

Identification of Neural Programmed Cell Death Through the Detection of DNA Fragmentation In Situ and by PCR

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ABSTRACT

Programmed cell death is a fundamental process for the development and somatic maintenance of organisms. This unit describes methods for visualizing both dying cells in situ and for detection of nucleosomal ladders. A description of various current detection strategies is provided, as well as support protocols for preparing positive and negative controls and for preparing genomic DNA. *Curr. Protoc. Neurosci.* 48:3.8.1-3.8.24. © 2009 by John Wiley & Sons, Inc.

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INTRODUCTION

A universal feature in the development of multicellular organisms is a physiological form of cell death termed programmed cell death (PCD). A subset of PCD is apoptosis, which is defined by characteristic cellular morphological changes and genomic DNA fragmentation producing nucleosomal ladders. To understand how PCD operates in a developing tissue or in a tissue following an experimental procedure, dying cells must be identified in relation to their surviving neighbors. One way to accomplish this is to visualize fragmented DNA in situ, in combination with independent approaches such as gel electrophoresis of isolated DNA to visualize the nucleosomal ladders associated with apoptosis.

Two protocols for assessing apoptosis are presented in this unit. In situ end-labeling plus (ISEL+), a technique that originated from studies of central nervous system (CNS) tissue, identifies dying cells in tissue sections or cell culture (optimized for embryonic samples; see Basic Protocol 1). Ligation-mediated polymerase chain reaction (LMPCR) identifies nucleosomal ladders from intact tissues (see Basic Protocol 2). Also included are protocols for preparing thymocyte cell cultures for use as controls in the ISEL+ procedure (see Support Protocol 1) and for isolating genomic DNA for LMPCR (see Support Protocol 2).

NOTE: All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

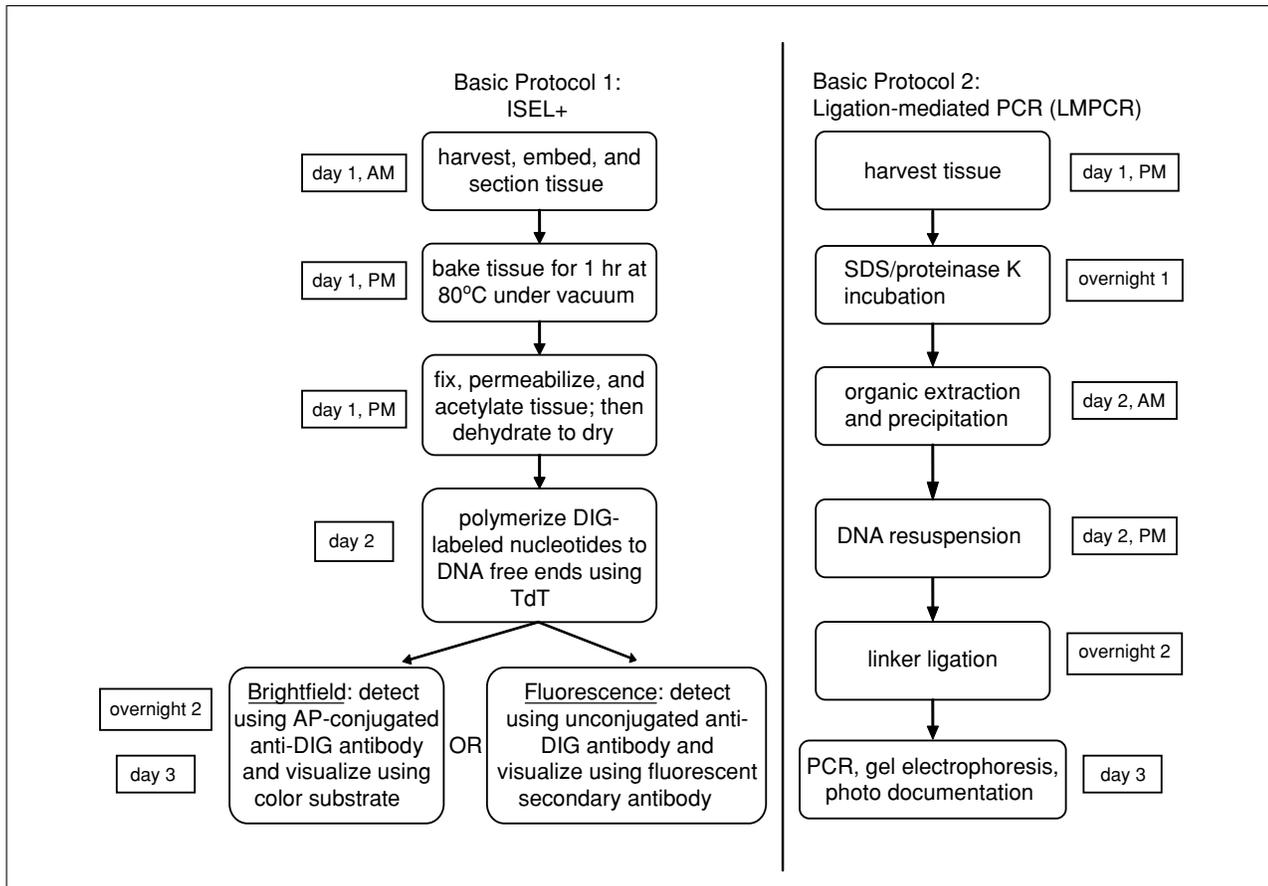
NOTE: Use of human tissues requires appropriate IRB approval. Possible biosafety and biohazardous materials must also be similarly considered by the appropriate oversight committee.

STRATEGIC PLANNING

Table 3.8.1 gives an overview of the features of different methods currently used to visualize cell death in histological sections. ISEL+ is currently the most sensitive method for

Table 3.8.1 Comparison of ISEL+, TUNEL (As Originally Reported), and Hybrid Techniques

	ISEL+	TUNEL	Hybrid (ISEL)
Tissue Isolation	Snap-freeze	Fixation	Fixation
Sectioning	Frozen and vacuum baked	Paraffin embedded	Frozen or paraffin embedded
Fixation	Post-sectioning 4% paraformaldehyde (fresh)	Pre-sectioning 4% paraformaldehyde or formalin 1-7 days	Pre-sectioning 4% paraformaldehyde or formalin
Post-fixation	Triton X-100 permeabilization Dehydration	De-paraffinization Proteinase K digestion Peroxidase treatment	De-paraffinization, Sodium citrate antigen retrieval, Proteinase K digestion
Hapten	Digoxigenin	Biotin	Digoxigenin
Visualization	Alkaline phosphatase, fluorescence	Peroxidase	Alkaline phosphatase, fluorescence
Relative sensitivity	10× (thousands of ends)	1×	Intermediate

**Figure 3.8.1** Diagram of workflow for Basic Protocol 1 and Basic Protocol 2 and time requirements.

detecting dying cells, up to 10× more sensitive than TUNEL, as originally reported. Over the past decade, hybrid protocols (generally still referred to as TUNEL or ISEL) offer a combination of flexibility and convenience for sample preparation and visualization. Figure 3.8.1 is a flow chart outlining the major steps of Basic Protocol 1 and Basic Protocol 2 and their time requirements.

DETECTION OF PROGRAMMED CELL DEATH BY IN SITU END-LABELING PLUS (ISEL+)

Tissue is rapidly and carefully dissected, frozen, and sectioned by cryostat. Sections are quickly thawed onto charged glass slides, air dried, then baked in a vacuum oven, fixed in paraformaldehyde, permeabilized in Triton X-100, acetylated, dehydrated through graded concentrations of ethanol, dried, and either stored desiccated at -80°C or used immediately. Slides to be processed further are end-labeled with terminal deoxynucleotidyl transferase (TdT) in the presence of labeled nucleotides, and the polymerized label is visualized by immunohistochemistry. Modifications for use of ISEL+ with cells in culture are also noted. The protocol is optimized for mouse tissues.

Materials

Pregnant mice *or* tissue culture cells grown on 12-mm glass coverslips coated with an appropriate adhesive (e.g., poly-L-lysine, *APPENDIX 2A*, or Cell-Tak from Becton Dickinson)

DMEM or other dissection medium, 4°C

OCT compound (e.g., Tissue-Tek II from Sakura) or other water-soluble embedding medium, 4°C

Appropriate controls (see step 1 annotation)

Liquefied Histo-Freeze (Fisher Sci), CryoKwik (Damon), *or* liquid nitrogen

Dry ice, finely crushed (optional)

Fixative (see recipe)

2× SSPE (*APPENDIX 2A*)

2× SSPE/0.6% Triton X-100 (see recipe)

0.1 M triethanolamine (TEA; see recipe)

Acetic anhydride (Sigma, cat. no. 91204)

DNase-free water

30%, 50%, 70%, 95%, and 100% ethanol, prepared with DNase-free water

ISEL+ TdT solution (see recipe)

Parafilm

MABS buffer (see recipe)

Blocking buffer (see recipe)

AP-conjugated sheep anti-DIG antibody (Roche, cat. no. 11093274910)

Alkaline phosphate buffer (see recipe)

Alkaline phosphatase color substrate buffer (see recipe)

Nuclear fast red solution (see recipe)

Permanent mounting medium (e.g., Crystal Mount from Biomedica and DPX from Fluka)

Unconjugated sheep anti-DIG antibody (Roche, cat. no. 11333089001)

Phosphate-buffered saline (*APPENDIX 2A*)

Fluorescent tagged anti-sheep antibody (e.g., AlexaFluor 488 tagged; Invitrogen, cat. no. A-11015)

4',6-diamidino-2-phenylindole (DAPI; Sigma, cat. no. D9542)

Fluorescence mounting medium (e.g., Vectashield from Vector Labs)

BASIC PROTOCOL 1

Dissecting instruments including:

- Fine forceps
- Fine scissors
- Blunt forceps and spatula

Freezing molds (Fisher Scientific, optional)

Cryostat

Superfrost Plus glass slides (Fisher Scientific)

Slide-warmer (Barnstead Thermolyne), 50°C

Slide-processing holders and vessels

80°C vacuum oven with house vacuum/pump

24-well microtiter plates (if using coverslip-mounted cells as controls)

Airtight containers for slide storage with desiccant (Tupperware)

Humidified chamber for microscope slides

Coverslips

Microscope, preferably equipped for brightfield, DIC, and/or fluorescence

CAUTION: Paraformaldehyde, triethanolamine, acetic anhydride, and potassium cacodylate (in the TdT buffer) are toxic and must be used in accordance with safety standards.

