TdT Microplate Apoptosis Detection Kit

For in situ colorimetric detection of apoptosis

Catalog No. 30-1206, 96 tests

Instructions

Multiple storage temperatures required.

For laboratory research use only. Not for human, clinical, or diagnostic use.

American Type Culture Collection
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Instruction Manual

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INTRODUCTION

Background

The DNA fragmentation that occurs during apoptosis provides the basis for several assays that detect apoptosis in situ. During apoptosis DNA is cleaved, most typically within the linker regions of DNA between nucleosomes, generating free 3'-hydroxyl residues that can be utilized by terminal deoxynucleotidyl transferase (TdT) in end-labeling reactions. Incorporation of biotinylated nucleotides into the DNA by TdT allows detection with streptavidin-linked conjugates (1).

ATCC's TdT Microplate Apoptosis Detection Kit is designed specifically for quantitation of apoptosis in suspension and monolayer cell cultures. Following incorporation of biotinylated dNTPs into cellular DNA, streptavidin-conjugated horseradish peroxidase is added which in turn reacts with the Sapphire Label colorimetric substrate. The assay provides quantitation of apoptosis in cultured cells without direct counting of labeled cells and the 96-well plate format allows analysis of a large number of samples at one time.

Precautions

Exercise caution and use gloves, lab coats, and eye protection when using chemical reagents and kit components.

KIT COMPONENTS

Store the following reagents at –20°C or colder:

Reagent	Quantity
Nuclease Mix	15 µl
Proteinase K	100 µl
TdT dNTP Mix	35 µl
TdT Enzyme	35 µl
50x Manganese Cation	100 µl

Store the following reagents at 2 to 8°C:

Strep-HRP	30 µl
Strep-HRP Diluent	7.5 ml
Nuclease Buffer	1.5 ml
Cytonin Reagent	6 ml
10x TdT Labeling Buffer	20 ml
10x TdT Stop Buffer	20 ml
Sapphire Label	10 ml

MATERIALS REQUIRED BUT NOT SUPPLIED

Molecular grade water (ATCC catalog no. 60-2450)
37% formaldehyde
Phosphate buffered saline without calcium or magnesium (ATCC catalog no. 30-2200)
100% ethanol
Tween 20
Sucrose
30% hydrogen peroxide
Methanol
50% phosphoric acid or 2N HCl
Microwell plate reader with 450-nm and 630-nm filters
Multichannel pipettor, 1-50 ml
Microcentrifuge tubes
Humidity chamber
Centrifuge with microplate adapters
Micropipettors and tips
37°C incubator
96-well microplate (conical and flat-bottomed)
10-ml serological pipets

PROTOCOLS

Experimental Overview

Cells grown in suspension or prepared from dissociated tissues can be fixed in batches and then transferred to 96-well plates. Alternatively, suspension or adherent cells may be grown and fixed directly in the plates. Cells are labeled and then analyzed with a microplate reader.

- It is important to read through the instructions before preparing cell samples for labeling.
- All incubations at 37°C should be carried out in a humidity chamber to prevent evaporation see appendix).
- Prepare and label suspension cells in conical well plates and transfer to flat-bottom plates before reading.
- Cells can be stored after fixation and postfixation steps. See the appendix for instructions, p.11.
- Experimental results may vary depending on the type of cells, cell treatment, length of storage and storage conditions. Observe cell morphology prior to assay (2) or use a second method to confirm apoptosis (see Related Products, p. 13).

Controls

Appropriate controls should be included in each experiment and performed in duplicate or triplicate.

Control 1: Nuclease-treated control to generate DNA breaks in every cell. This will confirm that labeling reaction has worked.

Control 2: Unlabeled control to assess background associated with nonspecific binding of Strep-HRP. Incubate in reaction mix without TdT enzyme.

Control 3: Experimental negative control (untreated or normal cells).

