks; single-base overhangs; PCR fragments; apoptosis detection; DNA damage detection; in situ labeling; in situ ligation; tissue sections

1. INTRODUCTION

The *in situ* ligation technique is often used for specific labeling of apoptotic cells in tissue sections. The technique employs the enzyme T4DNA ligase for the attachment of oligoprobes to the ends of double-strand DNA breaks in fixed cells (1, 2, 3). These probes are hairpin-shaped oligonucleotides bearing a single fluorophore or biotin. During labeling of DNA breaks a single fluorophore is placed at the end of each break. The sensitivity of such labeling, when using a non confocal fluorescent microscope for observation, is approximately 45,000 FITC molecules in the area occupied by a nucleus 0.01 mm in diameter (4). This is sufficient for labeling apoptotic cells because the amount of breaks in apoptosis rises from about 50,000 per genome, at the initial high molecular weight DNA degradation at the earliest stages of apoptosis, to 3×10^6 during the later internucleosomal DNA fragmentation stage (5). Consequently the molecular probes, which carry a single fluorophore per probe, permit visualization of the full spectrum of breaks present in apoptosis from the initial early stage, to the massive DNA cleavage stage.

However, probes with a signal which is orders of magnitude higher than that of hairpin-shaped oligos would be advantageous in several cases. This refers to the described instances where apoptosis occurs with very few DNA breaks, and to cases where small numbers of

apoptotic cells are present in a tissue. Probes with stronger signal can definitively visualize even solitary apoptotic cells, including those at the earlier stages of apoptosis.

One way to increase the intensity of signal is by placing multiple tags into each *in situ* ligation probe. This can be accomplished by using PCR-derived probes instead of hairpin probes. The additional attractive feature of these probes is that they are inexpensive and can be easily produced when needed in any molecular biology laboratory.

PCR-derived probes were introduced in our first paper describing *in situ* ligation (6). We used 226-bp double-stranded DNA fragments containing digoxigenin or Texas Red to label DNA breaks. In our subsequent work we also used longer 460-bp probes and a variety of fluorescent and conventional labels.

Soon thereafter we introduced a different type of probe based on hairpin-shaped oligonucleotides, but the PCR-based probes still remained popular (7–10). There are several reasons for this popularity. The probes are very sensitive, easy to produce and very inexpensive. Here we present the detailed protocol for their generation and application for apoptosis detection in the tissue section format.

Generally, two forms of double-stranded PCR-derived DNA fragments are used as probes for *in situ* ligation. A Taq polymerase-synthesized fragment has single-base 3' overhangs, whereas a Pfu polymerase-synthesized fragment has blunt-ends. Both types of fragments can be ligated to DNA breaks in apoptotic nuclei, indicating in such nuclei the presence of double-strand DNA breaks with either single-base 3' overhangs or blunt ends.

Compared to hairpin probes, the PCR-labeled fragments generate a much stronger signal. This signal is proportional to the probe length because longer probes contain higher numbers of labeled nucleotides.

A wide range of PCR probes was successfully used by different laboratories. PCR fragments as short as 146 bp (9, 11) and as long as 441 bp (12) were all successfully employed. Our initial PCR probes were 226 bp-long (6) and, in most cases, the PCR probes used in other laboratories stayed close to 200 bp. Some examples include PCR probes of 174 bp (13, 14); 200 bp (10) and 245 bp (7, 8, 15), etc. This is because the much longer probes are impractical as they can break during purification and storage, producing smaller fragments with nonspecific configurations of ends.

In general, digoxigenin-11-dUTP or biotin -dUTP are incorporated with high efficiency if the ratio of substituted-dUTP to dTTP in the labeling mixture is 1:2 (16). Therefore usually ~ 35% of a dTTP in the PCR reaction mix is substituted by its tagged analog. This is expected to translate into the substitution of ~35% of thymidines in our 226-bp probe by tagged nucleotides and would result in the insertion of 26 tags.

In this chapter, we present the *in situ* ligation procedure which uses PCR-derived probes for detection of two types of DNA breaks in fixed tissue sections. We also present the PCR protocols for probes preparation. The described probes are applicable for fluorescence and conventional detection of both blunt-ended DNA breaks and breaks with 3' overhangs for selective labeling of apoptotic vs. necrotic cells (6).