Prepare tissue

1. Harvest embryos (or other tissues) using fine scissors and forceps by laparotomy, uterine exposure, and careful incision of the uterus. Remove one embryo at a time, taking care not to rupture the amniotic sacs or transect the umbilical cords (and umbilical arteries/veins) of other embryos, so as to keep individual embryos blood-perfused as long as possible. For tissues requiring dissection, place tissue in ice-cold DMEM (or similar dissection medium). Rapidly isolate the tissue (within minutes, faster is better) and place it in ice-cold embedding medium preferably in a freezing mold using blunt forceps and/or spatula; embryos too small to dissect easily can be placed directly into embedding medium.

Care and speed are essential in isolating tissues. As with lengthy post-mortem intervals, physical damage to tissues may create detectable artifacts associated with induced cell death.

Positive and negative controls, particularly internal controls processed on the same slide as the experimental tissues, are useful in establishing this procedure or in troubleshooting. For a positive control in tissue sections, use developing thymus (4 weeks postnatal) or adult small intestine; these tissues contain many dying cells and will produce a robust and unambiguous signal (see Commentary). In a pinch, thymocytes in culture (see below) may be used instead. For negative controls, showing relatively little PCD, use adult (>4 months) neocortex, embryonic day 10 (E10) cerebral cortex, or adult liver (liver may produce increased background because of cytosolic alkaline phosphatase labeling, but this can be distinguished from true positive nuclear labeling; it can be reduced or eliminated by improved fixation and vacuum baking).

For positive controls in tissue culture, use a primary thymocyte culture treated with dexamethasone, where a marked increase in cell death is observed, beginning at step 5 below. For a negative control in cell cultures, use any healthy cell line (e.g., 3T3 fibroblast or undifferentiated P19 cells). Some cell death is always observed in culture (typically up to a few percent of total cells), but this is easily distinguished from the >80% of thymocytes induced to die by dexamethasone exposure. Untreated thymocytes can also be used in conjunction with dexamethasone-treated thymocytes. However, the large number of normally dying cells in the untreated thymus results in a substantial level of dying cells in controls, which should be expected.

2. Position the tissue by floating it in either a drop of ice-cold embedding medium or, optionally, in a rectangular plastic mold. Freeze the sample in liquefied Histo-freeze, CryoKwik, or a liquid nitrogen bath; powdered dry ice may also be used for small

samples. If possible, include positive and negative controls in the same mold so that they appear on the same slide as the tissue in question.

When using dry ice as an alternative method, crush the dry ice blocks into a fine powder to expedite cooling (a hammer is often used for this). During the isolation procedure, the tissue should be kept as cool as possible; the entire procedure, from harvesting to freezing, should take no more than 5 min, preferably less. Rapid freezing is essential, to avoid artifacts associated with cell death during isolation procedures through the freezing step.

Once frozen, blocks of embedded tissue can be stored up to several weeks at -80°C , preferably in an airtight container.

3. Cut sections using a cryostat with a sharp blade to produce 10- μm -thick sections (see UNIT 1.1).

Substandard cutting blades can result in artifacts produced by tissue damage.

4. After cutting, position the slide carefully over the section, thaw in place, and place on the 50°C slide warmer until ready for the next section. When all the slides have been collected, place slides in a slide rack and bake them in a preheated vacuum oven for 1 hr at 80°C under house vacuum.

Initial drying of slides at 50°C inhibits nucleases that may otherwise produce detectable, artifactual DNA damage. Further drying in a vacuum oven ensures complete dehydration, particularly important in humid climates. Coverslip-mounted culture cells used as controls (see Support Protocol 1) do not have to be baked.

Fix, permeabilize, and acetylate tissue sections

5. Incubate slide-mounted tissue sections in slide-processing vessels containing *fresh* fixative for 5 min at room temperature. If using dexamethasone-treated primary thymocyte cultures as controls, process the coverslip-mounted cells in parallel with the experimental slides from this point on.

Typically, 24 slides per rack are processed; coverslips are processed in the wells of a 24-well microtiter plate. Use fresh solutions whenever possible.

6. Wash twice in $2\times$ SSPE, 5 min each time, at room temperature.
7. Permeabilize in $2\times$ SSPE/0.6% Triton X-100 for 30 min at room temperature.
8. Wash slides twice in $2\times$ SSPE, 5 min each time, at room temperature. During this wash step, prepare for acetylation by combining 300 ml of 0.1 M TEA with 750 μl acetic anhydride while stirring until acetic anhydride globules have dissolved. Submerge slide rack immediately in TEA/acetic anhydride and incubate slides 10 min at room temperature.

Cultures do not need to be acetylated. Acetylation decreases nonspecific background, which is more problematic with tissue sections, particularly from embryonic brain.

9. Wash in $2\times$ SSPE for 5 min followed by a wash in dH_2O for 5 min, both at room temperature. Dehydrate through graded ethanol solutions diluted in dH_2O where needed: 30%, 50%, 70%, 95%, 100%, 100% (v/v), 2 min each.

It is vital to ensure that no TEA or SSPE remains in the tissue section, as TdT is inhibited by Na^+ , NH_4^+ , Tris buffer, and metal chelators such as EDTA. Phosphates can cause precipitation of the cobalt in the TdT buffer.

10. Dry slides 20 min at 50°C . (Optional: Dry in vacuum oven 20 min at 50°C under house vacuum).

Slides and cultures can now be stained immediately or stored in well-sealed slide containers with desiccant for up to 6 months at -80°C .

Polymerize digoxigenin-labeled nucleotides to free DNA ends

11. To each slide, without prewetting, add 50 μ l ISEL+ TdT solution. Overlay sections gently with precut Parafilm and incubate in a humidified chamber 1 hr at 37°C.

For coverslip-mounted cultures, add ISEL+ TdT solution directly to wells of 24-well microtiter plate and incubate as for slides. The Triton X-100 in the TdT solution and Parafilm allow for even distribution of a minimum volume of buffer. Incubate on a level surface, free from falling debris or other objects. Keep free of bubbles.

12. To terminate the reaction, remove Parafilm and wash with 2 \times SSPE for 5 min, then wash twice in MABS buffer for 5 min each.

MABS is a transition buffer that washes out phosphate from the previous SSPE buffer.

Detect labeled nucleotides/AP activity for brightfield visualization

- 13a. Incubate in blocking buffer 1 hr at room temperature in a humidified chamber.

Typical volumes are ~500 μ l per microscope slide and 300 μ l per coverslip-mounted culture.

- 14a. Replace blocking solution with sheep (or other species) anti-DIG/AP diluted 1:2000 in blocking buffer and incubate overnight at 4°C in a humidified chamber.

- 15a. Wash twice in MABS and three times in alkaline phosphate buffer, 5 min each at room temperature.

- 16a. Replace buffer with alkaline phosphatase color substrate buffer. Allow color (bluish precipitate) to develop for 1 to 2 hr, up to overnight, in darkness for maximum labeling, and terminate the reaction by transferring slides into dH₂O. Monitor color reaction every 15 min until complete.

Typical volume of color substrate buffer for both slides and cultures is ~500 μ l.

Nuclei of cells in culture appear to provide a much more accessible target for TdT and subsequent immunological and enzymatic amplification steps (somewhat analogous to amounts of antibody required for immunoblots versus immunohistochemistry). Because of this, the reaction times are often much shorter (e.g., several minutes to completion), and the investigator should initially monitor the reaction using appropriate controls.

- 17a. Repeat dH₂O wash three times, 10 min each.

- 18a. Lightly counterstain nuclei (if desired) using nuclear fast red solution for 5 min, then wash slides in running tap water for 2 min.

- 19a. Dehydrate sections in 70%, 95%, 95%, 100%, 100% (v/v) ethanol, 2 min each, and dry at 50°C for 2 min.

- 20a. Overlay sections with a thin film of permanent mounting medium (e.g., Crystal Mount), spread gently using a piece of clean Parafilm, dry at 50°C for 5 min, then coverslip using DPX and appropriate coverslip. View samples under a light microscope.

Crystal Mount creates a strong permanent seal for the sections and further mounting with DPX and coverslip allows for oil-immersion microscopy. Slides prepared in this manner will last for years.

Detect labeled nucleotides/DIG label for fluorescence visualization

- 13b. Incubate in blocking buffer 1 hr at room temperature in a humidified chamber.

- 14b. Replace blocking solution with polyclonal sheep (or other) anti-DIG antibody diluted 1:500 in PBS and incubate overnight at 4°C in a humidified chamber.

- 15b. Wash three times, 5 min each, in PBS at room temperature.

