Roche Molecular Biochemicals

Apoptosis and Cell Proliferation

2nd revised edition

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Overview of this Guide

How this guide can help you study cell death and cell proliferation?

When and why do cells die? Does the concentration of environmental pollutants exert cytotoxic or cytostatic effects on cells? What factors influence the rate and timing of cell proliferation? Researchers in basic, industrial, and medical research are asking these questions and looking for answers. Understanding the normal regulation of cell death and cell proliferation will be critical e.g., for the development of new and more successful therapies for preventing and treating cancer and for the screening of new anti-cancer compounds.

Many assays exist to measure cell death and cell proliferation. However, if you have only recently become interested in cell death or cell proliferation, you may find the diversity of such assays bewildering. You may not be able to determine what each assay measures nor decide which assays are best for your purposes. This guide is designed to help you make such decisions. It presents a brief overview of cell death and cell proliferation, along with the major assays currently available to measure each. In addition, it clearly lists the advantages and the disadvantages of these assays.

For those who want to eliminate radioactivity from their laboratories, this review also describes a number of non-radioactive assays that can serve as alternatives to radioactive assays. Wherever possible, the review will compare the sensitivity of the radioactive and non-radioactive assays.

What is new in this second edition?

Since the first edition of this guide appeared in 1995, apoptosis research has made much progress. Apoptosis now is recognized as an essential mechanism of physiological cell death. The basic mechanisms of apoptosis have been clarified.

This second edition of the guide reflects that progress in apoptosis research. It contains more information on apoptosis and describes more Roche Molecular Biochemicals products to make apoptosis research easier and faster.

This edition of the guide also describes new kits for the field of cell proliferation, which continues to be an important research area.

Some of the highlights of this edition are:

- Several new products for the measurement of apoptosis such as a Caspase Assay, Annexin, Anti-Fas and Anti-PARP.
- An apoptosis pathways chart, which summarizes information from many laboratories, and a brief literature guide for those interested in learning more about apoptosis research (see Section 1.1.3, on page 4)
- Method selection guides at the beginning of the apoptosis section and the cell proliferation chapter, to help you quickly find the Roche Molecular Biochemicals product that best fits your research needs (see Section 1.2., page 6, and Section 2.1, page 70)
- A separate section, within the cell death chapter, which spotlights those kits that can be used to measure cytotoxicity, regardless of whether the measured cell death is due to apoptosis or necrosis (see Section 1.3, page 52)
- More information on the use of flow cytometry to answer questions about cell death and cell proliferation
- An appendix, which presents supplementary technical information on such important techniques as TUNEL (**T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abeling)
- An introduction to the Apoptosis Chapter by Professor Andrew H. Wyllie, co-author of the first publication on apoptosis.

As we added new information, however, we always kept the original purpose of the guide in mind. As with the first edition, this second edition is still designed to answer one question: What is the best way for you to get the answers you need in your apoptosis or cell proliferation research?

To answer that question, we have retained the features that users told us they liked, such as the flow charts which give an overview of each assay and numerous examples of "typical assay results". We have also added a summary of the main characteristics of each assay and more references to literature describing applications of the assay.

CELL DEATH by Andrew H. Wyllie

Over the past five or six years there has been a near-exponential increase in publications on apoptosis. Around 30 new molecules have been discovered whose known functions are exclusively to do with the initiation or regulation of apoptosis. A further 20 molecules at least, although already associated with important roles in signalling or DNA replication, transcription or repair, have been recognised as affecting the regulation of apoptosis. This article is dedicated to young scientists thinking of entering this exploding area of biology, and to those more mature ones who happened to be looking elsewhere when the blast reached them, and consequently are in need of a rapid introduction to the present state of affairs.

The term apoptosis first appeared in the biomedical literature in 1972, to delineate a structurally-distinctive mode of cell death responsible for cell loss within living tissues[1]. The cardinal morphological features are cell shrinkage, accompanied by transient but violent bubbling and blebbing from the surface, and culminating in separation of the cell into a cluster of membrane-bounded bodies. Organellar structure is usually preserved intact, but the nucleus undergoes a characteristic condensation of chromatin, initiated at sublamellar foci and often extending to generate toroidal or cap-like, densely heterochromatic regions. Changes in several cell surface molecules also ensure that, in tissues, apoptotic cells are immediately recognised and phagocytosed by their neighbours. The result is that many cells can be deleted from tissues in a relatively short time with little to show for it in conventional microscopic sections.

This remarkable process is responsible for cell death in development, normal tissue turnover, atrophy induced by endocrine and other stimuli, negative selection in the immune system, and a substantial proportion of T-cell killing. It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral infection. It is a major factor in the cell kinetics of tumors, both growing and regressing. Many cancer therapeutic agents exert their effects through initiation of apoptosis, and even the process of carcinogenesis itself seems sometimes to depend upon a selective, critical failure of apoptosis that permits the survival of cells after mutagenic DNA damage. Apoptosis probably contributes to many chronic degenerative processes, including Alzheimer's disease, Parkinson's disease and heart failure. So how does it work?

Molecular genetic studies on the hard-wired developmental cell death programme of the nematode Caenorhabditis elegans led to discovery of a set of proteins, widely represented by homologues in other species, and responsible for turning on or off the final commitment to death[2]. In the nematode these proteins include the products of the *ced3* and *ced4* genes (which initiate cell suicide), *ced9* (which prevents it) and a series of some seven genes involved in recognition and phagocytosis of the doomed cell.

CED3 is the prototype of a family of around a dozen mammalian proteases, called caspases because of the obligatory cysteine in their active site and their predilection for cutting adjacent to aspartate residues. Mammalian caspases appear to constitute an autocatalytic cascade, some members (notably caspase 8 or FLICE) being "apical" and more susceptible to modification by endogenous regulatory proteins, whilst others (notably caspase 3 – also called CPP32, Yama and apopain) enact the final, irreversible commitment to death. Study of caspase substrates is providing interesting insights into the ways in which cells dismantle their structure and function. Such substrates include – not surprisingly – cytoskeletal proteins such as actin and fodrin and the nuclear lamins, but also an array of regulatory and chaperone-like proteins whose function is altered by cleavage in subtle and suggestive ways[3]. A recent example is the nuclease chaperone ICAD, whose cleavage permits nuclear entry by a distinctive apoptosis nuclease responsible for chromatin cleavage to oligonucleosome fragments[4].

Caspases appear to be present in most if not all cells in inactive pro-enzyme form, awaiting activation by cleavage. One of the killing mechanisms of cytotoxic T cells is a protease, granzyme B, that is delivered to the target cell by the T cell granules and triggers these latent pro-enzymes. There are endogenous triggers also, and the first to be discovered – the *C. elegans* CED4 protein and its mammalian homologue – is particularly intriguing because of its mitochondrial origin[5]. Thus CED4 could be the signal that initiates apoptosis under conditions of shut-down of cellular energy metabolism, or when there is a critical level of cell injury affecting mitochondrial respiration. In this way CED4 may act as the link between agents long known to be associated with mitochondrial injury, such as calcium and reactive oxygen species, and the initiation of apoptosis.

A second mitochondrial protein of enormous significance in apoptosis is BCL2, a mammalian homologue of the nematode CED9 protein. BCL2 has the tertiary structure of a bacterial pore-forming protein, and inserts into the outer membrane of mitochondria. It abrogates apoptosis, probably through binding CED4 and another protein BAX, with which it forms heterodimers and which, like CED4, is also a "killer" protein[6]. Both BCL2 and BAX have several structurally and functionally similar homologues and some of this family at least also tap into other cell membranes such as the outer nuclear membrane and the endoplasmic reticulum.

So are there other sources of death transducers, activating the caspase cascade because of injury to or signals arising in other parts of the cell than mitochondria? There are already examples that show that the answer is yes. Thus, the oncosuppressor protein p53 is activated following some types of DNA damage and can trigger apoptosis. One way – but only one of several – whereby this happens is through transcrip-

tional activation of BAX7. The second messenger ceramide, a product of membrane-linked acid sphingomyelinase activation, may act as a signal for plasma membrane damage[8]. And a powerful caspase-activating system is mediated by cytokine receptors of the tumor necrosis factor family, notably fas/apo-1/CD95, TNF receptor I, and others. These receptors, on receiving a death stimulus from binding their ligand, initiate a series of protein-protein interactions, building a complex (the death initiating signalling complex or DISC) which eventually recruits and activates caspase 8[9].