2. MATERIALS

2.1. PCR Probe Preparation and Labeling (see Note 1)

1. PCR primers. Dissolve in distilled water to 100 pmoles/ μ L.

Primer 1 - (27-mer): 5' GCT GGT CTG CCG CCG TTT TCG ACC CTG 3'

Primer 2 - (21-mer): 5' TGG CCT GCC CAA GCT CTA CCT 3'

2. TE buffer: 10mM Tris-HCl, pH 7.5, 1mM EDTA.
3. Uncut pBluescript-bSDI 1 plasmid (Vivid Technologies, Houston, TX). Dissolve in TE to 100 pg/μL (*see* Note 2).
4. Pfu DNA polymerase 2.5 U/μL (Stratagene, La Jolla, CA).
5. Taq DNA polymerase 5 U/μL (Invitrogen, Carlsbad, CA).
6. 10 × buffer for Pfu polymerase (Stratagene, La Jolla, CA): 200mM Tris-HCl, pH 8.8, 100mM KCl, 100mM ammonium sulfate, 20mM MgSO₄, 1% Triton X-100, 1mg/mL BSA.
7. 10x buffer for Taq polymerase: 100mM Tris-HCl, pH 8.35, 500 mM KCl
8. 25mM MgCl₂ stock solution.
9. Digoxigenin-11-dUTP (1 mM) (Roche Molecular Biochemicals, Indianapolis, IN) (*see* Note 3).
10. dATP, dGTP, dCTP, dTTP in separate vials 100 mM stock solutions (Roche Molecular Biochemicals, Indianapolis, IN).

Make 20x dNTPs-dig-dUTP labeling mix containing 1mM dATP; dGTP, dCTP; 0.65 mM dTTP, 0.33mM digoxigenin-11-dUTP (*see* Note 4) . Store at − 20°C.
11. High PureTM PCR Product Purification Kit (Roche Molecular Biochemicals, Indianapolis, IN).
12. Agarose.
13. Ethidium bromide at 0.5μg/mL.
14. Spectrophotometer.
15. PCR cyclor.

¹This protocol describes Taq and Pfu polymerase-based labeling of 226-bp fragments, used as *in situ* ligation probes. The fragment is amplified from pBluescript-bSDI 1 plasmid, which can be obtained from Vivid Technologies, Houston, TX, but almost any other plasmid or a DNA fragment, for which appropriate primers are available to amplify a 200–500 bases-long fragment, can be used instead.

²Although the pBluescript-bSDI1 plasmid is, possibly, the most used and tested template for preparation of the PCR-derived probes for *in situ* ligation (6, 17– 21), other commercially-available plasmids can be used instead with similar results. The examples of the plasmids and primers used by other laboratories include: 1) Bluescript II KS^{+/−} (Stratagene, La Jolla, CA) with primers complementary to the T3 and T7 promoters in the multi-cloning site, resulting in 146 bp probes (9, 11); 2) pOCME1B plasmid with primers 5'-ATGCTCTTCAGTTCGTGTGT-3' and 5'-CTGACTTGGCAGGCTTGAGG-3' amplifying 174 bp fragment (13, 14); 3) pBK-CMV plasmid (Stratagene, La Jolla, CA), as a source of a 200-bp DNA fragment corresponding to its multiple cloning site (10); 4) pBluescript SK(-) plasmid with primers 5'-CTCATAGCTCACGCTGTAGG-3' and 5'-AGTGTAGCCGTAGTTAGGCC-3' amplifying a 245bp fragment (7, 8, 15); 5) pBluescript SK(-) plasmid with primers 5'-TCGAGGTGACGATTTCGATG-3' and 5'-CCGCTCTAGAACTAGTGGATC-3' amplifying a 441bp fragment (12).

³Texas Red-12-dUTP, FITC-12-dUTP, biotin-dUTP and other modified nucleotide analogs can be used instead. However sometimes probes labeled with these tags had higher background staining. In our experiments, the probes labeled with digoxigenin-11-dUTP were less prone to nonspecific background staining.

