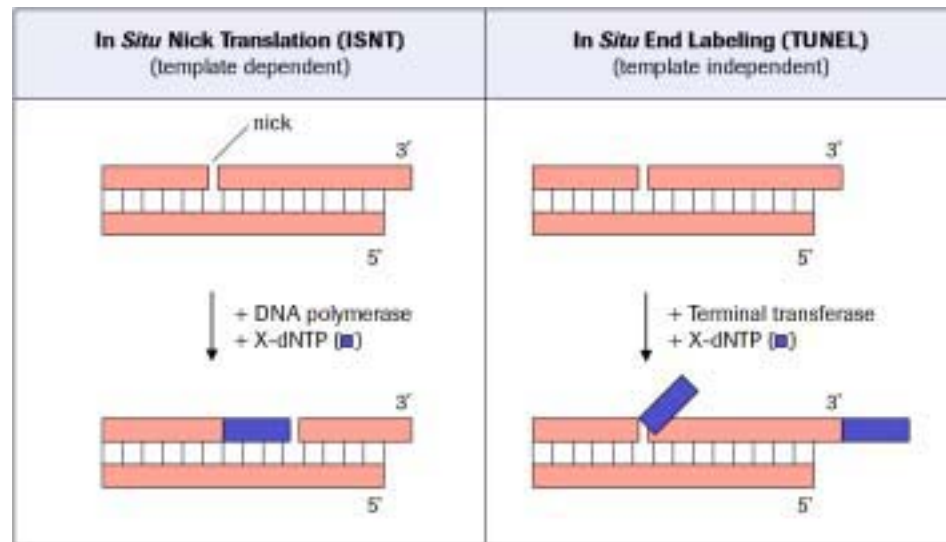


2.2.1 The TUNEL enzymatic labeling assay

Extensive DNA degradation is a characteristic event which occurs in the late stages of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand breaks (“nicks”) in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides (X-dUTP, X = biotin, DIG or fluorescein). Suitable labeling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labeling) (Figure 21).



▲ Figure 21: Schematic illustration of two enzymatic DNA labeling methods nick translation and end labeling.

Nick translation

DNA polymerase I catalyzes the template dependent addition of nucleotides when one strand of a double-stranded DNA molecule is nicked. Theoretically, this reaction (*In Situ* Nick Translation, ISNT) should detect not only apoptotic DNA, but also the random fragmentation of DNA by multiple endonucleases occurring in cellular necrosis.

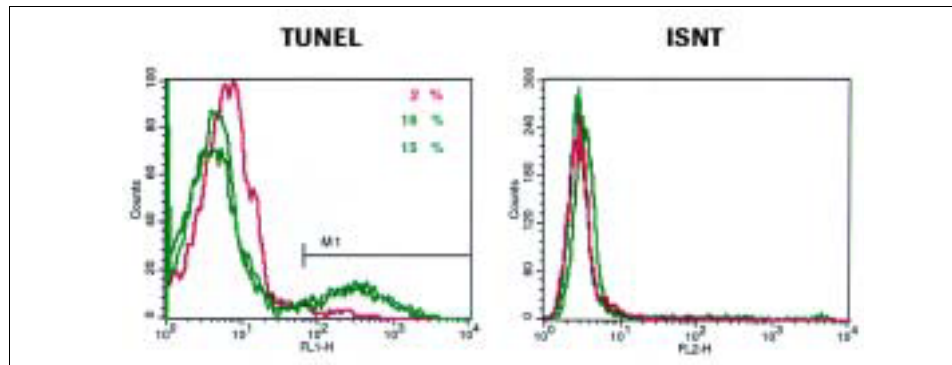
End labeling

Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double-stranded DNA breaks independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated X-dUTP nick end labeling)¹⁸.

The TUNEL method is more sensitive and faster than the ISNT method. Cells undergoing apoptosis were preferentially labeled by the TUNEL reaction, whereas necrotic cells were identified by ISNT. Thus, experiments suggest the TUNEL reaction is more specific for apoptosis and the combined use of the TUNEL and nick translation techniques may be helpful to differentiate cellular apoptosis and necrosis¹⁹.

Note: For a comparison of results with the TUNEL and ISNT methods, see Figure 22.

To allow exogenous enzymes to enter the cell, the plasma membrane has to be permeabilized prior to the enzymatic reaction. To avoid loss of LMW DNA from the permeabilized cells, the cells have to be fixed with formaldehyde or glutaraldehyde before permeabilization. This fixation crosslinks LMW DNA to other cellular constituents and precludes its extraction during the permeabilization step.



▲ **Figure 22: Comparison of TUNEL and ISNT methods for detecting apoptosis in CD8⁺ T cells from TcR transgenic mice.** F5 mice are transgenic for a T cell receptor (TcR) specific for a peptide derived from a nucleoprotein of influenza virus ANT/1968. In this experiment, the nucleoprotein was injected into F5 mice to activate T cells *in vivo*. After 4 days, mice were sacrificed and lymphoid organs were removed. Cell suspensions were prepared and incubated 4 h at 37°C. To allow detection of T cells which were dying after the *in vivo* immune response [Pihlgren, M., Thomas, J. and Marvel, J. (1996) *Biochemica*, No. 3, 12–14], cells were stained for CD8 (with a fluorescent antibody), fixed, permeabilized, and then labeled by either the TUNEL (TdT-mediated dUTP Nick End Labeling) or the ISNT (*In Situ* Nick Translation) method. Labeled and control cells were analyzed by flow cytometry, with CD8⁺ cells gated. Spleen cells from a control (not immunized) mouse (red) and from two mice immunized 4 days earlier (green) are shown.