These mechanisms for coupling cell injury to apoptosis have mostly depended on activation of pre-formed proteins. Apoptosis can also be initiated (and forestalled) by transcriptional mechanisms, although rather little is known about most of them. An outstanding example is the Drosophila gene *reaper*, transcriptionally activated around two hours prior to developmental and injury-induced deaths in this organism. Drosophila apoptosis can occur without *reaper* transactivation, but requires very substantially enhanced stimuli, suggesting that *reaper* adjusts a threshold for apoptosis initiation[10]. Another gene whose transcription can initiate death is the familiar immediate early gene *c-myc*[11]. Transcriptional activation of *c-myc* initiates entry into DNA synthesis and is required for sustained re-entry in repeated cell cycles, but *c-myc* activation in the absence of concurrent cytokine support triggers apoptosis. This can also be interpreted as a "threshold regulatory" effect: – *c-myc* expression increases the cellular requirement for survival factors such as IGF-1.

Impressive confirmation of the significance of these pathways to apoptosis is available from study of transforming viruses. These are hardened survivors in the labyrinth of cell regulation, and have found keys to allow escape from cell death in a variety of ways. Thus the transforming papovavirus SV40, adenovirus type 12, Human Papilloma Virus type 16 and Epstein-Barr Virus all have proteins that inactivate apoptosis through inactivation of p53 or binding of BAX[12]. Even lytic viruses possess mechanisms to postpone death, such as the cowpox crmA serpin protein and the baculovirus p35 protein, which are caspase inhibitors.

So far so good: there are transcriptional and non-transcriptional pathways for activation of apoptosis, and they play through common effector events mediated by caspases and regulated by members of the BCL2 family. Underlying this simple scheme, however, is an extraordinary complexity. Thus, inactivation of fas signalling appears to neuter the ability of both *c-myc* and p53 to initiate apoptosis[13,14]. Maybe fas signalling is yet another example of "threshold regulation". New proteins have been discovered that are recruited to the DISC but appear to inhibit rather than activate death[15], some of them of viral origin. Many of the proteins mentioned above have alternative splice variants that have opposite effects. And we still have little idea of the relevance of intracellular location or of cell lineage to the activity of most of the apoptosis proteins. Susceptibility to apoptosis can be influenced by many other gene products, including oncoproteins such as RAS and ABL[16], but in some cases a single oncoprotein may either increase or decrease susceptibility depending on the context. Perhaps it is not surprising that a cellular function as important and irreversible as death should be subject to a huge range of coarse and fine controls. The reagents and protocols in this book should help unravel these.

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Cell Death–Apoptosis and Necrosis

1.1 Introduction

1.1.1 Terminology of cell death

Cell death can occur by either of two distinct[1, 2] mechanisms, necrosis or apoptosis. In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death.

Someone new to the field might ask, what's the difference between these terms? To clear up any possible confusion, we start with some basic definitions.

Necrosis and apoptosis
The two mechanisms of cell death may briefly be defined:

Necrosis ("accidental" cell death) is the pathological process which occurs when cells are exposed to a serious physical or chemical insult.

Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes.

Cytotoxicity
Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (cytotoxic T cell). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism.

For example, cell-mediated cytotoxicity (that is, cell death mediated by either cytotoxic T lymphocytes [CTL] or natural killer [NK] cells) combines some aspects of both necrosis and apoptosis[3, 4].

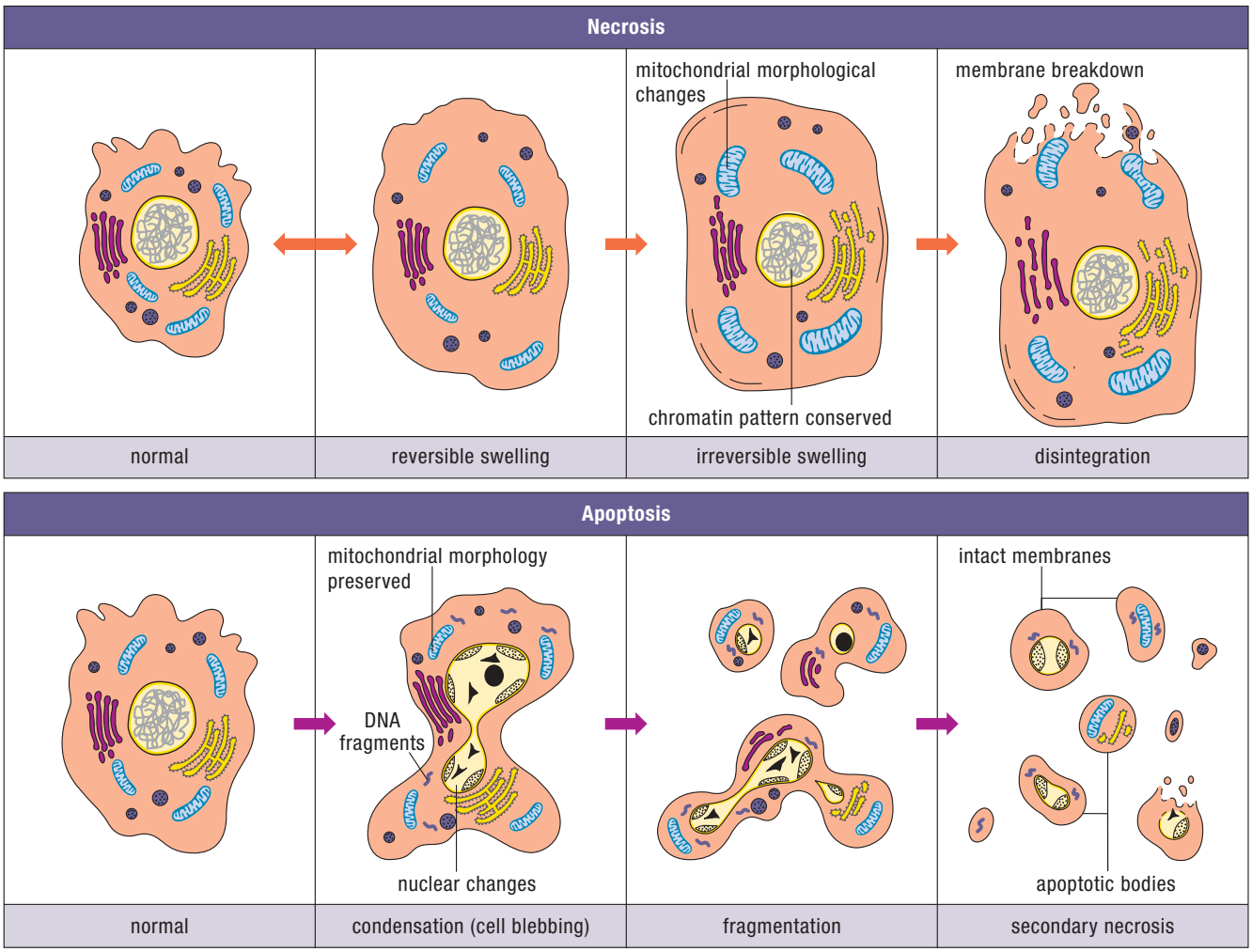


Figure 1: Illustration of the morphological features of necrosis and apoptosis.

1.1.2 Differences between necrosis and apoptosis

There are many observable morphological (Figure 1, Table 1) and biochemical differences (Table 1) between necrosis and apoptosis[2].

Necrosis occurs when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response[5].

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy.