⁴We found that significantly more labeled nucleotides can be inserted into the synthesized fragment during the Taq-driven PCR labeling when different formulations of dNTPs mixes are used. The successful labeling occurs when the concentration of digoxigenin-11-dUTP in the mixes is kept constant and is 16.5 μM, whereas concentration of dTTP is reduced from the commonly used 32.5 μM to 24.5 μM or to 16.5 μM to 8 μM or even to 0 μM. In the last case every dTTP in the PCR fragment is substituted by digoxigenin-11-dUTP. With our PCR fragments the efficiency of PCR labeling reaction was not visibly affected in all of these cases. Using FITC-12-dUTP instead of digoxigenin-11-dUTP in this reaction had similar results. Although the fragments generated in these PCR reactions contain more tags, it is untested whether they produce stronger signal as probes. This is due to the possibility of steric hindrance or, in case of fluorophores, of self-quenching, preventing detection of the closely-located labels.

2.2. In Situ Ligation in Tissue Sections

- 1 The 5-6 μ m-thick sections cut from paraformaldehyde-fixed, paraffin-embedded tissue blocks onto ProbeOnTM Plus charged and precleaned slides (Fisher Scientific, Pittsburgh, Pa). Other slide brands can also be used if they retain tissue well.
- 2 Xylene
- 3 80 and 96% Ethanol.
- 4 T4 DNA ligase 5 U/ μ L (Roche Molecular Biochemicals, Indianapolis, IN) (*see* Note 5).
- 5 10 $\times$ reaction buffer for T4 DNA ligase: 660 mM-Tris HCl, 50 mM MgCl₂, 10 mM dithioerythritol, 10 mM ATP, pH 7.5 (20°C) (Roche Molecular Biochemicals, Indianapolis, IN) (*see* Note 6).
- 6 30% (w/v) solution of PEG-8000 (Sigma, St. Louis, MO) in bidistilled water (*see* Note 7).
- 7 Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) 20 mg/mL stock solution in distilled water. Store at - 20°C. In the reaction use 50 μ g/mL solution in PBS, prepared from the stock. Do not reuse (*see* Note 8).
- 8 Phosphate-buffered saline (1x PBS): dissolve 9g NaCl, 2.76 g NaH₂PO₄·H₂O, 5.56 g Na₂HPO₄·7H₂O in 800 mL of distilled water. Adjust to pH 7.4 with NaOH, and fill to 1L with distilled water.
- 9 Washing buffer: 100mM Tris-HCl, pH 7.5, 100mM NaCl.
- 10 Blocking solution: 10% sheep serum in 100mM Tris-HCl, pH 7.5, 100mM NaCl.
- 11 1-StepTM 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.
- 12 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.
- 13 22x22mm or 22x40mm glass or plastic coverslips. Plastic coverslips are preferable as they are easier to remove from the section.

⁵T4 DNA ligase in lower stock concentrations (1 U/ μ L) can also be used. The labeling reaction is only marginally less efficient at lower concentrations of ligase in labeling solution (125-250 U/mL). However, the highly concentrated (5 U/ μ L) (Roche Molecular Biochemicals, Indianapolis, IN) ligase preparation gives the best signal.

⁶ATP in the ligase reaction buffer is easily destroyed in repetitive cycles of thawing-freezing. Aliquot the buffer in small 15-20 μ L portions and store at - 20°C. Use once.

⁷15 % PEG-8000 in the ligation mix strongly stimulates the ligation reaction increasing the effective concentrations of the probe and ligase by volume exclusion (22). Use water of the highest quality for all solutions, which come in contact with T4 DNA ligase. If nonspecific background staining is a problem, reducing PEG concentration or complete elimination of PEG from labeling solution significantly reduces nonspecific signal. However it also weakens the labeling reaction.

⁸Proteinase K is a very stable enzyme, when stored at concentrations higher than 1 mg/mL. However, autolysis of the enzyme occurs in aqueous solutions at low concentrations (~ 10 μ g/mL) (23).

