TECHNICAL BULLETIN

DeadEnd™ Colorimetric TUNEL System

Instructions for Use of Products
G7130 and G7360

Revised 1/19
TB199

DeadEnd™ Colorimetric TUNEL System

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The DeadEnd™ Colorimetric TUNEL System provides reagents for terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling of fragmented nuclear DNA. The system directly detects apoptotic cells in situ at the single-cell level in both tissue sections and cultured cells using a light microscope.

Most cells from higher eukaryotes have the ability to self-destruct by activation of an intrinsic cellular suicide program referred to as programmed cell death or apoptosis (1,2). Apoptosis is important in development, homeostasis and several diseases (2–5). It is characterized by certain morphological features, including membrane blebbing, nuclear and cytoplasmic shrinkage and chromatin condensation. Cells undergoing apoptosis fragment into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or neighboring cells without generating an inflammatory response. This is in contrast to the type of cell death known as necrosis, which is characterized by cell swelling, chromatin flocculation, loss of membrane integrity, cell lysis and generation of a local inflammatory reaction.

The morphological changes observed in the nucleus of apoptotic cells are, in part, due to the generation of DNA fragments through the action of endogenous endo-nucleases (6,7). Typically, the DNA of apoptotic cells is cleaved to a population of multimers of 180–200bp fragments, readily observed as a ladder on agarose gels. Apoptotic cells have been clearly labeled in vibratome sections of rat brain after axotomy-induced neuronal death in the lateral geniculate nucleus (LGN) (8–10), in Jurkat cells after anti-Fas treatment (9–11), and in HL-60 cells after anisomycin treatment (12). The DeadEnd™ Colorimetric TUNEL System labels fragmented DNA in situ and has been tested in all of these systems.

This Technical Bulletin includes protocols for detecting apoptosis in both tissue sections (Section 3.B) and in anisomycin-induced HL-60 cells (Sections 3.C–E).

Assay Principle

The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL method. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown and visualized with a light microscope. An overview of the protocol using cultured cells is shown in Figure 1 .

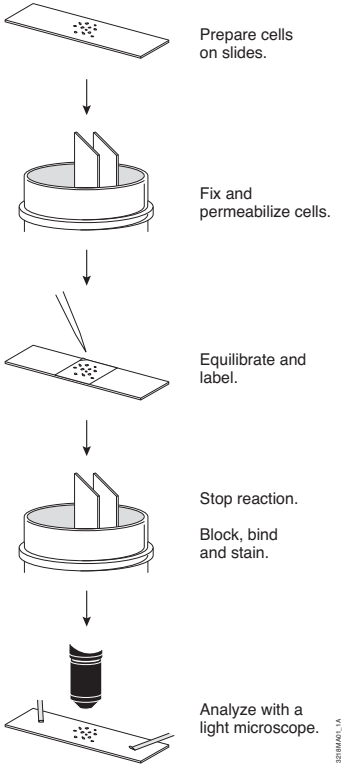

Figure 1. An overview of the DeadEnd™ Colorimetric TUNEL System using cultured cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
DeadEnd™ Colorimetric TUNEL System	**20 reactions**	**G7360**

Includes:

- 4.8ml Equilibration Buffer
- 20µl Biotinylated Nucleotide Mix (2 × 20µl)
- 20µl Terminal Deoxynucleotidyl Transferase, Recombinant
- 20ml SSC, 20X
- 10mg Proteinase K
- 40µl Streptavidin HRP (0.5mg/ml)
- 400µl DAB 10X Chromogen
- 3.6ml DAB Substrate 1X Buffer
- 20 Plastic Coverslips

PRODUCT	SIZE	CAT.#
DeadEnd™ Colorimetric TUNEL System	**40 reactions**	**G7130**

Includes:

- 9.6ml Equilibration Buffer
- 40µl Biotinylated Nucleotide Mix (2 × 20µl)
- 2 × 20µl Terminal Deoxynucleotidyl Transferase, Recombinant
- 70ml SSC, 20X
- 10mg Proteinase K
- 40µl Streptavidin HRP (0.5mg/ml)
- 400µl DAB 10X Chromogen
- 3.6ml DAB Substrate 1X Buffer
- 40 Plastic Coverslips (2 × 20)

Storage Conditions: Store the Equilibration Buffer, rTdT Enzyme, Biotinylated Nucleotide Mix and Proteinase K at –20°C. Store the Streptavidin HRP, DAB 10X Chromogen and DAB Substrate 1X Buffer at 4°C. Store the 20X SSC and Plastic Coverslips at room temperature.