- 16b. Add AlexaFluor 488 donkey anti-sheep antibody (or other immunofluorescent component of choice) diluted 1:500 (or appropriate dilution) in PBS for 1 hr at room temperature. Wash three times, 5 min each, in PBS at room temperature.
- 17b. Counterstain nuclei with 0.3 $\mu\text{g/ml}$ DAPI in dH_2O and coverslip using fluorescence mounting medium (e.g., Vectashield). View samples under a fluorescence microscope with appropriate filters.

Glycerol-based mounted samples (e.g., Vectashield) are less permanent than brightfield preparations. Samples are typically stored for up to 1 month at -20°C before discarding. Longer storage may result in fungal-contaminated slides or fading of fluorescence signals.

PREPARATION OF THYMOCYTE CELL CULTURES FOR ISEL+

To obtain primary thymocyte cultures, thymus cells are grown on 12-mm round glass coverslips (coated with a desired adhesive, e.g., poly-L-lysine or Cell-Tak). The cultures are then treated with dexamethasone to induce apoptosis. They can then be used as a positive control and compared with untreated thymocyte cultures being assessed for programmed cell death (see Basic Protocol 1).

Materials

- 4-week-old mice
- DMEM/F-12 (APPENDIX 2A), containing 0.0025% trypsin
- DMEM/F-12/5% FBS (APPENDIX 2A)
- 12-mm diameter round glass coverslips coated with poly-L-lysine (APPENDIX 2A) or Cell-Tak (Collaborative Research)
- Dexamethasone stock solution: 2 mM dexamethasone in 100% ethanol
- Long-nose Pasteur pipet, flame polished
- 24-well microtiter dishes
- Additional reagents and equipment for tissue culture (for cultured cells; see APPENDIX 3B)

1. Harvest thymi from appropriately anesthetized 4-week-old mice. Isolate and mince thymi in DMEM/F-12 containing 0.0025% trypsin, and then triturate with a flame-polished long-nose Pasteur pipet.

A single mouse thymus is sufficient, but several can be combined if desired.
2. Centrifuge cells 5 min at $800 \times g$, room temperature, remove trypsin medium, and resuspend resulting pellet in 2 ml DMEM/F-12/5% FBS.
3. Count cells (APPENDIX 3B), adjust concentration to 2×10^6 cells per well, and plate cells on coated 12-mm coverslips placed in 24-well microtiter dishes.
4. Add 1 μM dexamethasone (from 2 mM stock in ethanol) and incubate 4 hr in a humidified 37°C , 5% CO_2 incubator.
5. Use coverslip-plated cells as positive controls for the assessment of PCD in tissue sections (see Basic Protocol 1, steps 5 to 20).

The coverslip-plated cells are fixed and processed like the slides containing the sections being analyzed, with the exception that acetylation and baking are not required (since the cell monolayers are rapidly and thoroughly fixed on the coverslip).

Coverslip-plated cells may be stored at -80°C for later use, but it is preferable to use them within 24 hr.

SUPPORT PROTOCOL 1

DETECTION OF NUCLEOSOMAL LADDERS ASSOCIATED WITH PROGRAMMED CELL DEATH BY LIGATION-MEDIATED POLYMERASE CHAIN REACTION (LMPCR)

DNA ladders with nucleosomal-sized rungs are a hallmark of apoptosis, but ladders can be difficult to visualize in tissues where apoptosis is nonsynchronous, such as in developing tissues. In addition, a significant amount of starting tissue is generally required to isolate enough DNA for precise analyses. Most ladder rungs appear to be blunt ended and 5'-phosphorylated on each end, allowing their identification by ligation of artificial, dephosphorylated linkers followed by amplification using the polymerase chain reaction (PCR). The resulting amplified DNA is visualized by agarose gel electrophoresis and ethidium bromide staining.

Materials

- Isolated and quantified genomic DNA (see Support Protocol 2)
- Oligonucleotides for ligation, unphosphorylated:
 - 12-bp: 5'-TGCGGTGAGAGG-3'
 - 24-bp: 5'-AGCACTCTCGAGCCTCTCACCGCA-3'
- 10× T4 DNA ligase buffer (prepared fresh; see recipe)
- T4 DNA ligase (Roche; store up to 1 month at -20°C)
- DNase-free water
- PCR buffer (see recipe)
- Oligonucleotides for single-copy-gene PCR controls: e.g., for mouse *engrailed*:
 - 5'-AGGACAAGCGGCCTCGCACA-3'
 - 5'-CGGTGTCCGACTTGCCCTC-3'
- Taq DNA polymerase
- Agarose gels, analytical grade (see APPENDIX 1N), prepared in TBE buffer (APPENDIX 2A)
- Ethidium bromide staining solution (APPENDIX 2A)
- 0.5-ml microcentrifuge tubes
- Thermal cycler
- Gel photographic setup
- Additional reagents and equipment for agarose gel electrophoresis (APPENDIX 1N)

CAUTION: Ethidium bromide is a mutagen and should be handled, stored, and disposed of with appropriate care.

Ligate linkers

1. Combine in a 0.5-ml microcentrifuge tube:
 - ≤2.5 µg genomic DNA
 - ≤1 nmol 12-bp unphosphorylated oligonucleotide
 - ≤1 nmol 24-bp unphosphorylated oligonucleotide
 - 6 µl 10× T4 DNA ligase buffer
 - dH₂O to 60 µl.

Mix thoroughly.

Very small amounts of genomic DNA (≤1 ng) can also be used, depending on the extent of apoptosis in the tissue. Be sure that the T4 DNA ligase and ATP-containing T4 ligase buffer are fresh, and that salt concentrations are sufficiently low to allow ligation. Note that the 12- and 24-bp oligonucleotides must be equimolar.

2. Anneal oligonucleotides by heating ligation mixture 5 min at 55°C in thermal cycler.
3. Gently mix contents by flicking tube.

Never vortex DNA at this stage.

4. Heat mixture 5 min more at 55°C in the thermal cycler.
5. Slowly cool mixture to 10°C over a 55-min period, and incubate at least 10 min at 10°C.

Heating and cooling can be programmed into the thermal cycler.

6. Add 3 U T4 DNA ligase, mix, and briefly centrifuge contents to the bottom of the tube. Incubate 12 to 16 hr (i.e., overnight) at 16°C.

Be certain ligase is well mixed into the ligation solution.

7. Dilute reactions with DNase-free water to attain a final concentration of 2.5 to 5 ng genomic DNA/μl.

Samples can be stored at -20°C indefinitely, provided they are not contaminated with DNases, before analysis by PCR.

Analyze DNA by PCR

8. Set up PCR reaction for experimental samples at room temperature:

150 ng ligated DNA
 124 pmol 24-bp linker (as primer)
 10 μl PCR buffer
 H₂O to 100 μl.
 Mix thoroughly.

Smaller amounts of ligated DNA can also be used, and this step can be optimized by varying the amount of input DNA.

9. Set up PCR reaction for controls at room temperature:

150 ng ligated DNA
 100 pmol oligonucleotides for single-copy gene of choice PCR controls [e.g., *engrailed* as originally used in Staley et al. (1997)]
 10 μl PCR buffer
 H₂O to 100 μl.
 Mix thoroughly.

10. Heat tubes 3 min at 72°C in thermal cycler. Add 5 U *Taq* DNA polymerase to each reaction tube and incubate an additional 5 min at 72°C.

*The 3-min, 72°C heating step eliminates the 12-bp fragment, which can no longer hybridize to its now ligated partner, while the addition of *Taq* DNA polymerase allows the filling in of the resulting single-stranded, 5'-protruding ends of the ligated adapters during the 5-min step.*

11. Amplify experimental samples using the following cycling conditions:

20 to 30 cycles:	1 min	94°C
	3 min	72°C.

12. Amplify controls using the following cycling conditions:

25 to 30 cycles:	1 min	94°C
	1 min	70°C
	1 min	72°C.

*This will produce a single-band control (e.g., using *engrailed* primers, a 270-bp PCR product clearly visible upon ethidium bromide staining of the agarose gel). This control is to ensure loading of equal amounts of ligated DNA, making LMPCR semiquantitative. See Staley et al. (1997) for more information.*

- Analyze 15 μ l of each PCR product by electrophoresis through 1.2% agarose/TBE buffer gel (*APPENDIX 1N*). Stain with ethidium bromide and record photographically.

Using control tissue like the unmanipulated thymus, obvious ladders consisting of five to seven rungs should be observable after <30 cycles; test aliquots in 2-cycle increments.

ISOLATION OF GENOMIC DNA FROM TISSUES AND CULTURES

Analysis of DNA by LMPCR requires its isolation from intact tissues or cells in culture. Tissues are rapidly frozen in liquid nitrogen and pulverized using a liquid nitrogen-cooled mortar and pestle; then the cells are lysed in warmed SDS/proteinase K buffer. Cultured cells are not frozen; instead they are immediately lysed in SDS/proteinase K buffer, and the resulting slurry is incubated overnight and processed like tissues. In both cases, after an overnight incubation with agitation, the DNA solution is extracted with phenol/chloroform, precipitated, washed, resuspended, quantified, and used for LMPCR in Basic Protocol 2.

Materials

Mice *or* tissue culture cells (see *APPENDIX 3B*) grown on 12-mm glass coverslips coated with an appropriate adhesive (e.g., poly-L-lysine, *APPENDIX 2A*, or Cell-Tak, Collaborative Research)

Sodium dodecyl sulfate (SDS)/proteinase K buffer (see recipe)

Molecular biology-grade 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol equilibrated with Tris-Cl, pH 8.0 (*APPENDIX 2A*)

24:1 (v/v) chloroform/isoamyl alcohol

3 M sodium acetate, pH 5.0 (*APPENDIX 2A*)

100% ethanol, -20°C

70% ethanol

TE buffer, pH 8.0 (*APPENDIX 2A*)

RNase A solution, DNase-free (*APPENDIX 2A*; optional)

Ceramic mortar and pestle, precooled with liquid nitrogen

15- and 50-ml polypropylene tubes with caps

50°C oven with rocker platform

Fluorometer or spectrophotometer

Additional reagents and equipment for DNA quantitation using a spectrophotometer (see *APPENDIX 1K*)

- Harvest tissue from the anesthetized mouse. Rapidly dissect tissue and immediately freeze in liquid nitrogen.