- 15 Aqueous slide mounting media such as Aqua-Mount (Thermo Fisher Scientific, Waltham, MA) or ImmunoHistoMount (Santa Cruz Biotechnology, Santa Cruz, CA).
- 16 Microscope with appropriate filters and objectives.

3.METHOD

3.1 Preparation of PCR-labeled *in situ* ligation probes containing 3'A-overhangs

- 1 Set up the PCR reaction mix in thin-wall PCR tubes on ice [100μL volume]:
Combine in this order:
 - 75 μL – distilled water
 - 10 μL – 10x buffer for Taq polymerase
 - 6 μL – MgCl₂ (25 mM stock) {1.5mM final concentration}
 - 5μL - 20x dNTPs-dig-dUTP labeling mix { final concentrations dA, dC, dG -50μM, dT- 32.5 μM, dig-dUTP – 16.5 μM} (*see* Note 4)
 - 1μL - pBluescript-bSDI1 plasmid (10-100 pg/μL) (*see* Note 2)
 - 1μL - Primer 1 (100 pmoles/μL)
 - 1μL - Primer 2 (100 pmoles/μL)
 - 1μL - Taq polymerase (2.5 U/μL)
- 2 Perform PCR labeling for 40 cycles using the following cycle profile:
 - 95°C – 20 s
 - 61°C – 20 s
 - 74°C – 2 min

Post-amplification extension at 74°C – 5 min

Store at 4°C.

- 3 Take 2μL from the PCR reaction mix and run on a 1% agarose gel to verify successful labeling.
- 4 Purify using High Pure™ PCR Product Purification Kit. Elute in 50μL TE. (*see* Note 9). Store at –20°C. This solution can be directly used for *in situ* labeling.
- 5 Measure probe concentration using spectrophotometer. Dilute in TE to 0.5μg/μL.

3.2 Preparation of PCR-labeled blunt-ended probes for *in situ* ligation

- 1 Set up the PCR reaction mix in thin-wall PCR tubes on ice [100μL volume]:
Combine in this order:
 - 81 μL – distilled water
 - 10 μL – 10x buffer for Pfu polymerase
 - 5μL - 20x dNTPs-dig-dUTP labeling mix { final concentrations dA, dC, dG - 50μM, dT- 32.5 μM, dig-dUTP – 16.5 μM} (*see* Note 10)

⁹If not purified, the probe is prone to producing background staining.

1μL - pBluescript-bSDI1plasmid (10-100 pg/μL) (*see* Note 2)
 1μL - Primer 1 (100 pmoles/μL)
 1μL - Primer 2 (100 pmoles/μL)
 1μL - Pfu turbo polymerase (2.5 U/μL)

2 Perform PCR labeling for 40 cycles using the following cycle profile:

95°C – 20 s
 61°C – 20 s
 74°C – 2 min

Post-amplification extension at 74°C – 5 min.

Store at 4°C.

- 3** Take 2μL from the PCR reaction mix and run on a 1% agarose gel to verify successful labeling.
- 4** Purify using High Pure™ PCR Product Purification Kit. Elute in 50μL TE. (*see* Note 9). Store at –20°C. This solution can be directly used for *in situ* labeling.
- 5** Measure probe concentration using spectrophotometer. Dilute in TE to 0.5μg/μL.

3.3 In Situ Ligation in Tissue Sections

- 1.** Place the sections in a slide rack and dewax in xylene for 15 min, transfer to a fresh xylene bath for an additional 5 minutes.
- 2.** Rehydrate by passing through graded ethanol concentrations: 96% Ethanol – 2x5min; 80% Ethanol – 5min; water – 2x5 min.
- 3.** Digest section with Proteinase K. Use 100μL of a 50μg/mL solution per section. Incubate 15' at room temperature in a humidified chamber (*see* Note 11).
- 4.** Rinse in distilled water for 4x2 min.
- 5.** Apply 100 μL per section of the pre-incubation solution. Incubate for 15 min at room temperature (23° C). The pre-incubation solution consists of a 1x T4 DNA ligase reaction buffer supplemented with PEG-8000 to the final concentration of 15%. It contains 66 mM-Tris HCl, pH 7.5, 5mM MgCl₂, 1mM dithioerythritol, 1 mM ATP, and 15% polyethylene glycol (*see* Note 12).