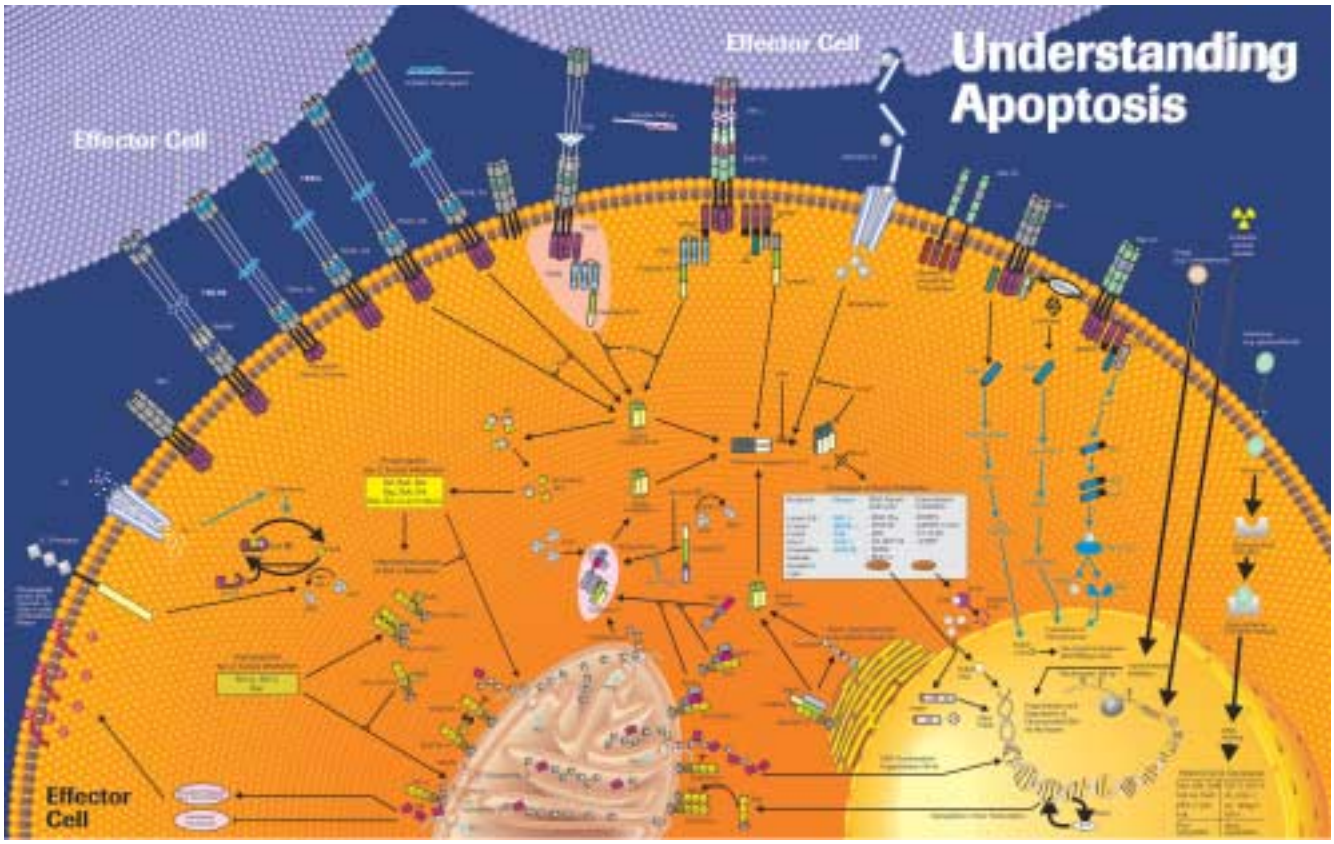
Cells undergoing apoptosis show characteristic morphological and biochemical features.[6] These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells.[7] Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis" (Figure 1).

Necrosis	Apoptosis
Morphological features	
● Loss of membrane integrity	● Membrane blebbing, but no loss of integrity ● Aggregation of chromatin at the nuclear membrane
● Begins with swelling of cytoplasm and mitochondria	● Begins with shrinking of cytoplasm and condensation of nucleus
● Ends with total cell lysis	● Ends with fragmentation of cell into smaller bodies
● No vesicle formation, complete lysis	● Formation of membrane bound vesicles (apoptotic bodies)
● Disintegration (swelling) of organelles	● Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family.
Biochemical features	
● Loss of regulation of ion homeostasis ● No energy requirement (passive process, also occurs at 4°C)	● Tightly regulated process involving activation and enzymatic steps ● Energy (ATP)-dependent (active process, does not occur at 4°C)
● Random digestion of DNA (smear of DNA after agarose gel electrophoresis)	● Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis)
● Postlytic DNA fragmentation (= late event of death)	● Prelytic DNA fragmentation ● Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria ● Activation of caspase cascade ● Alterations in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)
Physiological significance	
● Affects groups of contiguous cells ● Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemica, metabolic poisons) ● Phagocytosis by macrophages ● Significant inflammatory response	● Affects individual cells ● Induced by physiological stimuli (lack of growth factors, changes in hormonal environment) ● Phagocytosis by adjacent cells or macrophages ● No inflammatory response

▲ **Table 1:** Differential features and significance of necrosis and apoptosis.

1.1.3 Apoptotic Pathways

Scientists now recognize that most, if not all, physiological cell death occurs by apoptosis, and that alteration of apoptosis may result in a variety of malignant disorders. Consequently, in the last few years, interest in apoptosis has increased greatly. Great progress has been made in the understanding of the basic mechanisms of apoptosis and the gene products involved (Figure 2 below, Table 24, see Appendix, page 115).



▲ **Figure 2: Apoptotic pathways.** This apoptotic pathways chart represents a compendium of information on different cell lines, from various sources. As the dynamic field of apoptosis changes, the information shown here will likely change. Table 24 in the Appendix, page 115 contains a list of sources that can be consulted for more information about the items on this chart.

Key elements of the apoptotic pathway include:

Death receptors
Apoptosis has been found to be induced via the stimulation of several different cell surface receptors in association with caspase activation. For example, the CD95 (APO-1, Fas) receptor ligand system is a critical mediator of several physiological and pathophysiological processes, including homeostasis of the peripheral lymphoid compartment and CTL-mediated target cell killing. Upon cross-linking by ligand or agonist antibody, the Fas receptor initiates a signal transduction cascade which leads to caspase-dependent programmed cell death.

Membrane alterations
In the early stages of apoptosis, changes occur at the cell surface and plasma membrane. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell.

Protease cascade
Signals leading to the activation of a family of intracellular cysteine proteases, the caspases, (Cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. At least 11 different members of caspases in mammalian cells have been identified. Among the best-characterized cas-

pases is caspase-1 or ICE (Interleukin-1β-**C**onverting **E**nzyme), which was originally identified as a cysteine protease responsible for the processing of interleukin 1β.

Mitochondrial changes
Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of cytochrome C to the cytosol followed by subsequent depolarization of the inner mitochondrial membrane. Cytochrome C (Apaf-2) release further promotes caspase activation by binding to Apaf-1 and therefore activating Apaf-3 (caspase 9). AIF (apoptosis inducing factor), released in the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis.

DNA fragmentation
The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments.

Note: *For more information about the elements of the pathways as well as synonyms and abbreviations, please see Table 24 in the Appendix, page 115.*

1.2 Apoptosis Assay Methods

Originally, to study both forms of cell death, necrosis and apoptosis, cytotoxicity assays were used. These assays were principally of two types:

- Radioactive and non-radioactive assays that measure increases in plasma membrane permeability, since dying cells become leaky.
- Colorimetric assays that measure reduction in the metabolic activity of mitochondria; mitochondria in dead cells cannot metabolize dyes, while mitochondria in live cells can.

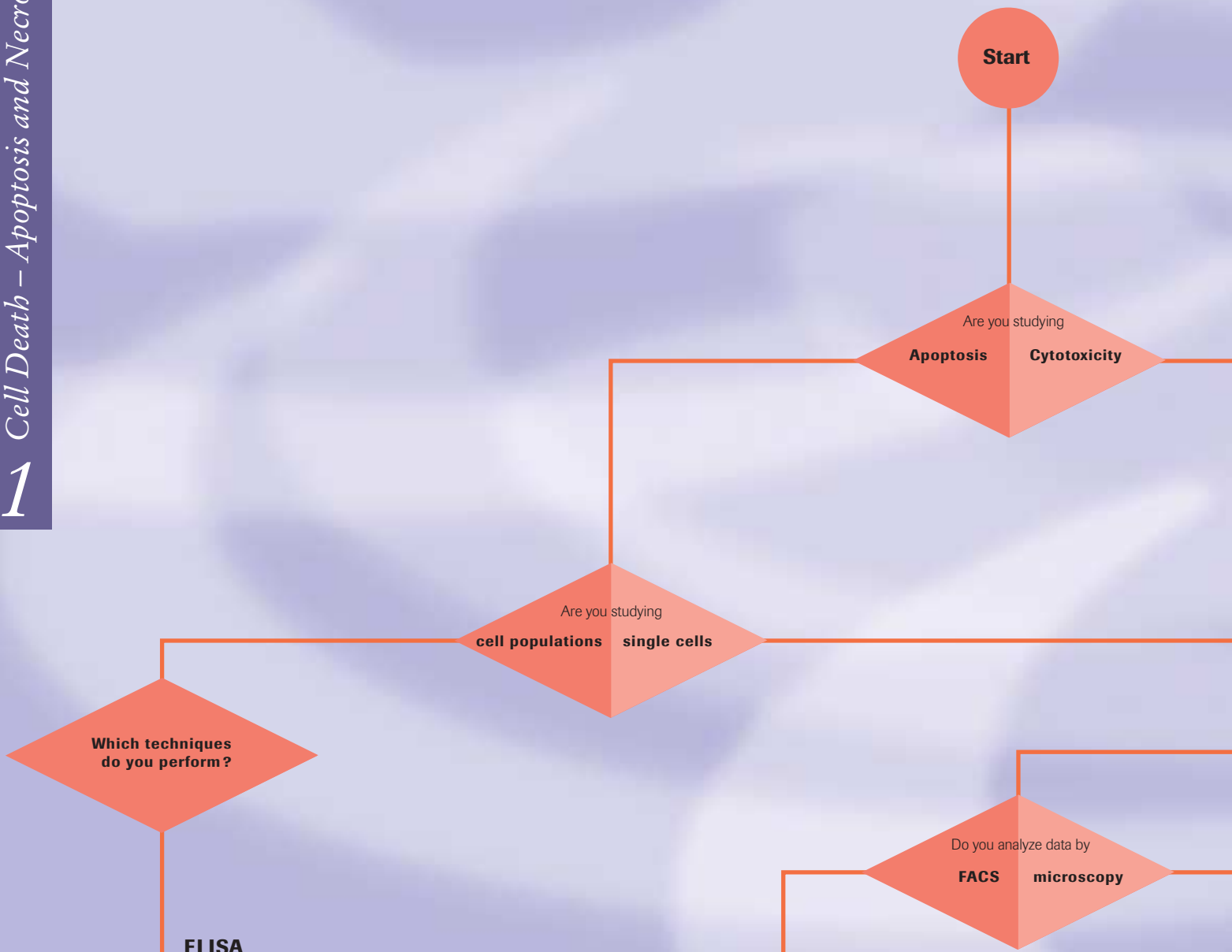
Note: *For a detailed discussion of both types of cytotoxicity assay, see Section 1.3, beginning on page 52 of this guide.*