Where multiple samples are to be combined, homemade foil cups partially submerged in liquid nitrogen can be used to accumulate samples. Keep the size of the tissue as small as possible before freezing (large pieces are difficult to crush).

When working with tissue culture cells, proceed directly to step 3.

- Grind frozen tissue into a fine powder with mortar and pestle.

Be sure to precool both mortar and pestle with liquid nitrogen, and then add small volumes of the liquid nitrogen during the crushing process. Wear eye protection and laboratory coat when grinding.

- Transfer powdered, frozen tissue to a 15-ml polypropylene tube containing 5 ml SDS/proteinase K digestion buffer per gram or less of tissue. For tissue culture cells, spin down cells and resuspend in 5 ml digestion buffer per 15-cm plate. Incubate 12 to 16 hr at 50°C with gentle agitation (e.g., using a rocker platform).

The tissue powder is most easily transferred as liquid nitrogen slurry (use a minimal amount of nitrogen; see caution below). Preheat buffer to 50°C before adding tissue powder. SDS will form a white precipitate if the solution is too cold; however, this is reversible upon heating.

CAUTION: *Be sure to warm tissue/buffer slurry to at least 37°C before sealing the top, as remaining liquid nitrogen, once vaporized, can cause the sealed tube to explode.*

- Using 50-ml polypropylene tubes, extract twice with one volume of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol and then once with one volume of 24:1 (v/v) chloroform/isoamyl alcohol.
- Precipitate DNA with 1/10 vol of 3 M sodium acetate, pH 5.0, and 2.5 vol precooled 100% ethanol. Centrifuge 15 min at $1500 \times g$, 4°C. Decant, wash pellet with 70% ethanol, and centrifuge as before. Decant solution, dry pellet, and resuspend in sterile water if examining them immediately. Resuspend in TE buffer for prolonged storage.

Other precipitation methods are acceptable, including standard spooling procedures, as long as they result in a final DNA product that is of sufficiently low salt concentration to allow efficient ligation.

- Quantify DNA samples using a DNA-specific fluorescent dye and fluorometer, or other accurate methodology. Digestion of RNA with DNase-free RNase A, followed by reprecipitation, and quantification using a spectrophotometer may also be used (see *APPENDIX 1K*).

REAGENTS AND SOLUTIONS

*Use DNase-free water for all recipes. Sterilization is optional except for PCR buffers. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.*

Alkaline phosphatase buffer

900 ml dH₂O
12.1 g Tris base (0.1 M)
5.8 g NaCl (0.1 M)
2.0 g MgCl₂·6H₂O (10 mM)
Mix well, titrate pH to 9.5 using Tris·Cl crystals (requires ~1 to 2 g).
Adjust volume to 1 liter
Store solution indefinitely at room temperature

Alkaline phosphatase color substrate buffer

Dilute 4-nitroblue tetrazolium chloride (NBT) liquid stock to 450 µg/ml and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) liquid stock to 17.5 µg/ml in alkaline phosphate buffer (see recipe). Make up 10 ml of solution fresh just before use.

NBT/BCIP stocks are also commercially available (e.g., from Millipore or Roche).

Blocking buffer

100 ml MABS solution (see recipe)
1 g blocking reagent (Roche)
100 µl Triton X-100
Store indefinitely at –20°C

Add blocking reagent to MABS. Heat in 60°C water bath to dissolve, vortex every 5 min until solubilized. Add Triton-X and stir gently to avoid foaming. [Alternative blocking solution: use 3 ml 1 mg/ml BSA (see recipe) or 5 g powdered milk.]

Bovine serum albumin (BSA; 1 mg/ml)

90 ml dH₂O
100 mg bovine serum albumin (Fraction V, Sigma)
Adjust pH to 8.8 with 6 M HCl
Add 700 µl 2-mercaptoethanol (100 mM)
Adjust volume to 100 ml
Store indefinitely at -20°C

Fixative (4% paraformaldehyde in 0.02M PBS, freshly depolymerized)

200 ml 0.02 M PBS, pH 7.4, microwave until just near boiling
8 g powdered paraformaldehyde (e.g., Sigma)
Stir until completely dissolved (3 to 5 min)
Filter with filter paper
Prepare fresh daily

CAUTION: *Paraformaldehyde is toxic; prepare solution in a chemical fume hood.*

ISEL+ TdT solution

784 µl dH₂O
200 µl 5× TdT buffer (Invitrogen)
10 µl DIG dUTP, 50 µM
10 µl 10% Triton X-100/dH₂O
6 µl TdT (Invitrogen; includes 5× buffer and enzyme)
Prepare fresh daily before use

MABS (0.1 M maleic acid buffer saline, pH 7.5)

980 ml dH₂O
Add 11.61 g maleic acid (Sigma)
Add 8.76 g NaCl (Fisher)
Add 6 g NaOH pellets
Stir to equilibrate, and then fine-adjust with 10 N NaOH solution to pH 7.5
Store at 4°C to minimize bacterial growth

CAUTION: *Maleic acid is toxic; wear a dust mask when weighing powder.*

MABST (MABS/0.1% Triton X-100)

100 ml MABS (see recipe)
100 µl Triton X-100
Stir gently to mix thoroughly, but avoid foaming

Nuclear fast red solution (0.1% w/v)

500 ml dH₂O
Add 25 g Al₂(SO₄)₃
Add 0.5 g nuclear fast red (Sigma, cat. no. N8002)

Heat water to boil, then stir and dissolve aluminum sulfate. Add nuclear fast red, and continue to stir at boiling temperature until color does not change (~10 min). Cool and filter before use. Store up to 1 year at room temperature.

PCR buffer

Prepare 10× stock:
8.1 g Tris-hydrochloride (670 mM)
0.41 g MgCl₂·6H₂O (20 mM)
2.11 g (NH₄)₂SO₄ (160 mM)

H₂O to 80 ml
Store up to 6 months at –20°C
Just before use, dilute to 1×, adding:
10 mM 2-mercaptoethanol
100 µg/ml BSA (from 1 mg/ml stock; see recipe)
32 µg/ml 10 mM 4-dNTP mix (320 µM final)
Alternatively, buffer may be included with the purchased Taq enzyme.

SDS/proteinase K buffer

80 ml H₂O
0.44 g NaCl (75 mM)
0.12 g Tris-hydrochloride (10 mM)
5 ml 0.5 M EDTA, pH 8.0 (APPENDIX 2A; 25 mM)
1 g SDS (1%)
Adjust pH to 8.0 with 1 M HCl
Add H₂O to 100 ml
Store indefinitely at room temperature
Add 0.4 g proteinase K per ml buffer just prior to use

SSPE/0.6% Triton X-100, 2×

100 ml 20× SSPE (APPENDIX 2A; 2×)
6 g Triton X-100 (0.6%)
H₂O to 1 liter
Store indefinitely at room temperature

T4 DNA ligase buffer, 10×

8 ml H₂O
0.80 g Tris-hydrochloride (660 mM)
0.10 g MgCl₂·6H₂O (50 mM)
0.1 ml 1 M DTT (10 mM)
0.055 g ATP, disodium salt (10 mM)
Adjust pH to 7.5 with concentrated HCl
Add H₂O to 10 ml
Store up to 1 month at –20°C

Alternatively, buffer may be included with the purchased enzyme.

Triethanolamine (TEA), 0.1 M

295 ml H₂O
4 ml triethanolamine (Sigma, cat. no. T1377)
520 µl concentrated (12 M stock) HCl
Add 750 µl acetic anhydride (Fluka, cat. no. 45830) just prior to acetylation step
Mix until globules are gone and use immediately
Prepare fresh each time.

COMMENTARY

Background Information

Programmed cell death (PCD) is the physiological elimination of cells by death (Wyllie, 1981; Ucker, 1991). Apoptosis is the best-known form of PCD and is characterized morphologically by nuclear size reduction, blebbing of the cellular membrane,

chromatin condensation, and the formation of nucleosome-sized fragments of genomic DNA, detectable by gel electrophoresis (Kerr et al., 1972, 1994; Morris et al., 1984). Other forms of PCD also exist. Autophagy (Bursch et al., 2000) is thought to be distinct from apoptosis by involving the combined destruction of

the Golgi apparatus, polyribosomes, and endoplasmic reticulum, but with preservation of mitochondrial and micro- and intermediate filaments. Caspase-independent forms of PCD also exist (Liang et al., 2008). Evidence for the occurrence of PCD has been found in every multicellular organism studied, from flies to worms to mammals (Oppenheim, 1985; Ellis and Horvitz, 1986; Raff et al., 1993; White et al., 1994; Blaschke et al., 1996; Staley et al., 1997). All forms of PCD involve some degree of DNA fragmentation.