¹⁰We found that significantly more labeled nucleotides can be inserted into the synthesized fragment during the Pfu-driven PCR labeling when different formulations of dNTPs mixes are used. The successful labeling occurs when the concentration of digoxigenin-11-dUTP in the mixes is kept constant and is 16.5 μM, whereas concentration of dTTP is reduced from the commonly used 32.5 μM to 24.5 μM or to 16.5 μM to 8 μM or even to 0 μM. In the last case every dTTP in the PCR fragment is substituted by digoxigenin-11-dUTP. With our PCR fragments the efficiency of PCR labeling reaction was not visibly affected in all of these cases. Using FITC-12-dUTP instead of digoxigenin-11-dUTP in this reaction had similar results. Although the fragments generated in these PCR reactions contain more tags, it is untested whether they produce stronger signal as probes. This is due to the possibility of steric hindrance or, in case of fluorophores, of self-quenching, preventing detection of the closely-located labels.

¹¹Proteinase K digestion time may need adjustment depending on the tissue type. Times of 15–25 min are usually used, though hard tissues might require longer digestion. Insufficient digestion may result in a weaker signal. Overdigestion, on the other hand, results in signal disappearance and section disruption.

¹²Pre-incubation with ligation buffer ensures even saturation of the section prior to 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 10x ligase buffer (Roche Molecular Biochemicals, Indianapolis, IN) to 40 μL of bidistilled water and 50 μL of 30% PEG-8000. Sometimes, in the case of very high background staining, the 1hr 30 min pre-block with 20% BSA instead of ligation buffer solves the problem.

6. Aspirate the preincubation solution and apply the full ligase reaction mix with the probe (750 ng/μL stock) and T4 DNA ligase (*see* Notes 13, 14). *In situ* ligation labeling solution (20 μL per section):

Prepare on ice in this order:

6 μL – bidistilled water

10 μL – PEG8000 (30% stock solution)

2 μL – 10x buffer for T4 DNA ligase

1 μL – PCR labeled probe (0.5μg/μL) (for smaller sections can be 0.5μL)

1 μL – T4 DNA ligase (5U/μL) (*see* Note 5)

The total volume of the labeling solution can be scaled up to accommodate bigger sections.

Incubate overnight at room temperature (23° C) (*see* Note 15) in a humidified chamber with a plastic coverslip. The incubation time can be reduced to 4 h, if a faster detection is needed, this, however, decreases the signal and has to be compensated by longer color development.

7. Remove coverslips by gently immersing the slides vertically in a Coplin jar containing water at room temperature. Then wash sections 3x10 min in distilled water.
8. Apply 100 μL per section of the blocking solution. Incubate for 15 min at room temperature (23° C) in humidified chamber. The blocking solution contains 10% sheep serum in 100 mM Tris-HCl, pH 7.5, 100mM NaCl.
9. Aspirate the preincubation solution and apply 50 μL per section of solution of anti-digoxigenin antibody - alkaline-phosphatase conjugate diluted 1:100 in the blocking solution. Incubate 10 min at room temperature (23° C) in humidified chamber.
10. Wash 2 x10min in 100 mM Tris-HCl, pH 7.5, 100mM NaCl.
11. Apply 1-Step™ NBT/BCIP substrate solution for alkaline phosphatase. Keep in dark. Monitor color development. The color usually develops in 5-20 minutes.
12. Stop reaction by rinsing sections in water. Add water-soluble mounting media such as Aqua-Mount or ImmunoHistoMount and coverslip.

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¹³A mock reaction is recommended as a regular control in order to rule out nonspecific background staining. In a mock reaction solution, an equal volume of 50% glycerol in water is substituted for T4 DNA ligase.

¹⁴The probe has exclusively 5'OH groups and is not self-ligatable because T4DNA ligase can only ligate the 5' PO4 end of one probe to the 3'OH of another (22).

¹⁵Lowering of the temperature to 16°C reduces the signal; a temperature increase to 37°C completely eliminates the signal.

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