! Different components of the system have different storage conditions.

Reconstitute the Proteinase K supplied with the system in 1ml of proteinase K buffer (see Section 5) before use. Store aliquots of this reconstituted 10mg/ml Proteinase K solution at –20°C, where the enzyme is stable for at least 6 months.

Safety Considerations: The Equilibration Buffer contains potassium cacodylate (dimethylarsinic acid). Avoid contact with skin and eyes. Wear gloves and safety glasses when working with this reagent.

DAB is a suspected carcinogen. Wear gloves and safety glasses when working with this reagent.

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3. Assay Protocol

3.A. Materials to be Supplied by the User and Reagent Preparation

Please read the material list below recommended for using the DeadEnd™ Colorimetric TUNEL System in your application.

Solution compositions are provided in Section 5.

- phosphate buffered saline (PBS)
- 0.3% hydrogen peroxide for blocking endogeneous peroxidases
- fixative (e.g., 10% buffered formalin, 4% paraformaldehyde, 4% methanol-free formaldehyde)
- mounting medium

Additional Materials Specific for Cultured Cells

- poly-L-lysine
- 0.2% Triton® X-100 solution in PBS
- DNase I (e.g., RQ1 RNase-Free DNase, Cat.# M6101)
- DNase buffer

Additional Materials Specific for Paraffin-Embedded Tissues Sections

- xylene or xylene substitute [e.g., Hemo-De® Clearing Agent (Fisher Cat.# 15-182-507A)]
- ethanol (100%, 95%, 85%, 70% and 50%) diluted in deionized water
- 0.85% NaCl solution
- proteinase K buffer
- DNase I (e.g., RQ1 RNase-Free DNase, Cat.# M6101)
- DNase buffer

Additional Materials for Both Tissue Sections and Cultured Cells

- poly-L-lysine-coated or silanized microscope slides, [e.g., Poly-Prep® Slides (Sigma Cat.# P 0425), Superfrost® Plus Glass Slides (Fisher Cat.# 12-550-15) or other appropriate pretreated slides]
- Coplin jars (a separate jar is needed for optional DNase I positive-control slides)
- forceps
- 37°C incubator
- micropipettors
- glass coverslips
- clear nail polish or rubber cement
- microscope

Reagent Preparation

1. Prepare a 20µg/ml Proteinase K solution from the 10mg/ml Proteinase K stock solution (Section 2) by diluting 1:500 in PBS.
2. Dilute the 20X SSC 1:10 with deionized water.

   Note: Ensure that all salts of the 20X SSC are in solution before diluting.

3.B. Procedure for the Analysis of Apoptosis in Tissue Sections

This procedure is suitable for tissue sections prepared in a variety of ways, including paraffin-embedded sections, frozen sections and vibratome sections. **For frozen and vibratome sections, begin at Step 4.**

Assay Controls: For a Positive Control, perform DNase treatment of a sample (Step 11). For a Negative Control, prepare Reaction Mix without TdT Enzyme (Step 13).

1. For paraffin-embedded sections, deparaffinize tissue sections (attached to microscope slides) by immersing the slides in fresh xylene in a Coplin jar for 5 minutes at room temperature. Repeat.
2. Wash by immersing the slides in 100% ethanol for 5 minutes at room temperature in a Coplin jar.
3. Repeat the 100% ethanol wash for 3 minutes, and then rehydrate the samples by sequentially immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%) for 3 minutes each at room temperature.
4. Wash by immersing the slides in 0.85% NaCl for 5 minutes at room temperature.
5. Wash by immersing the slides in PBS for 5 minutes at room temperature.
6. Fix the tissue sections by immersing the slides in 4% paraformaldehyde solution or 10% buffered formalin in PBS for 15 minutes at room temperature.
7. Immerse the slides in PBS for 5 minutes at room temperature. Repeat.
8. Remove the liquid from the tissue and place the slides on a flat surface. Add 100µl of the 20µg/ml Proteinase K solution (see Section 3.A for reagent preparation) to each slide to cover the tissue section and incubate slides for 10–30 minutes at room temperature.