A significant advance in the study of PCD has been the development of histological techniques that localize DNA strand breaks within dying cells (Gavrieli et al., 1992; Wijsman et al., 1993; Wood et al., 1993). The first such technique, TdT-mediated dUTP nick-end labeling (TUNEL; Gavrieli et al., 1992; Strater et al., 1995; Negoescu et al., 1996), employed the template-independent DNA polymerase terminal deoxynucleotidyl transferase (TdT) to attach biotin-labeled nucleotides to the free 3' ends of fragmented DNA. These labeled nucleotides could then be recognized by a peroxidase-conjugated avidin molecule and visualized through a substrate reaction that produced a colored precipitate within the nucleus. TUNEL and related techniques have been used to demonstrate the presence of PCD in many organisms, as well as in tissue culture (Surh and Sprent, 1994; White et al., 1994). In the nervous system, techniques conceptually related to TUNEL, but employing different polymerases and/or visualization techniques, have also been used to show evidence of PCD in the granule cell layer of the cerebellum (Wood et al., 1993) and in neurodegenerative disorders such as Huntington's disease (Thomas et al., 1995).

Procedures like TUNEL, as originally published, were unable to identify dying cells to any significant degree within the embryonic cerebral cortex, which, combined with prevailing views at the time, led some to conclude that cell death did not occur there. This disparity led to the development of techniques with improved sensitivity over TUNEL, resulting in *in situ* end-labeling plus (ISEL+; Blaschke et al., 1996, 1998) and the associated but independent technique of ligation-mediated PCR [LMPCR; Staley et al., 1997] of DNA ladders. These techniques have proved successful in both tissue preparations and cell cultures at identifying dying cells produced by PCD in the developing nervous system, as well as in other tissues (Staley et al., 1997; Weiner et al., 1998; Vaccarino et al., 1999; Weiner and Chun, 1999;

Contos et al., 2000; Gu et al., 2000; Pompeiano et al., 2000; Yan et al., 2000; Kingsbury et al., 2003; McConnell et al., 2004; Chao et al., 2006; Rehen et al., 2006). Multiple technical differences exist between ISEL+ and the original TUNEL technique (Table 3.8.1; see also Strategic Planning) that result in a significant increase in sensitivity. Many current commercial kits are in fact hybrids of TUNEL and ISEL+.

It is important to make a distinction between dying versus dead cells. ISEL+ is optimized to detect the former, whereas the original TUNEL protocol generally identifies the latter, e.g., corresponding with pyknotic nuclei. An experiment to examine the latency between DNA fragmentation detected by ISEL+ and actual cell death and removal was conducted in the small intestine, utilizing BrdU birthdating to follow cells after their last cell division (Pompeiano et al., 1998). The epithelium of the small intestine turns over completely in several days, and the age of epithelial cells increases as one ascends the villus (into the intestinal lumen). Use of this well-defined model system demonstrated that cells destined to die commenced DNA fragmentation 2 to 3 days before they were actually eliminated as dead cells at the villus tip.

The increased level of sensitivity produced by ISEL+ identified significant levels of developmentally variable dying cells, averaging around 50%, within the embryonic cerebral cortex (Blaschke et al., 1996), as well as throughout the neuraxis (Blaschke et al., 1998). At the time, this was a surprising result, as it contrasted with prevailing views. However, independent studies using genetic deletion of the pro-apoptotic proteins caspase-3 (Kuida et al., 1996; Pompeiano et al., 2000) or caspase-9 (Kuida et al., 1998; Zaidi et al., 2001) resulted in a striking hyperplasia of embryonic brain cells, consistent with the proven functions of the caspases in cell death, and consistent with at least partial rescue of the PCD that had been identified by ISEL+ and LMPCR. These results have been further supported by deletion of more pro-cell death genes, resulting in "big brain" phenotypes like the caspase-null mutants: Apaf 1 (Ceconi et al., 1998; Yoshida et al., 1998), Bax and Bak (Lindsten et al., 2003), Pten (Groszer et al., 2001), as well as novel pathways involving the ephrins (Depaepe et al., 2005). Similarly, anti-cell-death genes, when genetically removed, resulted in smaller brains or progenitor pools, as observed for Bcl-X (Motoyama et al., 1995), Survivin (Jiang et al., 2005), and

Mcl-1 (Arbour et al., 2008). Further evidence supporting extensive neural cell death comes from examination of single clones identified using a transgenic, mutant *lacZ* reversion construct (Wilkie et al., 2004). An unanticipated, marked decrease in the proportion of embryos with labeled clones was found during early neurogenesis, which is consistent with significant cell death during this period, as identified by ISEL+.

Sensitivity of ISEL+

The most significant advantage of ISEL+ is its sensitivity in detecting dying cells. ISEL+ is up to ten times more sensitive than TUNEL. Figure 3.8.2 shows a comparison between TUNEL and ISEL+ in an adult mouse thymus treated in vivo with the corticosteroid dexamethasone (DX). DX causes rapid apoptosis of all immature thymocytes within the cortex (outer portion) of the thymus (Surh and Sprent, 1994) and, under the employed experimental conditions, should kill the majority of thymic cortical cells. In identically treated thymi, TUNEL identified a number of dying cells in the thymic cortex (Fig. 3.8.2A), but ISEL+ labeling (Fig. 3.8.2B) showed many more dying thymocytes; thus, the extent of PCD was more accurately revealed by ISEL+. A parallel time-course study of TUNEL and ISEL+ labeling in DX-treated thymic cultures further demonstrated the relatively greater sensitivity of ISEL+ (Fig. 3.8.3). TUNEL (Fig. 3.8.3A-C) did not detect increased cell death

until 4 hr after DX addition, while ISEL+ (Fig. 3.8.3 D-F) detected the occurrence of increased apoptosis within 30 min of treatment. These results demonstrate that ISEL+ identifies dying cells with greater sensitivity than does TUNEL.

To quantify the relative difference in sensitivity between ISEL+ and TUNEL, the number of fragmented DNA ends that are required for visualization of a dying cell using each technique was estimated. A 1013-bp linearized fragment of plasmid DNA was labeled in solution by either TUNEL or ISEL+. Known amounts of labeled fragment were electrophoresed on an agarose gel and transferred to nitrocellulose. Labeled fragments were then detected either by TUNEL or by ISEL+. Using this assay (Fig. 3.8.4), 10 ng of labeled DNA were barely detectable by TUNEL, but prominently detected by ISEL+, which has a limit of detection approaching 1 ng (DNA content measured using a fluorometer). A conservative estimate was obtained of the number of fragmented ends that are required in a single cell for labeling by ISEL+, based on the following calculation. The number of ends in one nanogram of 1013-bp DNA (1.50×10^{-15} mol) is 1.80×10^9 . The area of the blotted DNA band was determined to be 2×10^{-6} m²; 6.37×10^5 cells (with a maximum cross-sectional diameter of 10^{-5} m, and an area of 3.14×10^{-12} m² each) would fit into that area. Dividing the number of free ends by the number of cells equates to 3000 ends that

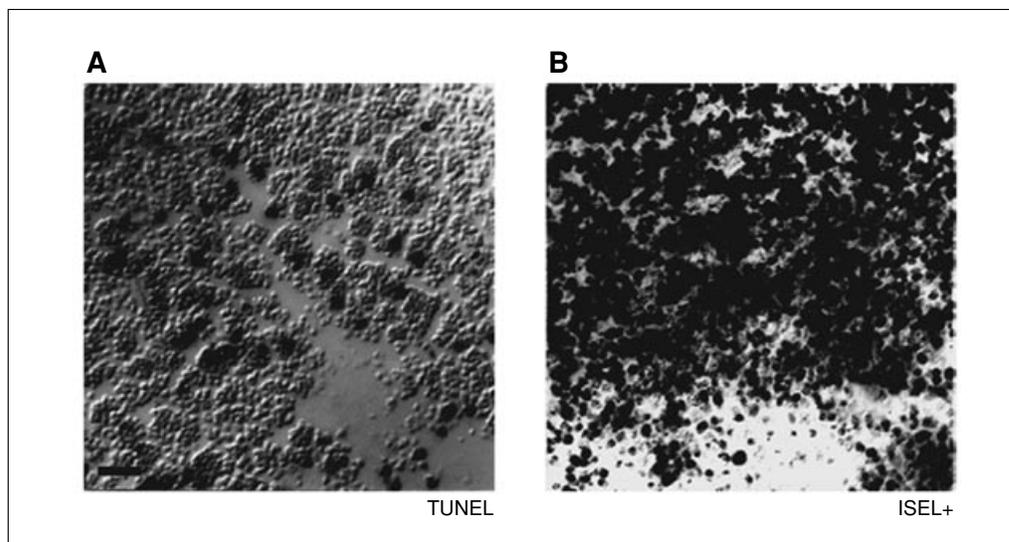


Figure 3.8.2 ISEL+ is more sensitive than TUNEL in identifying dying cells. Data are for thymi from 4-week-old mice treated with dexamethasone for 8 hr to induce massive PCD of cortical thymocytes, and then fixed and processed for ISEL+ or TUNEL. (A) A number of dying cells were identified by TUNEL in the cortex of the thymus (dark stain). (B) The ISEL+ procedure identified significantly more dying cells in the thymic cortex (dark stain). Scale bar = 20 μ m.