FIXATION PROTOCOLS

Preparation of suspension cells: Batch method

Step	Action
1	Count cells in suspension. Harvest cells by centrifugation at 500 x g for 5 minutes at room temperature. Between 2 x 10^4 and 1 x 10^5 cells/well will be required.
2	Resuspend cells in 200 µl PBS and collect by centrifugation as in step 1. Discard supernatant.
3	Prepare 3.7% buffered formaldehyde solution immediately before use; 20 ml is needed to process 96 samples. Sucrose 5 g PBS 20 ml 37% formaldehyde 2.5 ml Adjust volume to 25 ml with PBS.
4	Resuspend cells at 1 x 10^6 cells/ml in 3.7% buffered formaldehyde solution. Let stand for 7 minutes at room temperature (no longer than 10 minutes).
5	Centrifuge at 500 x g for 5 minutes at room temperature. Discard fixative
6	Wash cells once in PBS as in Step 2.
7	Post-fix sample in 100 µl 100% methanol for 20 minutes at room temperature.
8	Wash cells twice in 200 µl PBS as in Step 2.
9	Resuspend cells at 1 x 10^6 cells/ml in PBS.
10	Distribute cells at 2 x 10^4 to 1 x 10^5 cells/well in a conical-well plate. **Note:** The optimal number of cells will vary for each cell type and will need to be determined empirically.
11	Proceed to labeling procedure protocol, page 7.

Preparation of suspension cells: In-well method

Step	Action
1	Count cells and distribute at 2 x 10^4 to 1 x 10^5 cells/well. Or grow cells directly in a 96-well plate (conical bottom) to achieve 2 x 10^4 to 1 x 10^5 cells/well. **Note:** The optimal number of cells will vary by cell type and will need to be determined empirically.
2	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard medium.
3	Resuspend cells in 200 µl PBS. Collect by centrifugation at 1000 x g for 3 minutes. Discard PBS.
4	Prepare 3.7% buffered formaldehyde solution immediately before use; 20 ml is needed to process 96 samples. Sucrose 5 g PBS 20 ml 37% formaldehyde 2.5 ml Adjust volume to 25 ml with PBS.
5	Add 100 µl 3.7% Buffered Formaldehyde solution to each well. Let stand 7 minutes at room temperature (no longer than 10 minutes).
6	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard fixative.
7	Wash cells once with 200 µl PBS as described in Step 3.
8	Post-fix sample in 100 µl 100% methanol for 20 minutes at room temperature.
9	Wash cells twice with 200 µl PBS as described in Step 3.
10	Proceed to labeling procedure protocol, page 7.

Preparation of adherent cells

Step	Action
1	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard growth medium. **Note:** Grow cells in a flat-bottomed 96-well plate to a density of 2 x 10^4 to 1 x 10^5 cells/well. The optimal number of cells will vary by cell type and will need to be determined empirically.
2	Wash cells in 200 µl PBS. Collect by centrifugation at 1000 x g for 3 minutes at room temperature. Discard PBS.
3	Prepare 3.7% buffered formaldehyde solution immediately before use; 20 ml is needed to process 96 samples. Sucrose 5 g PBS 20 ml 37% formaldehyde 2.5 ml Adjust volume to 25 ml with PBS.
4	Add 100 µl 3.7% Buffered Formaldehyde solution to each well. Let stand 7 minutes at room temperature (no longer than 10 minutes).
5	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard fixative.
6	Wash cells once with 200 µl PBS as described in step 2.
7	Post-fix sample in 100 µl 100% methanol for 20 minutes at room temperature.
8	Wash cells twice with PBS as described in step 2.
9	Proceed to labeling procedure protocol, page 7.

LABELING PROTOCOL

Reagent Preparation
The following reagents can be prepared ahead of time. Others must be prepared immediately before use; directions are given within the protocol.

1x TdT Labeling Buffer (Prepare 25 ml for 100 samples.)
Add 2.5 ml of 10x TdT Labeling Buffer to 22.5 ml distilled water.
Remove 50 µl per sample for the labeling reaction and place on ice.
Leave remainder at room temperature until use.

1x TdT Stop Buffer (Prepare 20 ml to process 100 samples.)
Add 2 ml 10x TdT Stop Buffer to 18 ml distilled water.
Store at room temperature until use.

0.1% Tween 20 in PBS (Prepare 100 ml to process 100 samples.)
Add 100 µl Tween 20 to 100 ml PBS. Store at room temperature.