However, as more information on apoptosis became available, researchers realized that both types of cytotoxicity assays vastly underestimated the extent and timing of apoptosis. For instance, early phases of apoptosis do not affect membrane permeability, nor do they alter mitochondrial activity. Although the cytotoxicity assays might be suitable for detecting the later stages of apoptosis, other assays were needed to detect the early events of apoptosis.

In concert with increased understanding of the physiological events that occur during apoptosis, a number of assay methods have been developed for its detection. For instance, these assays can measure one of the following apoptotic parameters:

- Fragmentation of DNA in populations of cells or in individual cells, in which apoptotic DNA breaks into different length pieces.
- Alterations in membrane asymmetry. Phosphatidylserine translocates from the cytoplasmic to the extracellular side of the cell membrane.
- Activation of apoptotic caspases. This family of proteases sets off a cascade of events that disable a multitude of cell functions.
- Release of cytochrome C and AIF into cytoplasm by mitochondria.

For practical reasons, we have divided this chapter into two broad categories: assays that measure apoptosis in cell populations (Section 1.2.1 of this guide) and assays that measure apoptosis in individual cells (Section 1.2.2 of this guide).
For discussions of particular assays, turn to the pages indicated in the method selection guide (Figure 3).



ELISA

DNA fragmentation, quantitative

- Cell Death Detection ELISA^{PLUS} 1 774 425

Fluorescence MTP Reader

Caspase activity, quantitative

- Caspase 3 Activity Assay 2 012 952
- Homogeneous Caspases Assay, fluorimetric 3 005 372
  2 236 869

Western blotting

ICE protease activity

- Anti-PARP 1 835 238

Gel electrophoresis

DNA fragmentation, qualitative

- Apoptotic DNA Ladder Kit 1 835 246

DNA fragmentation

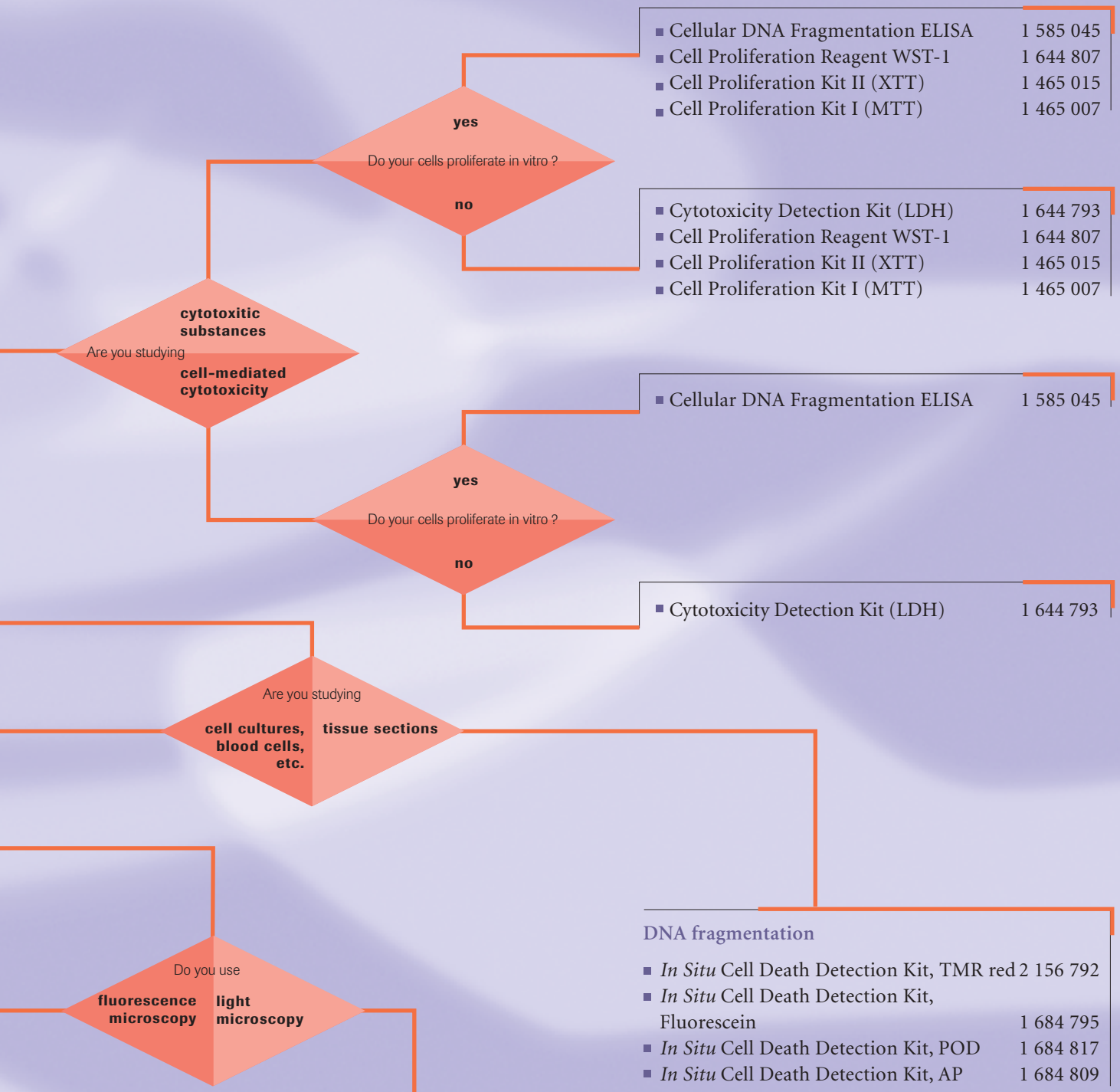
- *In Situ* Cell Death Detection Kit, TMR red 2 156 792
- *In Situ* Cell Death Detection Kit, Fluorescein 1 684 795

Membrane modification

- Annexin-V-Alexa 568 1 985 485
- Annexin-V-FLUOS 1 828 681
- Annexin-V-FLUOS Staining Kit 1 858 777
  1 988 549

Proteolytic activity

- M30 CytoDEATH, Fluorescein 2 156 857



- Cellular DNA Fragmentation ELISA 1 585 045
- Cell Proliferation Reagent WST-1 1 644 807
- Cell Proliferation Kit II (XTT) 1 465 015
- Cell Proliferation Kit I (MTT) 1 465 007

- Cytotoxicity Detection Kit (LDH) 1 644 793
- Cell Proliferation Reagent WST-1 1 644 807
- Cell Proliferation Kit II (XTT) 1 465 015
- Cell Proliferation Kit I (MTT) 1 465 007

- Cellular DNA Fragmentation ELISA 1 585 045

- Cytotoxicity Detection Kit (LDH) 1 644 793

DNA fragmentation

- *In Situ* Cell Death Detection Kit, TMR red 2 156 792
- *In Situ* Cell Death Detection Kit, Fluorescein 1 684 795
- *In Situ* Cell Death Detection Kit, POD 1 684 817
- *In Situ* Cell Death Detection Kit, AP 1 684 809

DNA fragmentation

- *In Situ* Cell Death Detection Kit, POD 1 684 817
- *In Situ* Cell Death Detection Kit, AP 1 684 809

Proteolytic activity

- M30 CytoDEATH 2 140 322 2 140 349
- M30 CytoDEATH, Fluorescein 2 156 857

Membrane modification

- Annexin-V-Biotin 1 828 690

Proteolytic activity

- M30 CytoDEATH 2 140 322

1.2.1 Methods for studying apoptosis in cell populations

A number of methods have now been developed to study apoptosis in cell populations. We focus on two key apoptotic events in the cell:

① Apoptosis and cell mediated cytotoxicity are characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. Because DNA cleavage is a hallmark for apoptosis, assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death. The DNA fragments may be assayed in either of two ways:

- As "ladders" (with the 180 bp multiples as "rungs" of the ladder) derived from populations of cells, e.g., with the Apoptotic DNA Ladder Kit (described on page 11 of this guide).
- By quantification of histone complexed DNA fragments with an ELISA (described on page 13 of this guide).