Result: The TUNEL method detected approximately 15% apoptotic cells among CD8⁺ T cells from the immunized mice. No positive cells were found in the control mouse. In contrast, the ISNT method was unable to detect any apoptotic cells, possibly due to the lower sensitivity of the technique.

If free 3' ends in DNA are labeled with biotin-dUTP or DIG-dUTP, the incorporated nucleotides may be detected in a second incubation step with (strept)avidin or an anti-DIG antibody. The immunocomplex is easily visible if the (strept)avidin or an anti-DIG antibody is conjugated with a reporter molecule (*e.g.*, fluorescein, AP, POD).

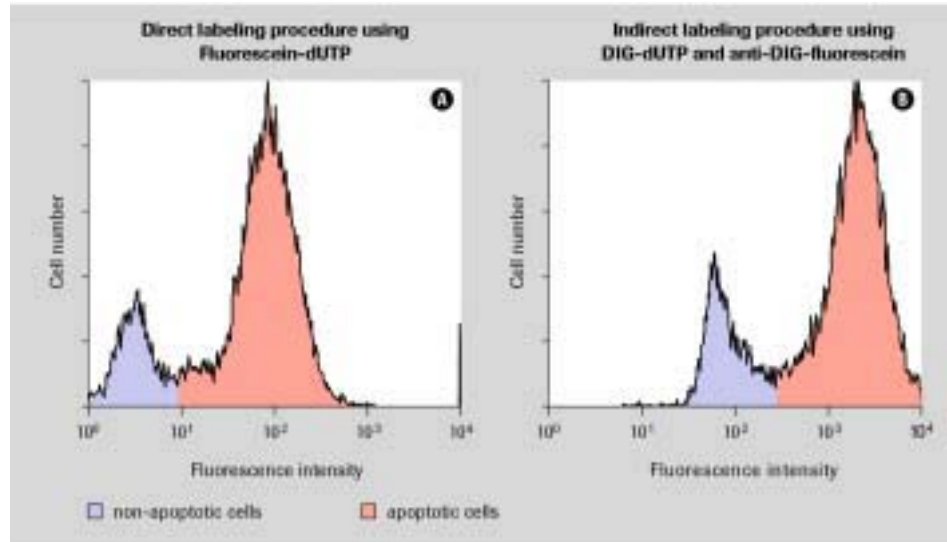
In contrast, the use of fluorescein-dUTP to label the DNA strand breaks allows the detection of the incorporated nucleotides directly with a fluorescence microscope or a flow cytometer²⁰. Direct labeling with fluorescein-dUTP offers several other advantages. Direct labeling produces less nonspecific background with sensitivity equal to indirect labeling (Figure 23) and, thus, is as powerful as the indirect method in detecting apoptosis. Furthermore, the fluorescence may be converted into a colorimetric signal if an anti-fluorescein antibody conjugated with a reporter enzyme (Table 5) is added to the sample.

Although the enzymatic labeling methods are time-consuming (due to multiple incubation and washing steps), they are very sensitive and specific²¹.

Caution: One has to keep in mind that these methods are based on the detection of DNA strand breaks. There are rare situations when apoptosis is induced without DNA degradation. Conversely, extensive DNA degradation, even specific to the internucleosomal linker DNA, may accompany necrosis. Thus, one should always use another independent assay, along with the TUNEL method, to confirm and characterize apoptosis.

Roche Applied Science offers four kits for the detection of DNA strand breaks in individual cells using the TUNEL method. Each is described on the following pages.

Note: For technical tips on the TUNEL method, see page 113 of the Appendix.



▲ Figure 23: Comparison of direct and indirect labeling of DNA strand breaks in apoptotic cells. PBL were incubated with 1 μ M dexamethasone for 24 h at 37°C and then labeled by TUNEL. Recordings were made at the same photomultiplier settings.

(Data were kindly provided by R. Sgonc, University of Innsbruck, Austria).

Result: Direct labeling is as sensitive as indirect labeling, but produces less non-specific background.

Method/RAS product	Label	Indirect (secondary) detection system	Analysis by
<i>In Situ</i> Cell Death Detection Kit, Fluorescein	Fluorescein-dUTP	None (direct detection)	Flow cytometry Fluorescence microscopy
<i>In Situ</i> Cell Death Detection Kit, TMR red	TMR-dUTP	None (direct detection)	Fluorescence microscopy
<i>In Situ</i> Cell Death Detection Kit, AP	Fluorescein-dUTP	Anti-Fluorescein-AP	Light microscopy
<i>In Situ</i> Cell Death Detection Kit, POD	Fluorescein-dUTP	Anti-Fluorescein-POD	Light microscopy

▲ Table 5: Four different kits for the enzymatic labeling of DNA and the secondary detection systems required.