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>1</td><td colspan="2">Determine the total number of samples (n) for analysis including controls.</td></tr>
<tr><td rowspan="2">2</td><td colspan="2">Permeabilize fixed cells.</td></tr>
<tr><td>Monolayer cells:
Add 50 µl of Cytonin Reagent to each well. Incubate for 15 minutes at room temperature.</td><td>Suspension cells:
Dilute Proteinase K solution: For 100 samples, dilute 100 µl Proteinase K into 5 ml of distilled water.

Add 50 µl of Proteinase K solution to each well. Incubate for 15 minutes at room temperature.</td></tr>
<tr><td>3</td><td colspan="2">Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard the buffer.</td></tr>
<tr><td>4</td><td colspan="2">Resuspend cells in 200 µl distilled water. Collect by centrifugation at 1000 x g for 3 minutes at room temperature. Remove and discard supernatant.</td></tr>
</table>

(continued on p. 8)

5	Prepare 50 µl Nuclease Solution for each nuclease-treated control. Store on ice. 1 sample / n samples Nuclease Buffer: 50 µl / n x 50 µl Nuclease Mix: 1 µl / n x 1 µl Add 50 µl Nuclease Solution to each well of Control 1. Cover the other samples with 50 µl PBS during this incubation. Incubate for 10 to 60 minutes at 37°C. **Note:** Tap the edge of the microplate gently every 15 minutes during incubation to keep cells in suspension. Do not use a pipette to mix cells as this may cause cell damage.
6	Centrifuge and wash samples for 2 minutes in 200 µl PBS as described in Step 4.
7	Quench endogenous peroxidase: a. Prepare 6 ml of 3% hydrogen peroxide for 100 samples by combining 0.6 ml 30% hydrogen peroxide and 5.4 ml methanol. b. Add 50 µl peroxide solution to each well. Incubate 5 minutes. (Do not exceed 5 minutes.) c. Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard peroxide solution.
8	Wash once with 200 µl distilled water as described in Step 4.
9	Add 150 µl 1x TdT Labeling Buffer to each well. Let stand for 5 minutes.
10	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Discard the buffer.
11	Prepare Labeling Reaction mix for n-1 samples just before use. Keep reaction mixture on ice. n=1 / n=100 TdT dNTP mix: 0.35 µl / 35 µl 50x Manganese Cation: 1 µl / 100 µl TdT Enzyme: 0.35 µl / 35 µl 1x cold TdT Labeling Buffer: 50 µl / 5 ml Prepare one reaction mix without TdT Enzyme for Control 2. **Note:** To maintain optimal enzyme activity, remove enzyme from freezer to remove the required volume then return immediately to freezer.

12	Add 50 µl Labeling Reaction mix **without** TdT enzyme to Control 2. Add 50 µl Labeling Reaction mix **with** TdT enzyme to remaining samples. Incubate 1 hour at 37°C in humidity chamber. **Note:** Tap the edge of the microplate gently every 15 minutes during incubation to keep cells in suspension. Do not use a pipette to mix cells as this may cause cell damage.
13	Add 150 µl 1x TdT Stop Buffer to each well. Incubate 5 minutes to stop labeling reaction.
14	Centrifuge plate at 1000 x g for 3 minutes at room temperature. Remove and discard the buffer.
15	Wash samples twice for 2 minutes with PBS as described in Step 4.
16	Prepare 50 µl Strep-HRP (1:1250) solution per well: n=1 n=100 Strep-HRP diluent 50 µl 5 ml Strep-HRP 0.04 µl 4 µl
17	Add 50 µl Strep-HRP Solution to each well. Incubate at room temperature 10 minutes.
18	Wash samples four times with 200 µl PBS + 0.1% Tween 20. Centrifuge as in Step 4 between washes.
19	Add 100 µl Sapphire Label at room temperature to each well. Ensure that cells are resuspended in the solution.
20	Incubate 30 minutes at room temperature in the dark. If possible, follow kinetics of reaction at 630 nm to determine linear range of reaction; you may need to stop the reaction sooner if it proceeds rapidly.
21	Stop reaction with 100 µl of 50% phosphoric acid **OR** 2N HCl per well.
22	Transfer suspension cells to a flat-bottom plate. Measure absorbance within 30 minutes of acid addition at 450 nm using microplate reader.

DATA INTERPRETATION

The controls allow optimization of in situ detection of apoptosis without expending valuable test samples.