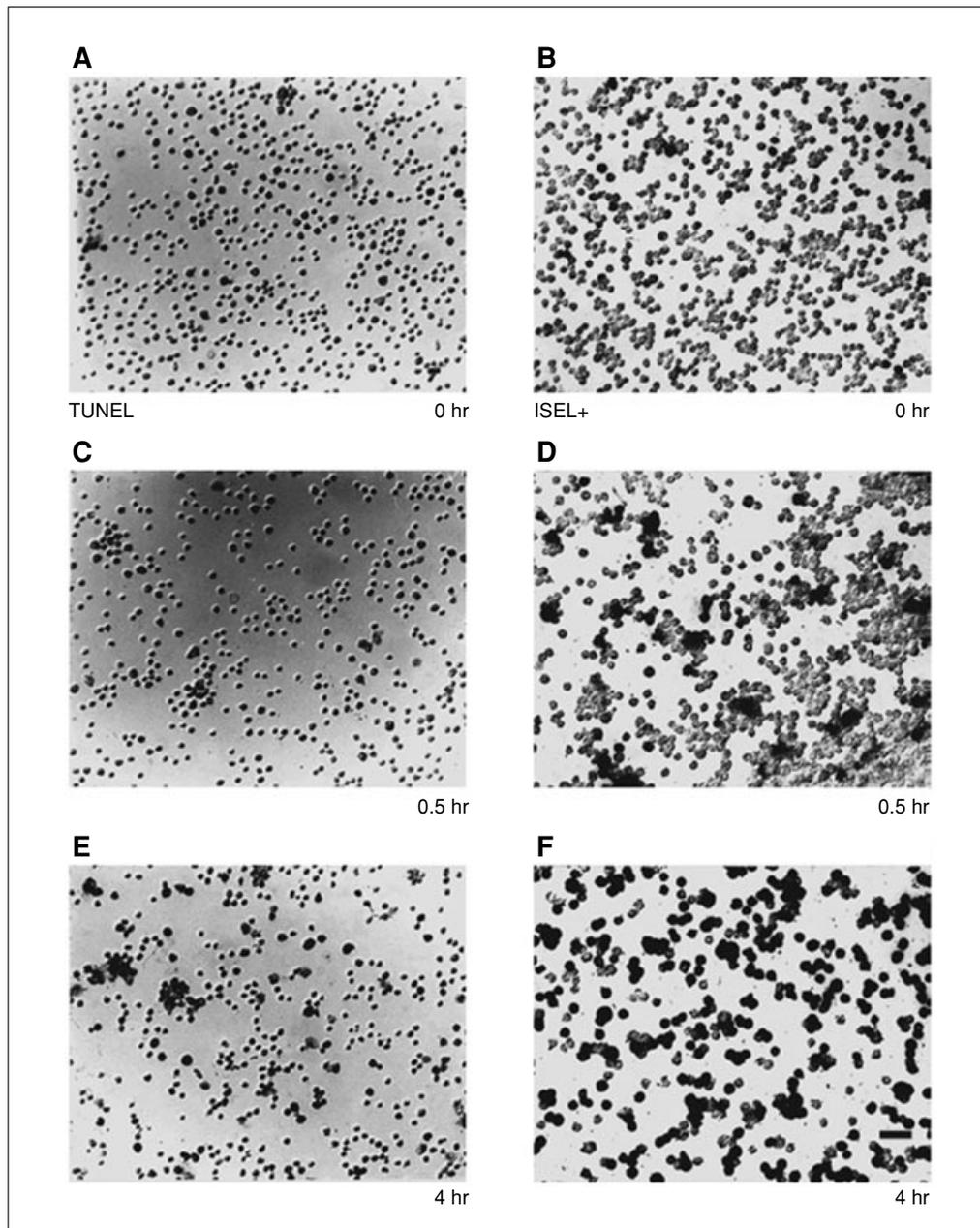


Figure 3.8.3 ISEL+ identifies dying cells earlier than TUNEL following the induction of PCD. Data are for thymic cultures prepared from 4-week-old mouse thymi, then treated with dexamethasone to induce PCD and fixed for either ISEL+ or TUNEL at various times after PCD treatment. Dying cells were identified by either the TUNEL (**A**, **C**, **E**) or ISEL+ (**B**, **D**, **F**) procedure. (**A**, **B**) 0 hr (control). Neither ISEL+ nor TUNEL identified many dying cells before dexamethasone treatment of cultures. (**C**, **D**) 0.5 hr. Although TUNEL (**C**) did not show an increase in dying cells, ISEL+ (**D**) detected an increase in cells undergoing PCD. (**E**, **F**) 4 hr. At this timepoint, TUNEL (**E**) detected an increase in dying cells produced by the dexamethasone treatment (at 1 hr and 2 hr there was still no increase in TUNEL-labeled cells; data not shown). Labeling looked similar to that shown by ISEL+ at 0.5 hr (**D**). ISEL+ labeling at 4 hr (**F**), however, showed a massive increase in dying cells, reflecting its much higher sensitivity. Scale bar = 20 μ m.

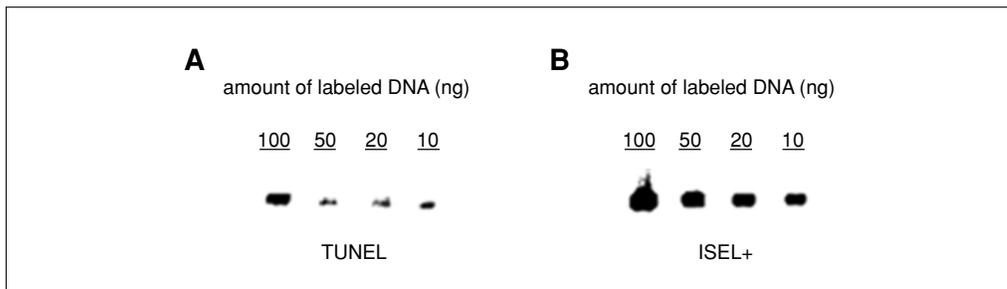


Figure 3.8.4 Labeling by ISEL+ is ~10 times more sensitive than TUNEL. Known quantities of a 1013-bp DNA fragment were labeled by either ISEL+ or TUNEL and electrophoresed on an agarose gel, then transferred to nitrocellulose and detected by ISEL+ or TUNEL. **(A)** TUNEL required 10 ng of labeled DNA per well for threshold detection. **(B)** ISEL+ detection of 10 ng labeled DNA is shown for comparison. This observation is consistent with thymic *in situ* data (see Fig. 3.8.2).

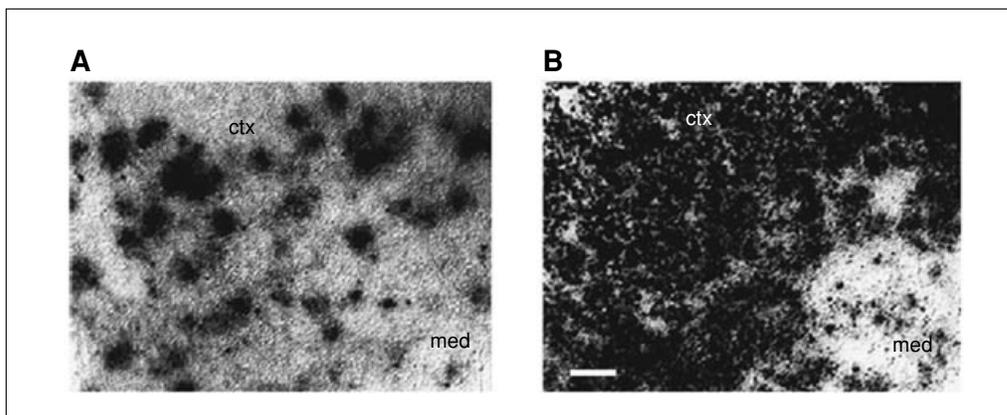


Figure 3.8.5 ISEL+ is specific in its identification of cells undergoing PCD. **(A)** ISEL+ identified relatively small numbers of dying cells in the cortex of the normal thymus, consistent with the normal rate of PCD in this tissue (dark stain). **(B)** After cortical thymocytes were induced to undergo PCD *en masse* by dexamethasone (Wyllie, 1981), ISEL+ labeled virtually all the thymocytes in the cortex. Scale bar = 50 μ m. Abbreviations: ctx, thymic cortex; med, thymic medulla.

are required per cell for labeling by ISEL+ at the 1-ng limit of detection, or 30,000 ends per cell at the 10-ng level. Therefore, ISEL+ is approximately ten times more sensitive than TUNEL.

Specificity

The specificity of ISEL+ in the detection of dying cells was examined using the thymus as a model. Under normal conditions, ~90% of immature thymocytes asynchronously undergo PCD in the cortex of the thymus (Egerton et al., 1990; Shortman et al., 1990). *In vivo* exposure of thymus to DX causes immature cortical thymocytes to undergo PCD *en masse* (Egerton et al., 1990; Shortman et al., 1990). This drug-induced apoptosis involves essentially all of the thymocytes in the cortex, which proceed synchronously through the cell death pathway. Mature thymocytes, located in the thymic medulla, are insensitive to DX and do not

undergo massive PCD (Caelles et al., 1994; Blaschke et al., 1996; Staley et al., 1997). A normal mouse thymus labeled by ISEL+ identifies a number of labeled cells, consistent with the asynchronous nature of PCD (Fig. 3.8.5A). In contrast, a thymus treated *in vivo* with DX and then labeled by ISEL+ identifies a majority of cells (Fig. 3.8.5B), consistent with the large-scale synchronous cell death induced by DX, as can be seen by the intense labeling of most cells in the thymic cortex. Thus, cells identified by ISEL+ specifically increase in number after a treatment known to increase the number of dying cells.

As an additional example of the specificity of ISEL+, the medulla of a DX-treated thymus is shown (Fig. 3.8.5B). In this portion of the thymus, cells are insensitive to DX, and, as would be expected, very few cells were labeled by ISEL+. The specificity of ISEL+ has also been addressed in an *in vitro* paradigm of PCD, in which death was induced in P19 embryonal

carcinoma cells by UV irradiation (Staley et al., 1997). All of these paradigms also show parallel increases in DNA ladders, identified by both standard and LMPCR techniques (Pompeiano et al., 1998). Further validation in the small intestine, mentioned above, also supports the specificity of the assay (Blaschke et al., 1996). These combined data show that ISEL+ is specific in labeling apoptotic cells under the employed biological conditions. It is possible that artifactual or non-biological production of DNA double-strand breaks could produce false-positive signals, as could poor tissue conditions associated with extended post-mortem interval, physical damage, toxicity, etc. Use of independent means of assessing PCD (e.g., appropriate genetic mutants or LMPCR) can minimize errors. Additional controls are shown and discussed in Young (1984), Crespo et al. (1985), and Sengelaub et al. (1986). Some examples are presented below.