② Further, researchers discovered that proteases were involved in the early stages of apoptosis. The appearance of these caspases sets off a cascade of events that disable a multitude of cell functions. Caspase activation can be analyzed in different ways:

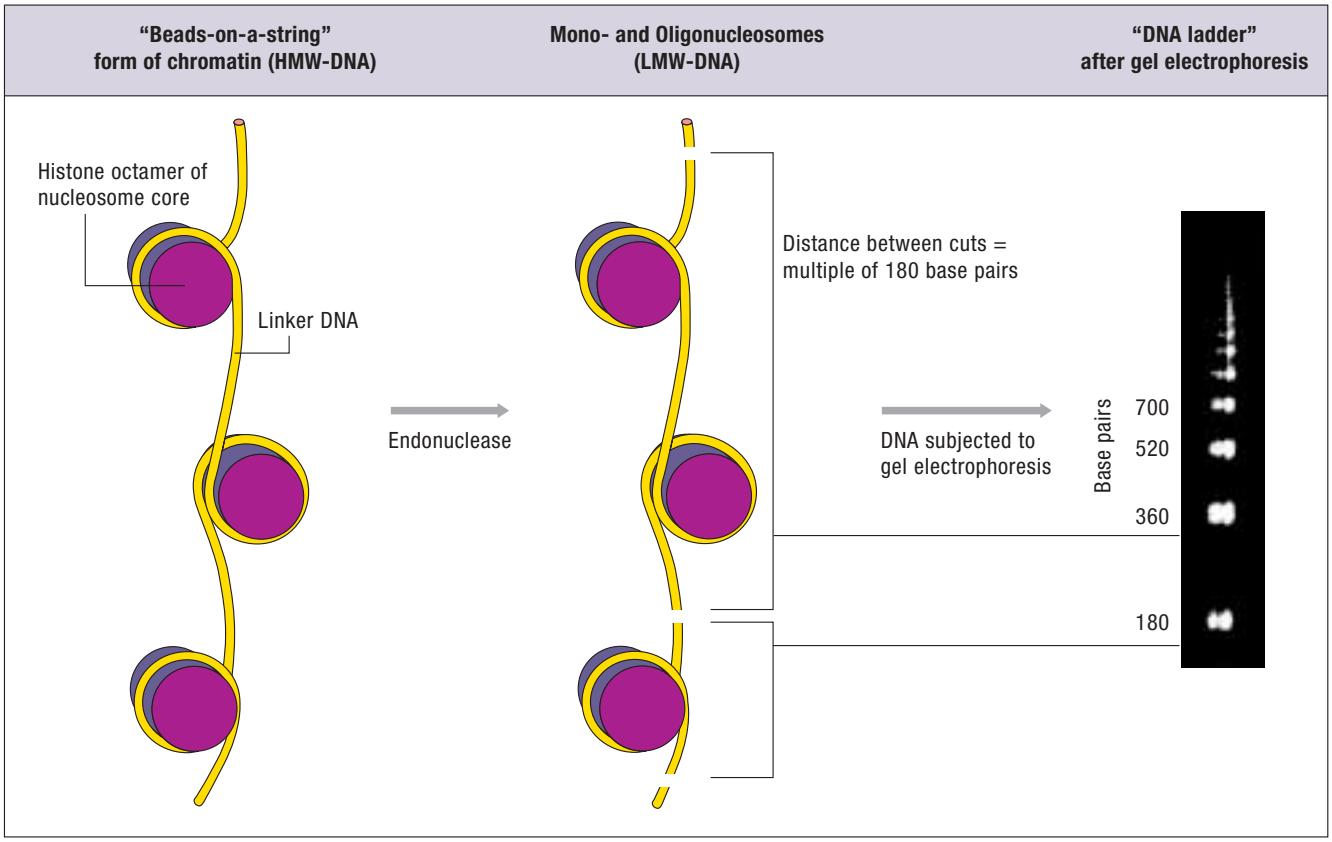
- By an *in vitro* enzyme assay. Activity of a specific caspase, for instance caspase 3, can be determined in cellular lysates by capturing of the caspase and measuring proteolytic cleavage of a suitable substrate (described on page 19 of this guide).
- By detection of cleavage of an *in vivo* caspase substrate. For instance caspase 3 is activated during early stages (as shown in Figure 2). Its substrate PARP (**P**oly-**A**DP-**R**ibose-**P**olymerase) and the cleaved fragments can be detected with the anti PARP antibody (described on page 24 of this guide).

If you're just starting out in the field, however, it may be difficult to decide how best to assay apoptosis in your system. Thus, in the following sections, we will describe details of each of these apoptosis assays.

1.2.1.1 Assays that measure DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die. In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments (Figure 4). These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit[8].

Radioactive as well as non-radioactive methods to detect and quantify DNA fragmentation in cell populations have been developed. In general, these methods are based on the detection and/or quantification of either low molecular weight (LMW) DNA which is increased in apoptotic cells or high molecular weight (HMW) DNA which is reduced in apoptotic cells (Figure 5). The underlying principle of these methods is that DNA, which has undergone extensive double-stranded fragmentation (LMW DNA) may easily be separated from very large, chromosomal length DNA (HMW DNA), e.g., by centrifugation and filtration.



▲ **Figure 4:** The biochemistry of DNA fragmentation and the appearance of the "DNA ladder".

For the quantification of DNA fragmentation, most methods involve a step in which the DNA of the cells has to be labeled: Prior to the addition of the cell death-inducing agent or of the effector cells, the (target) cells are incubated either with the [^{3}H]-thymidine ([^{3}H]-dT) isotope or the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU). During DNA synthesis (DNA replication) these modified nucleotides are incorporated into the genomic DNA. Subsequently, those labeled cells are incubated with cell death-inducing agents or effector cells and the labeled DNA is either fragmented or retained in the cell nucleus. Finally each type of DNA (HMW and LMW) is quantitated. Because the labeling of the cellular DNA has to be done prior to the induction of cell death, this labeling is also called "prelabeling".

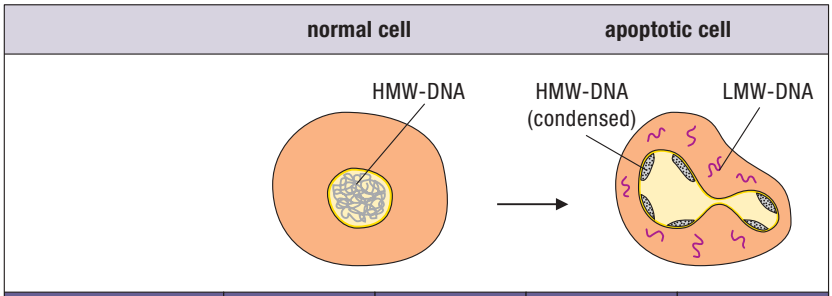
The prelabeling of one cell population (e.g., the target cells) allows the behavior of the labeled cells to be traced specifically when different cell populations are mixed.

Note: *Because cell-mediated cytotoxicity (CMT) proceeds, at least in part, by apoptotic mechanisms, the DNA fragmentation assay may also be used as a CMT assay.*

In a study of cell-mediated cytotoxicity the target cell population is labeled before the effector cells (e.g., CTL) are added. Subsequently, due to pore formation in the target cell plasma membrane, the fragmented LMW DNA is released from the cytoplasm of the target cell into the culture supernatant (Table 2). The cytotoxic potential of the effector cells is measured by quantification of the label released from the damaged target cells.

Because this metabolic prelabeling of the genomic DNA requires DNA synthesis, only cells proliferating *in vitro* (e.g., cell lines) may be labeled in this way; cells which do not proliferate *in vitro* (e.g., primary cell cultures, tumor cells *ex vivo*) do not replicate their DNA and therefore, do not incorporate labeled nucleotides (see also Section 1.3.2.1. "Cellular DNA Fragmentation ELISA" page 56).