Nuclease-treated Control 1: Readings will be higher than the experimental values and will provide a maximum value.

Unlabeled Control 2: Should have low or negligible absorbance.

Negative Control 3: May have a small number of apoptotic cells resulting in a low level of labeling.

Duplicate or triplicate samples will allow statistical validation of results. Refer to the Troubleshooting section (page 11) for recommendations if the controls do not provide the expected results.

Figures 1 and 2 show typical results.

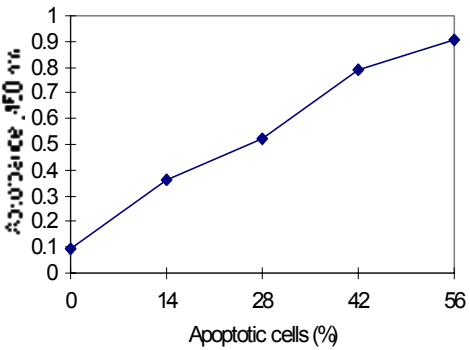

Figure 1. Quantitation of apoptosis in fixed ML-1 cells, treated with 1 µM staurosporine for 24 hours to induce apoptosis. Cells were incubated with Sapphire Label for 30 minutes. The percentage of apoptotic cells in the culture was estimated by enumeration using the TdT In Situ Apoptosis Detection kit – DAB (ATCC catalog no. 30-1301). The apoptotic culture was diluted with a nonapoptotic cell culture to obtain different concentrations of apoptotic cells.

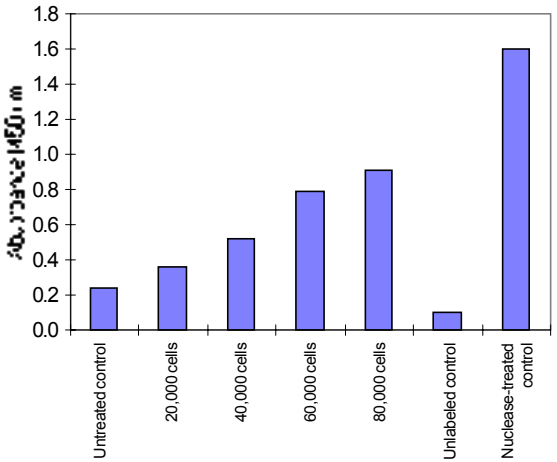

Figure 2. Quantitation of apoptosis in increasing numbers of staurosporine-treated ML-1 cells. Cells were incubated with Sapphire Label for 30 minutes. Control wells contained 10^5 cells that were untreated (without apoptosis inducer), unlabeled (without the TdT enzyme), and nuclease-treated, respectively.

APPENDIX

Storage Of Cells After Fixation

After fixation and postfixation steps, cells can be stored in 80% ethanol at 4°C or –20°C for several weeks or months before labeling. For short-term storage of up to one month, cells can be stored in Cytonin Reagent at 2 to 8°C. An adhesive plate seal (ATCC catalog no. 60-2400) is recommended to prevent contamination and evaporation.

For labeling after storage, wash the cells with 80% ethanol, then wash three times with PBS and proceed to the Labeling Protocol (page 7). **Note**: When cells are fixed using alcohol, signal intensity may diminish with time due to loss of small DNA fragments.

Humidity Chamber

To prevent evaporation, use a humidity chamber when incubating at 37°C. A humidity chamber can be made using a plastic box with a tight-fitting lid and two glass rods or other support. Place a paper towel on the bottom of the box and wet thoroughly with water. Lay the glass rods parallel to each other and less than one 96-well plate length apart on the wet towel. Position the plate on the glass rods and place the plastic box (with lid) in a 37°C incubator. Ensure that the plate is kept horizontal throughout the incubation time.

TROUBLESHOOTING

Problem: No labeling in nuclease-treated sample (Control 1).