Examples of PCD detected by ISEL+

PCD has been studied in a number of neural cell types, and ISEL+ was designed to identify PCD in the nervous system, as shown in several examples from the murine nervous system (Fig. 3.8.6A-C).

The death of cells in the ganglion layer of the early postnatal rodent retina is well documented (Young, 1984; Sengelaub et al., 1986), and PCD is prominent during the first 11 days (Flanagan, 1969; Lance-Jones, 1982; Oppenheim, 1985, 1989). In the P1 retina, reportedly a site of high PCD, >60% of ganglion cells were labeled by ISEL+ (Fig. 3.8.6A), consistent with prior studies.

During the period of limb innervation, spinal cord motor neurons undergo PCD between embryonic days 13 (E13) and E18, and the peak of motor neuron cell death is on E14 (Davies and Lumsden, 1984; Buchman and Davies, 1993). Many cells are labeled by ISEL+ in the area where motor neurons are differentiating at E14 (Fig. 3.8.6B).

The developmental period of PCD for trigeminal ganglia is E13 to E18 (Pompeiano et al., 1998), which corresponds to the period during which synaptic connections are formed. A trigeminal ganglion at E16 shows a consistent amount of ISEL+ labeling (Fig. 3.8.6C).

Critical Parameters and Troubleshooting

ISEL+

Tissue isolation: Once deprived of normal nutrient exchange, via diffusion or blood sup-

ply, tissues begin to die. It is critical, therefore, to minimize cell death produced by tissue handling. Rapid dissection and use of ice-cold buffers during tissue preparation can help to minimize damage produced during tissue isolation.

Clearance time of labeled cells: The sensitivity of ISEL+ allows one to identify dying cells sooner than with other techniques. Like other techniques, ISEL+ provides static information from a tissue section at a single age from a single animal. Rigorous determination of how a population of cells changes with time, which would require real-time analysis of the same cells in the same animal, cannot therefore be made. Within a tissue section, a cell observed to be labeled on a single day may be just starting to die, midway through its death, or about to be eliminated. The half-life of dying neuroblasts or post-mitotic neurons identified by ISEL+ could range from a few hours to several days, and therefore the actual number of cells eliminated on a given day is uncertain. Based on studies in the small intestinal villus (Pompeiano et al., 1998), cells labeled by ISEL+ can require up to several days to be eliminated following the onset of PCD, or can be eliminated within hours at the end of the cell death program, which currently serves as the best estimate for interpreting ISEL+ labeling.

Tissue sectioning: Cryostat sections must be of excellent quality. Sections should be cut with a sharp blade to minimize tissue damage and potential artifactual labeling. Thickness should be kept in the range of 10 μm ; greater thickness may prevent access of labeling reagents into all cells. Other variables, such as sample and box temperatures within the cryostat, blade angle, anti-roll bar positioning, size of cut block, and ambient temperature and humidity outside the cryostat can all affect tissue sectioning.

Fixation and permeabilization of tissue sections: Over-fixation can block access of labeling reagents, while under-fixation can produce artifactual labeling. Using Basic Protocol 1, a wide variety of tissues has been examined with consistent results; however, fixation can be re-examined empirically to further optimize results. No proteinase treatments are used in this procedure (in contrast to the original TUNEL procedure); the Triton X-100 permeabilization step is therefore critical, because it allows access through both the outer cell and nuclear membranes for DNA labeling.

Acetylation of tissue sections: Background signal primarily arises from nonspecific retention of marker molecules (e.g., dUTP or

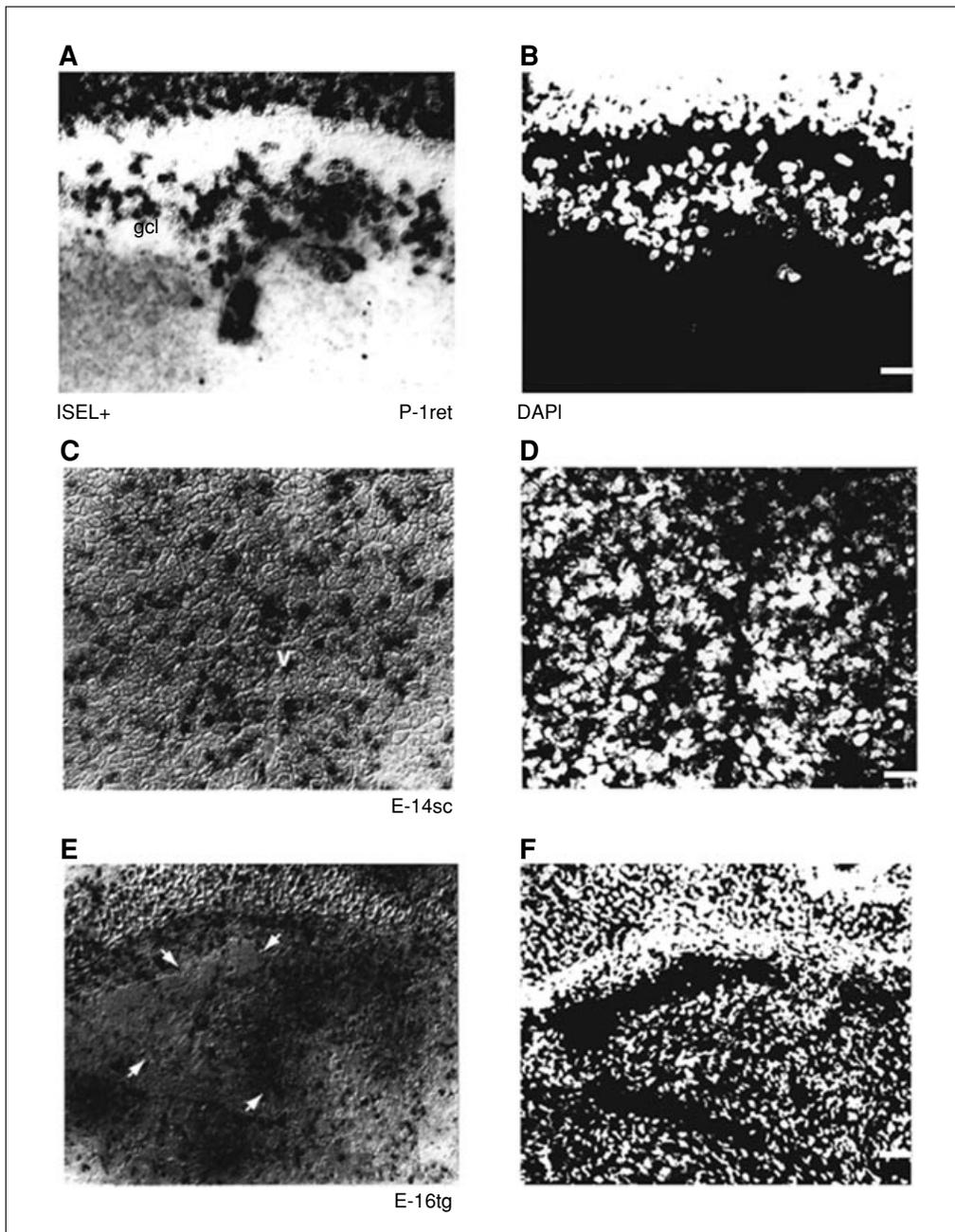


Figure 3.8.6 ISEL+ identifies dying cells in known models of neuronal PCD. Slides were viewed by ISEL+ (**A, C, E**) and DAPI counterstaining (**B, D, F**) for comparison. (**A, B**) P1 is the peak of PCD in the ganglion cell layer, and here >60% of cells were labeled by ISEL+. (**C, D**) ISEL+ identified dying motor neurons in the embryonic mouse (E14) spinal cord. (**E, F**) In the trigeminal ganglion at E16, dying neurons were easily identified by ISEL+ despite their low incidence at this age. Arrowheads indicate ganglion borders. Scale bar = 20 μ m. Abbreviations: ret, retina; gcl, ganglion cell layer; sc, spinal cord; v, ventricle; tg, trigeminal ganglion.

anti-DIG/AP antibody) in tissue sections. Several chemical functional groups in proteins (such as amine and carboxylate groups) are believed to promote nonspecific binding. In addition, marker molecules may become entrapped within the three-dimensional tissue structure. This source of background can be minimized by treating tissue slides with acetic anhydride

to acetylate basic groups. Acetic anhydride is very hygroscopic and hydrolyzes in the presence of water to form acetic acid, thereby neutralizing its acetylation ability. Therefore, fresh acetic anhydride (preferably using newly opened vials) should be used during the preparation of TEA/acetic anhydride.

Terminal deoxynucleotidyl transferase (TdT): The unit activity of TdT differs between suppliers; even different batches from one supplier can be quite variable. Enough TdT from the same supplier and batch should be obtained to complete an entire experiment without switching batches or suppliers. Like all enzymes, TdT should be stored in a reliable, non-defrosting -20°C freezer, and maintaining a dedicated stock of the enzyme is recommended.