Proteinase K helps permeabilize tissues, but prolonged incubation may cause sections to come off the slide. For best results, optimize the length of incubation. Use shorter incubations for thin tissue sections (e.g., 5–10µm paraffin sections) and longer incubations for thick sections (e.g., 50µm vibratome sections).

9. Wash by immersing the slides in PBS for 5 minutes at room temperature in a Coplin jar.
10. Refix the tissue sections after washing by immersing the slides in 4% paraformaldehyde solution or 10% buffered formalin in PBS for 5 minutes at room temperature.
11. Wash by immersing the slides in PBS for 5 minutes at room temperature. Repeat.
    Note: If required (Step 11), prepare a positive control by treating a sample with DNase I to cause DNA fragmentation. See Section 3.D for a protocol for DNase treatment.

3.B. Procedure for the Analysis of Apoptosis in Tissue Sections (continued)

12. Remove excess liquid by tapping the slides. Cover the cells with 100µl of Equilibration Buffer. Equilibrate at room temperature for 5–10 minutes.

13. While the sections are equilibrating, thaw the Biotinylated Nucleotide Mix on ice and prepare sufficient rTdT reaction mix for all experimental and control reactions. Keep on ice. One hundred microliters of reaction mix per slide will adequately cover the section. See Table 1 for details on preparation of rTdT reaction mix.

Table 1. Preparation of rTdT Reaction Mix.

Buffer Component	Component Volume per Standard 100µl Reaction		Number of Reactions (Experimental Reactions + Optional Positive Controls)		Component Volume
Equilibration Buffer	98µl	×	_____	=	_____µl
Biotinylated Nucleotide Mix	1µl	×	_____	=	_____µl
rTdT Enzyme	1µl	×	_____	=	_____µl

For negative controls: Prepare a control incubation buffer without rTdT Enzyme by combining 98µl of Equilibration Buffer, 1µl of Biotinylated Nucleotide Mix and 1µl of autoclaved, deionized water. Process through Steps 14–24.

14. Blot around the equilibrated areas with tissue paper and add 100µl of rTdT reaction mix to the sections on a slide. Do not allow the sections to dry.

15. Cover the sections with Plastic Coverslips to ensure even distribution of the reagent. Incubate the slides at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur.

16. Remove the Plastic Coverslips and terminate the reactions by immersing the slides in 2X SSC (see Section 3.A for reagent preparation) in a Coplin jar for 15 minutes at room temperature.

17. Wash by immersing the slides in fresh PBS for 5 minutes at room temperature. Repeat this wash twice to remove unincorporated biotinylated nucleotides.

18. Block the endogenous peroxidases by immersing the slides in 0.3% hydrogen peroxide in PBS for 3–5 minutes at room temperature.

19. Wash by immersing the slides in PBS for 5 minutes at room temperature. Repeat twice.

20. Dilute the Streptavidin HRP solution 1:500 in PBS. Add 100µl to each slide and incubate for 30 minutes at room temperature.

21. Wash by immersing the slides in PBS for 5 minutes at room temperature. Repeat twice.

22. Prepare the DAB Solution just prior to use. Add 100µl of DAB 10X Chromogen to 900µl of DAB Substrate 1X Buffer. Add 100µl of DAB Solution to each slide and develop until there is a light brown background (typically approximately 10 minutes, but the time may need to be optimized). Do not allow the background to become too dark.

Keep the DAB Solution away from light and use within 30 minutes.