To detect fragmented DNA in cells which do not replicate *in vitro*, the DNA has to be isolated and analyzed by agarose gel electrophoresis ("DNA ladder assay", Figure 6, see also Figure 4). Roche Molecular Biochemicals offers a kit, the Apoptotic DNA Ladder Kit, that simplifies this assay.



compartiment	HMW	LMW	HMW	LMW
Nucleus	+	–	↓	↑
Cytoplasm	–	–	–	↑

▲ **Figure 5:** Compartmentalization of HMW and LMW DNA in normal and apoptotic cells. (↓ = decreasing, ↑ = increasing)

	Apoptosis		Cell mediated cytotoxicity	
Compartment	HMW DNA	LMW DNA	HMW DNA	LMW DNA
Nucleus	+	+	+	+
Cytoplasm	–	+	–	+
Supernatant	–	–	–	+

▲ **Table 2:** Distribution of HMW and LMW DNA in cells undergoing apoptosis and target cells during cell mediated cytotoxicity.

Note: *In the early phases of apoptosis, no DNA is released into the supernatant (prelytic DNA fragmentation). However, in vitro, the apoptotic cells will lyse ("secondary necrosis"). Therefore, LMW DNA is found in the supernatant late in apoptosis.*

An alternative method which circumvents the isolation and electrophoretic analysis of DNA is the immunological detection of LMW DNA (histone-complexed DNA fragments) by an immunoassay (Cell Death Detection ELISA^{PLUS}, see page 13).

This non-radioactive immunoassay, offered by Roche Molecular Biochemicals can quantitate that hallmark of apoptosis. The Cell Death Detection ELISA^{PLUS} has been designed to quantify DNA fragmentation in cells which do not proliferate *in vitro* (since the kit requires no prelabeling of the cells). This kit measures the enrichment of histone-complexed DNA fragments (mono- and oligonucleosomes) in the cytoplasm of apoptotic cells.

Each of the methods to detect and measure apoptosis has its advantages and limitations. Because the cellular mechanisms that result in apoptosis are complex, most published methods cannot by themselves detect apoptosis unambiguously.

To ensure that the mode of cell death in the individual cell system or experiment is apoptotic, one also has to consider other criteria like the cellular morphology. Morphologic criteria for apoptotic cell death include, for example, chromatin condensation with aggregation along the nuclear envelope and plasma membrane blebbing followed by separation into small, apoptotic bodies. When internucleosomal DNA fragmentation is accompanied by these morphological features it provides an additional useful criterion to define cell death as apoptotic.

Apoptotic DNA Ladder Kit

Cat. No. 1 835 246 20 tests

Type	DNA purification kit
Useful for	Preparation of apoptotic DNA fragments for display on electrophoretic gels
Samples	Whole blood or cells in culture
Method	Cell lysis, followed by binding of cellular DNA on glass fiber, removal of impurities, and DNA recovery
Time	DNA preparation: < 20 min (after induction of apoptosis)

Significance of kit: This kit offers the easiest way to isolate apoptotic DNA fragments for DNA ladder analysis. The purification method outlined in the kit is much faster than other DNA purification methods (e.g., phenol/chloroform extraction, DNA precipitation). Purified DNA may be mixed directly with gel loading buffer and analyzed on an agarose gel.

Test principle: Apoptotic DNA binds quickly to glass fiber fleece in the presence of a chaotropic salt, guanidine hydrochloride (guanidine HCl). After cellular impurities are washed off the fleece, the DNA is released from the fleece with a low salt buffer. The procedure (see Flow Chart 1) involves:

1. Incubating an aliquot of apoptotic cells with an equal volume of binding/lysis buffer. After the incubation, the lysed sample is poured into a filter tube containing glass fiber fleece.
2. Using centrifugation to separate the DNA in the lysate (which binds to the glass fiber fleece) from unbound lysate components (which flow through the fleece into a collection tube).
3. Washing the bound DNA twice.
4. Eluting the purified DNA from the filter tube and collecting it by centrifugation.

Sample Preparation

Treat sample with apoptosis-inducing agent (1–24 h)

↓

Incubate treated sample with binding/lysis buffer (10 min, RT°C)

↓

Mix isopropanol with sample and pipette mixture into filter tube

↓

Centrifuge tube assembly (8000 rpm) and discard the flow-through (1 min, RT)

↓

Add wash buffer to the filter tube, then centrifuge as before (1 min, RT)

↓

Repeat the wash step, then add a final high speed spin (13,000 rpm) (1 min, then 10 sec, RT)

↓

Insert the filter tube into a 1.5 ml centrifuge tube, and add warm elution buffer to the filter tube

↓

Collect the eluted DNA by centrifugation (1 min, RT)

DNA Ladder Assay

Mix the eluted DNA sample with gel loading buffer

↓

Apply sample to a 1% agarose gel which contains ethidium bromide

↓

Run the gel in TBE (Tris-borate EDTA) buffer at 75 V (1.5 h, RT)

↓

Place the gel on a UV light box to visualize the DNA ladder pattern

▲ **Flow Chart 1:** Assay procedure, Apoptotic DNA Ladder Kit.

Sample size: 200–300 µl whole blood or cell suspension (for instance, 2 x 10^6 cells). The kit allows simultaneous processing of multiple samples.

Yield

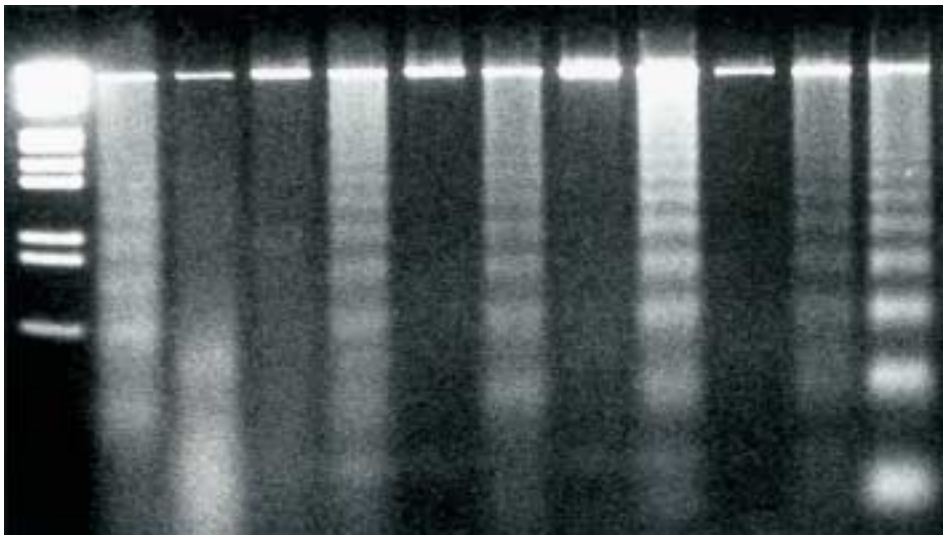
Sample	Sample volume	Yield of purified DNA
Whole blood (human)	200 µl	3–6 µg
Cultured cells (K562)	2 x 10^6 cells	10 µg

Specificity: Only nucleic acid will bind to the glass fiber filters under the conditions outlined in the kit. Salts, proteins, and other cellular components do not bind.

Kit contents

1. Nucleic acid binding/lysis buffer, ready-to-use
2. Washing buffer (ethanol to be added before use)
3. Elution buffer, ready-to-use
4. Glass fiber filter tubes, 700 µl capacity
5. Polypropylene collection tubes, 2 ml (for washes)
6. Positive control, apoptotic U937 cells, lyophilized

Typical results: see Figure 6.