Immunohistochemical procedures: Two steps are particularly important. First, overnight incubation with anti-digoxigenin gives a substantially stronger and cleaner signal than a 1 hr (or intermediate) incubation, and can be used for maximum sensitivity. Prolonged incubation for color development should be conducted in darkness. Second, alkaline phosphatase, especially associated with a bluish (versus reddish) reaction product, produces the best sensitivity. Other labels can be used (e.g., fluorescence, horseradish peroxidase), but they are less sensitive than the blue alkaline phosphatase product (Fig. 3.8.7A,B). Fluorescence labeling, however, does allow for detection and co-visualization of other proteins with tagged antibodies.

Sample storage: Embedded samples can be stored at -80°C for up to several weeks. Cut sections can be stored desiccated for at least several months, provided they are not repeatedly thawed and refrozen. Once reacted for alkaline phosphatase detection, samples should be coverslipped (see protocols above) and can be stored indefinitely.

Labeling of DNA not associated with cell death: In theory, any free DNA end could be labeled by ISEL+. Biologically relevant free ends such as Okazaki fragments do not appear to be labeled, as shown in studies comparing cells undergoing DNA synthesis (identified by BrdU incorporation) to cells labeled by ISEL+ (Blaschke et al., 1996; Staley et al., 1997). Mitochondrial DNA is circular and does not appear to be labeled. DNA cleavage by procedures like acid nicking (e.g., for anti-BrdU immunohistochemistry) can produce artifactual labeling and should be accounted for before using ISEL+.

LMPCR

All oligonucleotides should be gel-purified to ascertain their correct size before ligation; they may also need to be desalted. Of course, the primers must *not* be phosphorylated. Be sure to work from the same batch of primers for all the analyzed samples, as differences in

ligation efficiency between batches of primers have been observed. It is advisable to use fresh ligase and dNTPs. DNA samples must be washed thoroughly in 70% ethanol to remove salts that can inhibit ligation. Be sure to resuspend the DNA completely after precipitation, washing, and drying. Various thermal cyclers may produce slightly different ramping profiles and they should be assessed accordingly. Use a standard DNA sample with which to compare the tissue in question. In the mouse, DNA from the adult small intestine always produces a very strong signal with 20 cycles or less, while DNA from 2.5-week-old thymus produces a signal after ~ 22 cycles.

Anticipated Results

The ISEL+ procedure will result in the visualization of more PCD compared to TUNEL. As discussed above, the clearance time is not well defined for cells labeled by ISEL+. The seemingly high level of PCD identified by ISEL+ has been shown to be a more accurate description of PCD compared to other methodologies, as discussed above. In conjunction with ISEL+, LMPCR provides independent confirmation of the operation of apoptotic mechanisms. Clear nucleosomal ladders can be observed in nervous system tissue similar to those seen in the thymus (Fig. 3.8.8); the adult PCD reflects glial and/or subependymal cell PCD (A.J. Blaschke and J. Chun, unpub. observ.).

Time Considerations

ISEL+

Starting in the morning, the entire procedure can be completed within 3 days; with optimization, and depending on the level of sensitivity required, this should be viewed as an upper limit.

Day 1: Tissue isolation and slide processing

Day 2: TdT polymerization and heat inactivation

Overnight 2: Anti-digoxigenin incubation

Day 3: Visualization of bound antibody and analysis.

LMPCR

Starting in the afternoon, the entire procedure can be completed in 3 days.

Day 1, p.m.: Tissue isolation

Overnight 1: SDS/proteinase K incubation

Day 2, a.m.: Organic extraction and precipitation

Day 2, p.m.: DNA resuspension

Overnight 2: Linker ligation

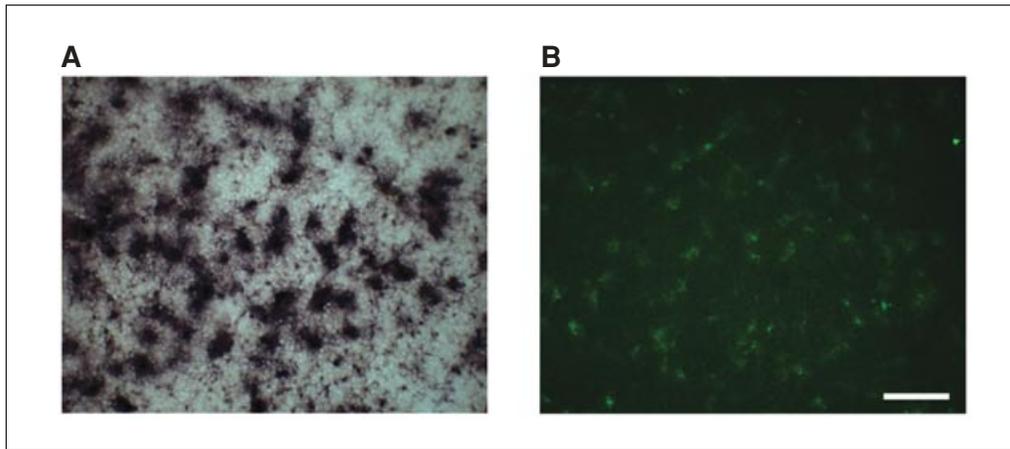


Figure 3.8.7 ISEL+ using AP and NPT/BCIP colorimetric detection is more sensitive than fluorescence detection. ISEL+ was run in parallel for identical lengths of time using both **(A)** AP and NPT/BCIP colorimetric detection and using **(B)** an AlexaFluor 488 (green) tagged secondary antibody on E12 mouse fetal liver to detect clusters of lymphocytes. The signal strength was stronger, the background was cleaner, and more dying cells were detected using colorimetric detection. Positive signal is denoted by dark stain and green fluorescence in the left and right panels, respectively. Scale bar = 100 μ m. For color version of this figure go to <http://www.currentprotocols.com/protocol/ns0308>.

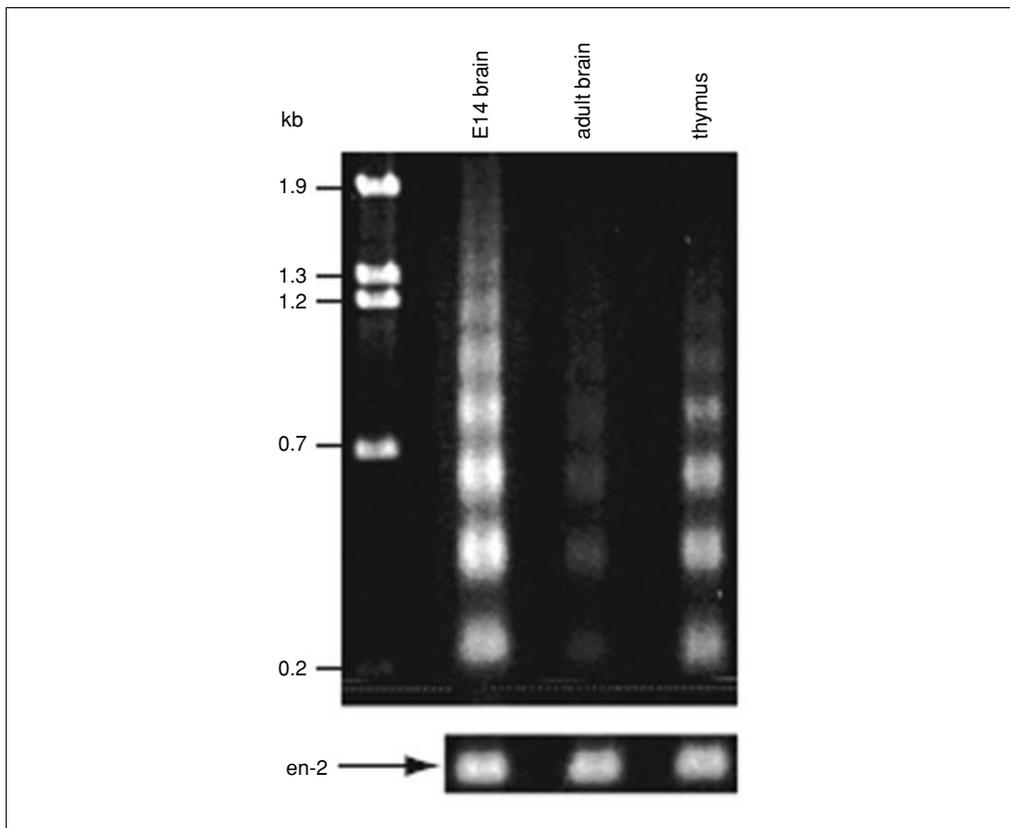


Figure 3.8.8 Ligation-mediated PCR (LMPCR) identifies DNA nucleosomal ladders from tissues undergoing apoptosis. Mouse embryonic day E14 and adult brain tissue, as well as 4-week-old mouse thymus, were examined using LMPCR for the presence of nucleosomal ladder as an independent indication of apoptosis. Clear nucleosomal ladders can be seen for embryonic brain and adult thymus tissue, while adult brain showed significantly less intense laddering. A single-copy gene PCR control is also shown (for *engrailed*, *en*), confirming that equivalent amounts of genomic DNA were used for each sample. See Staley et al. (1997) for additional details.

Day 3: PCR, gel electrophoresis, and photographic documentation.

As with ISEL+, the procedure can be optimized and truncated to reduce the duration of the procedure.

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Key References

- Blaschke et al., 1996. See above.
First use of ISEL+ and the detection of programmed cell death in the embryonic cortex.
- Gavrieli et al., 1992. See above.
First technique to utilize labeling of DNA ends to detect programmed cell death.
- Raff et al., 1993. See above.
An informative discussion of nervous system programmed cell death.
- Staley et al., 1997. See above.
First use of ligation-mediated PCR to demonstrate apoptotic ladders in normal tissues.
- Wyllie, 1981. See above.
First demonstration of nucleosomal ladders associated with apoptosis.