Cause	Remedy
Poor permeabilization and/or excessive fixation with cross-linking fixative, preventing enzyme access.	Optimize Proteinase K treatment or optimize time in Cytonin Reagent; reduce time in fixative to 5 minutes.
No DNA left in sample due to hydrolysis (poor storage of samples).	Cells should be prepared and fixed according to the fixation protocol (p. 4). If storing cells, see the appendix (above).
Excessive or inadequate nuclease treatment.	Optimize time for nuclease treatment (from 5 minutes to 2 hours).
TdT Enzyme is inactive. The enzyme is the most labile component in the kit.	Enzyme must be stored at –20°C. The freezer should not be frost-free. Do not bring enzyme up to ice temperature. Place in –20°C freezer block or remove aliquot from tube directly to freezer.

Problem: No labeling in experimental sample.

Cause	Remedy
No apoptosis (or necrosis) occurring in the sample.	If all controls gave the expected results and were processed at the same time as the experimental sample, there may be no DNA fragmentation in the cells within the sample. Examine the morphology of cells (2) or use a second assay to confirm apoptosis.

Problem: Excessive background in negative control (Control 2).

Cause	Remedy
Residual unlinked Strep-HRP.	Wash cells at least 4 times with PBS + 0.1% Tween 20.
Nonspecific binding of Strep-HRP.	Incubate Strep-HRP with a blocking reagent such as 5% (w/v) nonfat dried milk or fetal bovine serum in PBS, 0.1% Tween 20.
Endogenous peroxidase not sufficiently quenched.	Make sure fresh hydrogen peroxide was used. If in doubt, obtain a new bottle.

Problem: Poor duplicate or triplicate values.

Insufficient centrifugation.	Centrifuge after every wash.
Poor removal of buffer.	Use care when pipetting off buffer. Plates may also be inverted to remove buffer from all wells at once.
Loss of cells after or during washes.	Use conical 96-well plate to perform assay on suspension cells and transfer to flat bottom plate after incubation with Sapphire Label. Perform all centrifugations at 1000 x g.

REFERENCES

1. Negoescu A et al. In situ apopototic cell labeling by the TUNEL method: improvements and evaluation on cell preparation. J. Histochem. Cytochem. 44(9): p. 959-968, 1996.
2. Kerr JF et al. Anatomical methods in cell death. Methods Cell Biol. 46: 1-27, 1995.

RELATED PRODUCTS

Apoptosis Detection Products		
Annexin V Fluorescent Detection Kit	30-1236	100 tests
Anti-G3PDH Antibody	30-1255	100 µl
Anti-PARP Monoclonal Antibody	30-1253	50 µl
Anti-Phosphorylated Histone H2AX Polyclonal Antibody	30-1251	20 µl
Anti-Phosphorylated Histone H2AX Polyclonal Antibody	30-1252	100 µl
Apoptotic Cell DNA Laddering Kit	30-1231	20 tests
CV Caspase 3 & 7 Substrate Kit	30-1344	100 tests
FAM Caspase 3 & 7 Binding Kit	30-1304	100 tests
FAM Caspase 8 Binding Kit	30-1306	100 tests
FAM Caspase 9 Binding Kit	30-1308	100 tests
FAM Poly-Caspase Binding Kit	30-1302	100 tests
Mitochondrial Potential Assay Kit	30-1233	100 tests
PARP Activity Assay Kit	30-1235	50 tests
SR Caspase 3 & 7 Binding Kit	30-1324	100 tests
SR Poly Caspase Binding Kit	30-1322	100 tests
TdT Flow Cytometry Apoptosis Detection Kit	30-1207	60 tests
TdT In Situ Apoptosis Detection Blue Kit	30-1202	30 tests
TdT In Situ Apoptosis Detection DAB Kit	30-1201	30 tests
TdT In Situ Replenisher Kit	30-1205	30 tests
TdT Microplate Apoptosis Detection Kit	30-1206	96 tests
Stains and Reagents		
Erythrosin B Stain Solution	30-2404	40 ml
Trypan Blue Stain Solution	30-2402	40 ml
Dulbecco's Phosphate Buffered Saline (PBS)	30-2200	500 ml
Molecular Grade Water	60-2450	1 liter
Clone Sets		
Human Apoptosis Clone Set	MBA-89	
Mouse Apoptosis Clone Set	MBA-90	

ATCC also has apoptotic inducible cell lines, cell line model systems, and hybridomas, as well as a full line of products for cell culture. See our Web site at www.atcc.org for details.

Please refer to the Material Transfer Agreement and packing slip enclosed with the product for terms and conditions.

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