▲ **Figure 6:** DNA ladder assayed with the Apoptotic DNA Ladder Kit
Lane Identification:
M = Size marker
– = Control cells without camptothecin
+ = Cells treated with camptothecin
C = Positive control from the kit

Cell Death Detection ELISA^{PLUS}

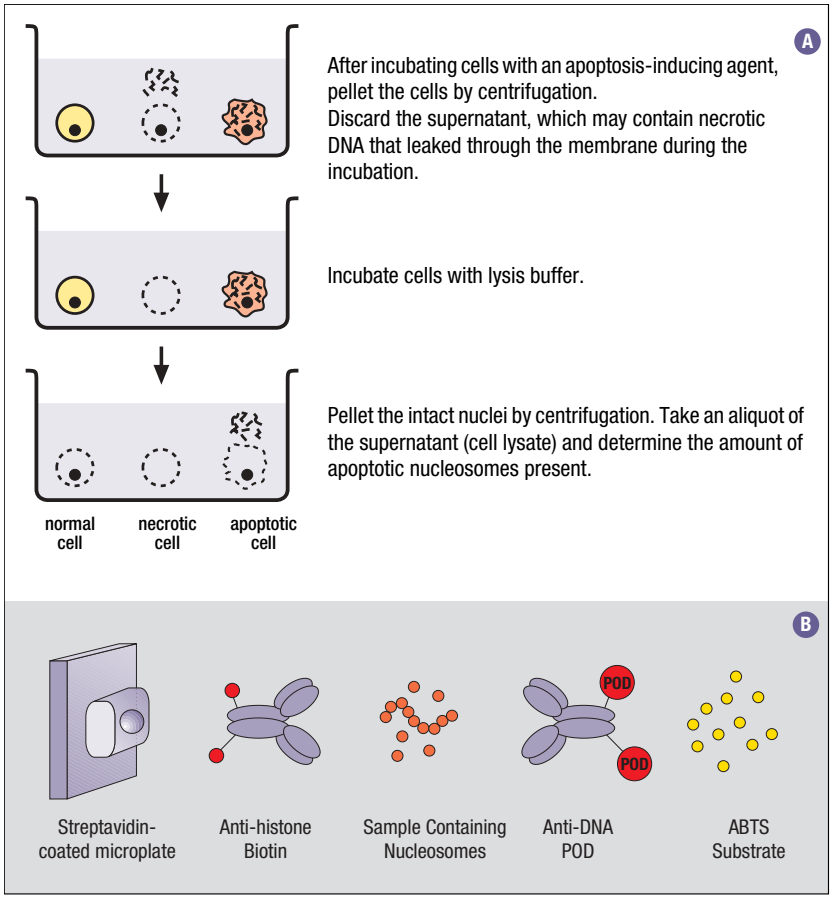
Cat. No. 1 774 425 96 tests
Cat. No. 1 920 685 10 x 96 tests

Type	One-step sandwich ELISA, colorimetric
Useful for	Quantitation of apoptosis without cell labeling; differentiating apoptosis from necrosis
Samples	Cell lysates, cell culture supernatants, serum, or plasma
Method	Cell lysis, followed by immunochemical determination of histone-complexed DNA fragments in a microplate well (*Note: For detection of necrosis, histone-complexed DNA fragments are detected directly in the culture supernatant, without cell lysis)*
Time	Approx. 3 h (after induction of apoptosis)

Significance of kit: This kit quantifies histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of cells after the induction of apoptosis or when released from necrotic cells. Since the assay does not require prelabeling of cells, it can detect internucleosomal degradation of genomic DNA during apoptosis even in cells that do not proliferate *in vitro* (for example, freshly isolated tumor cells). The antibodies used in the assay are not species-specific, so the kit may be used to assay cells from a wide variety of species (see "Other applications" in this article).

Test principle: The assay uses an one-step sandwich immunoassay to detect nucleosomes. The procedure (Figure 7 and Flow Chart 2) involves:

1. Incubating cells in a microplate well (for instance, 10^4 human cells in 200 µl culture) with an agent that induces cell death (for example, campothecin). After the incubation, the cells are pelleted by centrifugation and the supernatant is (containing DNA from necrotic cells that leaked through the membrane during incubation) discarded.
2. Resuspending and incubating cells in lysis buffer. After lysis, intact nuclei are pelleted by centrifugation.
3. Transferring an aliquot of the supernatant to a streptavidin-coated well of a microplate.
4. Binding nucleosomes in the supernatant with two monoclonal antibodies, anti-histone (biotin-labeled) and anti-DNA (peroxidase-conjugated). Antibody-nucleosome complexes are bound to the microplate by the streptavidin.
5. Washing the immobilized antibody-histone complexes three times to remove cell components that are not immunoreactive.
6. Incubating sample with peroxidase substrate (ABTS).
7. Determining the amount of colored product (and thus, of immobilized antibody-histone complexes) spectrophotometrically.



▲ **Figure 7:** How the Cell Death Detection ELISA^{PLUS} works.
Panel A: Sample preparation
Panel B: ELISA

Treat cells with apoptosis-inducing agent in the well of a microplate (1–24 h, 37°C)

Centrifuge microplate (200 x *g*) and remove supernatant (10 min, RT)

Incubate treated cells with lysis buffer (30 min, RT)

Repeat microplate centrifugation (200 x *g*) (10 min, RT)

Transfer aliquot of supernatant (lysate) to streptavidin-coated microplate

Incubate supernatant with immunoreagent (containing anti-histone and anti-DNA) (2 h, RT)

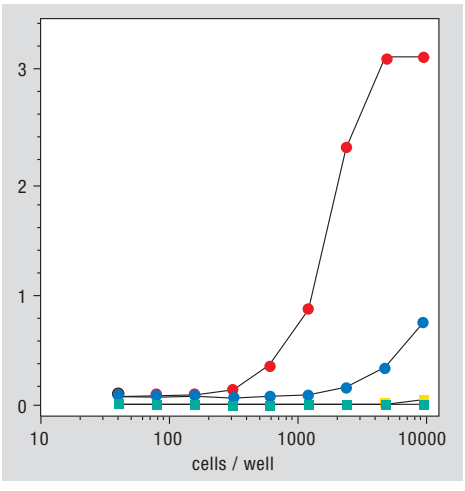
Wash microplate wells three times with incubation buffer at RT

Add substrate solution to wells and incubate (approx. 15 min, RT)

Measure absorbance at 405 nm

▲ **Flow Chart 2:** Assay procedure, Cell Death Detection ELISA^{PLUS}.

Sensitivity: In a model system, nucleosomes were detectable in as few as 600 camptothecin-induced U937 cells (Figure 8). However, the lower limit for detecting dying/dead cells in a particular sample varies with the kinetics of the apoptotic process, the cytotoxic agent used, and the number of affected cells in the total cell population.



▲ **Figure 8: Sensitivity of Cell Death Detection ELISA^{PLUS}.** Different cell concentrations of U937 cells were incubated with CAM (2 µg/ml) or without CAM for 4 h at 37°C. 20 µl of cell culture supernatant and cell lysates were analyzed in the ELISA. Substrate reaction time: 10 min. ● Lysate with CAM, ● Lysate without CAM, ■ Supernatant with CAM, ■ Supernatant without CAM
Result: The ELISA can clearly detect apoptosis-related nucleosomes in as few as 600 cells.

Specificity: The ELISA is specific for nucleosomes containing single- or double-stranded DNA (Figure 9). It is not species specific.

Can be used to assay:

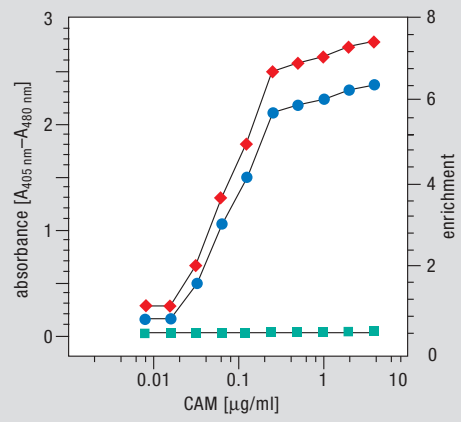
- Adherent cells
- Cells in suspension culture
- Cell culture supernatant
- Lysates of cells obtained *ex vivo*
- Serum, or plasma

Kit contents

1. Anti-histone antibody (clone H11-4), biotin-labeled
2. Anti-DNA antibody (clone M-CA-33), peroxidase-conjugated
3. DNA-histone complex (positive control)
4. Incubation buffer, ready-to-use
5. Lysis buffer, ready-to-use
6. Substrate buffer, ready-to-use
7. ABTS substrate tablets
8. Microplate modules (12 x 8 wells)
9. Adhesive plate cover

Typical results: see Figure 9

Other applications: For more examples of how the Cell Death Detection ELISA^{PLUS} can be used in the lab, see Appendix, page 121.



▲ **Figure 9: Dose-response experiment analyzed by the Cell Death Detection ELISA^{PLUS}.** U937 cells (10^4 cells/well, in 200 µl) were incubated with different concentrations of CAM for 4 h at 37°C. Before and after lysis, cells were centrifuged and a 20 µl aliquot of the supernatant was analyzed with the Cell Death Detection ELISA^{PLUS}. Results were plotted as dose vs. response. Substrate reaction time: 5 min. ♦ Lysate, ■ Supernatant, ● Enrichment factor of the lysate.
Result: Amounts of cytoplasmic oligonucleosomes (an indicator of apoptosis) increase as CAM concentration increases. Cell culture supernatants removed from the cells after treatment (but before lysis) gave no signal, indicating that there are no necrotic cells during the treatment.