23. Rinse several times in deionized water.

24. Mount slides in an aqueous or permanent mounting medium [e.g., 100% glycerol or Permount® Mounting Medium (Fisher Cat.# SP15-100)]. For the aqueous mounting medium, seal the edges of the coverslip with nail polish. Use a light microscope to observe staining.

3.C. Procedure for the Detection of Apoptosis in Cultured Cells

Prepare sufficient poly-L-lysine-coated slides for all experimental samples and controls.

Preparation of Poly-L-Lysine-Coated Slides

Pipette an aqueous solution of poly-L-lysine (Sigma Cat.# P 8920, diluted 1:10 in water) onto the surface of each precleaned glass slide. Distribute a thin layer of the poly-L-lysine solution throughout the areas to be used for fixing cells. Immediately after the slides have dried, rinse in deionized water and then allow the coated slides to air-dry for 30–60 minutes. Poly-L-lysine-coated slides may be stored at 4°C for 7 days before use. To grow adherent cells on poly-L-lysine-coated slides, use poly-L-lysine without preservative (Sigma Cat.# P 9155).

Preparation of Cells on Slides

Centrifuge control or apoptosis-induced cells, wash in PBS, resuspend and add to the poly-L-lysine-coated slides. Let the cells air-dry in a tissue culture hood for approximately 15 minutes before fixing. Alternatively, grow adherent cells on Lab-Tek® Chamber Slides. Following control or experimental treatment to induce apoptosis, wash the slides twice with PBS and process directly in the apoptosis detection assay follows.

Apoptosis Detection

1. Fix cells by immersing slides in 10% buffered formalin, 4% paraformaldehyde solution or 10% buffered formalin in PBS in a Coplin jar for 25 minutes at room temperature.

2. Wash by immersing in fresh PBS for 5 minutes at room temperature. Repeat.
   Note: After completion of Step 2, slides may be stored in PBS at 4°C or in 70% ethanol at –20°C.

3. Permeabilize cells by immersing the slides in 0.2% Triton® X-100 solution in PBS for 5 minutes at room temperature.

4. Rinse slides by immersing in fresh PBS for 5 minutes at room temperature. Repeat.

5. Follow Section 3.B, Steps 12–24. An optional positive control slide may be prepared at this point as described in Section 3.D.

3.D. Procedure for DNase Treatment of Positive Controls (optional)

A positive control for detection of DNA fragmentation may be included in each experiment. For cultured cells, follow Steps 1–4 as described in Section 3.C, then prepare a positive control slide by treating the cells with DNase I (not included with the system) as described in this section.

Note: DNase I treatment of the fixed cells results in fragmentation of the chromosomal DNA and exposure of multiple 3´-OH DNA ends at which Biotinylated Nucleotides can be incorporated. The protocol outlined below generally results in the majority of the treated cells demonstrating peroxidase labeling.

! Use a separate Coplin jar for positive control slides. Residual DNase I activity from the positive control slide may introduce high background to the experimental slides.

1. Add 100µl of DNase I buffer (Section 5) to the fixed cells and incubate at room temperature for 5 minutes.
2. Tap off the liquid and add 100µl of DNase I buffer containing 5–10unit/ml of DNase I (Cat.# M6101, RQ1 RNase-free DNase; when using other DNases an optimization step may be required). Incubate for 10 minutes at room temperature.
3. Remove excess liquid by tapping the slide, and wash the slide extensively 3–4 times in deionized water in a Coplin jar dedicated for the positive control.
4. Wash by immersing the slides in PBS for 5 minutes at room temperature.
5. Process the positive control as described in Section 3.B, Steps 12–24, using separate Coplin jars.

3.E. Procedure for Anisomycin-Induced Apoptosis in HL-60 Cells

Treatment with the protein synthesis inhibitor anisomycin induces apoptosis in the human promyelocytic cell line HL-60 (10).

1. Grow HL-60 cells in RPMI-1640 medium containing 10% fetal bovine serum in a humidified 5% CO_2 incubator at 37°C.
2. Adjust the cell density to 5×10^5 cells/ml and treat with anisomycin at a final concentration of 2µg/ml (dissolved in DMSO). Incubate for 2 hours in a humidified 5% CO_2 incubator at 37°C. Treat negative control cells with an equal volume of DMSO and incubate under the same conditions.
3. Harvest the cells and resuspend in PBS to 1.5×10^6 cells/ml before adding a thin layer to the poly-L-lysine-coated slides. Then follow Steps 1–5 as described in Section 3.C for the analysis of apoptosis.

4. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
High background (i.e., strong staining of nonapoptotic cells)	Nonspecific incorporation of biotinylated nucleotides. Do not allow cells to dry out.
	At Section 3.B, Step 17, slides may be washed 3 times for 5 minutes with PBS containing 0.1% Triton® X-100 and 5mg/ml BSA, followed by a single PBS wash step to remove nonspecific background.
	DNA degraged during tissue preparation. Make sure that tissue samples are fixed or frozen promptly.
High general background	Sample incubated too long with DAB Solution. Decrease DAB development time.
	Endogenous peroxidases not blocked. Increase blocking time with 0.3% hydrogen peroxide.
Little or poor staining	Insufficient permeabilization with Triton® X-100 or Proteinase K. Optimize permeabilization step by adjusting the incubation time with the permeabilization agent.
Loss of tissue section from slide	Tissue section enzymatically digested from slide. Decrease Proteinase K incubation time.

5. Composition of Buffers and Solutions

1X PBS (pH 7.4)

137mM	NaCl
2.68mM	KCl
1.47mM	KH_2PO_4
8.1mM	Na_2HPO_4

20X SSC

87.7g	NaCl
44.1g	sodium citrate

Dissolve in 400ml of deionized water. Adjust pH to 7.2 with 10N NaOH and bring volume to 500ml.

2X SSC

Dilute 20X SSC 1:10 with deionized water before use.

4% paraformaldehyde solution

Weigh out 4g paraformaldehyde in a fume hood, add PBS and bring to 100ml. Dissolve by heating the closed bottle in a water bath at 65°C for 2 hours. Store the solution at 4°C, where it is stable for at least 2 weeks.

DNase I Buffer

40mM	Tris-HCl (pH 7.9)
10mM	NaCl
6mM	$MgCl_2$
10mM	$CaCl_2$

Equilibration Buffer

200mM	potassium cacodylate (pH 6.6 at 25°C)
25mM	Tris-HCl (pH 6.6 at 25°C)
0.2mM	DTT
0.25mg/ml	BSA
2.5mM	cobalt chloride

proteinase K buffer

100mM	Tris-HCl (pH 8.0)
50mM	EDTA

6. Related Products

Product	Size	Cat.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
	125µl	G7462
Caspase Inhibitor Z-VAD-FMK	50µl	G7231
	125µl	G7232
Caspase Inhibitor Ac-DEVD-CHO	100µl	G5961
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-PARP p85 Fragment pAb	50µl	G7341
CellTiter-Glo® 2.0 Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	20ml	G8080
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	200 assays	G3582
CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay	1,000 assays	G5421
CellTiter 96® AQ_{ueous} MTS Reagent Powder	250mg	G1112
CellTiter 96® Non-Radioactive Cell Proliferation Assay	1,000 assays	G4000
CellTox™ Green Cytotoxicity Assay	10ml	G8741
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
CytoTox 96® Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780
rhTNF-α	10µg	G5241
RQ1 RNase-Free DNase	1,000u	M6101
Terminal Deoxynucleotidyl Transferase, Recombinant	300u	M1871
Proteinase K (Lyophilized)	100mg	V3021

For Research Use Only. Not for Use in Diagnostic Procedures. Additional Sizes Available.

7. References

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8. Summary of Changes

The following changes were made to the 1/19 revision of this document:

1. Product description has been updated to include information about how the detection is performed with a light microscope.
2. Product components list has been updated to reflect new names and sizes.
   a. Hydrogen Peroxide 20X is no longer included in this system and references in the protocol have been removed.
3. Reagent Preparation information has been added to Section 3.A.
4. New Related Products have been added and discontinued products have been removed.