1.2.1.2 Assays that measure apoptosis-induced proteases (caspases)

Several caspases (see Table 24, in the Appendix, page 115) are thought to mediate very early stages of apoptosis[10]. For instance, one of these, caspase 3 (CPP32) is required for the induction of apoptosis by certain effectors [especially tumor necrosis factor and the cytotoxic T cell ligand effector, CD95 (also called Fas)] Enari et al. *(1996)* , *Nature* **380**, 723–726.

These proteases cleave numerous substrates at the carboxy site of an aspartate residue. All are synthesized as pro-enzymes; activation involves cleavage at aspartate residues that could themselves be sites for the caspase family. As caspases are probably the most important effector molecules for triggering the biochemical events which lead to apoptotic cell death, assays for determination of caspase activation can detect apoptosis earlier than many other commonly used methods.

The most elucidatory assay for these caspases involves western blot detection of proteolytic cleavage products found in apoptotic cells. An antibody, Anti-PARP, sold by Roche Molecular Biochemicals, can be used in such an assay. The antibody can detect intact and cleaved forms of Poly-ADP-Ribose Polymerase, a target for some caspases.

For specific and quantitative measurement of caspase activity Western blotting is not suitable. To quantify caspase activation enzyme activity assays based on detection of cleaved caspase substrates have been developed recently. However most of the caspase substrates are not exclusively cleaved by a specific caspase but only preferentially, while other members of the caspases family act on these substrates to a lower extent. Roche Molecular Biochemicals offers a casapase 3 activity assay with highest specificity by the use of an immunosorbent enzyme assay principle.

M30 CytoDEATH*

Cat. No. 2 140 322 50 tests
Cat. No. 2 140 349 250 tests

M30 CytoDEATH, Fluorescein

Cat. No. 2 156 857 250 tests

Type	Monoclonal antibody, clone M30, IgG2b, mouse
Useful for	Detection of apoptosis in epithelial cells and tissues (formalin grade)
Samples	Adherent cells, tissue samples (routinely fixed and paraffin-embedded tissue sections, cryostat sections)
Method	Detect apoptosis by applying the M30-antibody to fixed samples, then using secondary detection systems. Suitable for immunohistochemistry, immunocytochemistry, and flow cytometry
Time	2 h for immunofluorescence on cells, 3.5h for staining of tissues (excluding dewaxing)

Background: During Apoptosis, vital intracellular proteins are cleaved. The proteases that mediate this process are called caspases (**C**ysteinyl-**asp**artic acid prote**ases**). Caspases are expressed as zymogenes, which are activated by different apoptosis inducers. Once activated, a single caspase activates a cascade of caspases.

Until recently cytokeratins, in particular cytokeratin 18, were not known to be affected by early events of apoptosis. Recently, it has been shown that the M30 antibody recognizes a specific caspase cleavage site within cytokeratin 18 that is not detectable in native CK18 of normal cells (Leers et al., in preparation). Consequently, the M30 CytoDEATH antibody is a unique tool for the easy and reliable determination of very early apoptotic events in single cells and tissue sections.

Significance of reagent: Use the M30 CytoDEATH antibody for the determination of early apoptotic events in cells and tissue sections by detection of a specific epitope of cytokeratin 18 that is presented after cleavage by caspases.

Test principle

for formalin-embedded tissue:
1. Dewax formalin-fixed, paraffin-embedded tissue sections.
2. Retrieve antigen by heating in citric acid buffer.
3. Add M30 antibody.
4. Add Anti-Mouse-Biotin.
5. Add Streptavidin-POD.
6. Add substrate solution (DAB or AEC).
7. Counterstain with Harries hematoxilin.
8. Analyze under a light microscope.

for immunofluorescence on cells:
1. Fix cells.
2. Add M30 antibody.
3. Add Anti-Mouse-Ig-Fluorescein.
4. Analyze under a fluorescence microscope.

Specificity: The M30 CytoDEATH antibody binds to a caspase-cleaved, formalin-resistant epitope of the cytokeratin 18 (CK 18) cytoskeletal protein.

The immunoreactivity of the M30 CytoDEATH antibody confined to the cytoplasma of apoptotic cells.

Antibody supplied as: Mouse monoclonal antibody (clone M30), lyophilized, stabilized. Formalin grade.

Typical results: see Figures 10–12.

Staining procedure for immunohistochemistry and flow cytometry (FCM)

Wash cells with PBS

↓

Fix cells in ice-cold pure methanol (–20°C) for 30 min

↓

Wash cells twice with PBS/BSA

↓

Incubate with M30 working solution (for 30 min, at RT)

↓

Wash cells twice with PBS

↓

Incubate with Anti-Mouse-Ig-Fluorescein (for 30 min, at RT)

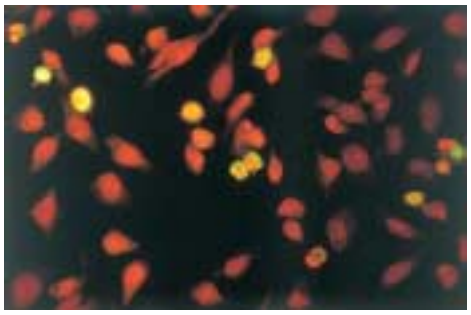
↓

Wash cells twice with PBS

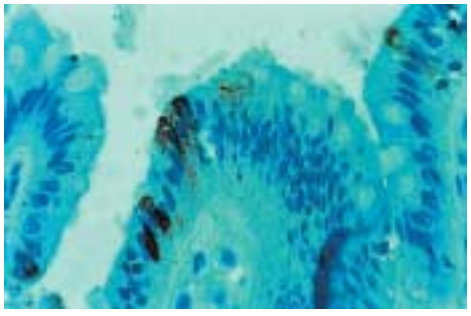
↓

Flow cytometry, dilute cells in PBS, and store the samples in the dark until analysis.

* The M30 CytoDEATH antibody is made under a license agreement form BEKI AB/BEKI Diagnostics AB, Sweden.



▲ **Figure 10: Detection of apoptosis in HeLa cells, treated with TNF and actinomycin D, using M30 CytoDEATH.** Secondary detection with Anti-Mouse-Fluorescein and propidium iodide.



▲ **Figure 11: Detection of apoptosis in human colon using M30 CytoDEATH (blue filter).** Secondary detection with Anti-Mouse-Biotin, Streptavidin-POD and AEC as substrate, counterstained with hematoxilin.

Staining procedure for immunohistochemistry

Incubate paraffin-embedded sections (over night) at 37°C

↓

Dewax formalin-fixed, paraffin-embedded tissue sections: 2x xylol, 2x ethanol (96%), 1x ethanol (70%), 1x methanol/H_2O_2 (3%) (10 min, RT). Rinse for 10 min in demineralized water

Antigen retrieval:

Prepare 500 ml of 10 mM citric acid buffer. Incubate in a microwave oven at 750 W until boiling. Place slides into the heated citric acid solution. Incubate once more at 750 W. When solution is boiling, turn setting of microwave oven to "keep warm" (about 100 W). Incubate for 15 min. Cool the slides down (for 5 min, at RT)

↓

Rinse three times in PBS; incubate 2 min in a separate jar of PBS

↓

Block with PBS + 1% BSA (for 10 min, at RT)

↓

Remove blocking solution. Add M30 working solution (1 h, RT)

↓

Wash slides three times in PBS

↓

Cover with Anti-Mouse-Biotin (for 30 min, at RT)

↓

Wash slides three times in PBS

↓

Cover with Streptavidin-POD (for 30 min, at RT)

↓

Wash slides three times in PBS

↓

Incubate slides in a freshly prepared substrate solution (DAB or AEC at RT) until a clearly visible color develops

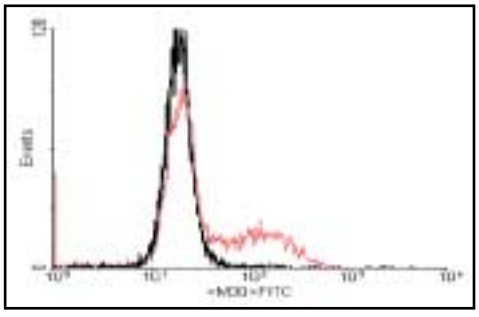
↓

Counterstain with hematoxilin, and mount the section

↓

Analyze by a light microscope

▲ **Flow Chart 3:** Assay procedure, M30 CytoDEATH, immunohistochemistry and cytometry.



▲ **Figure 12: Detection of apoptosis in HeLa cells, using M30 CytoDEATH.** Secondary detection with Anti-Mouse-Fluorescein. Blue: untreated control cells. Red: Cells treated with TNF and actinomycin D.

Caspase 3 Activity Assay

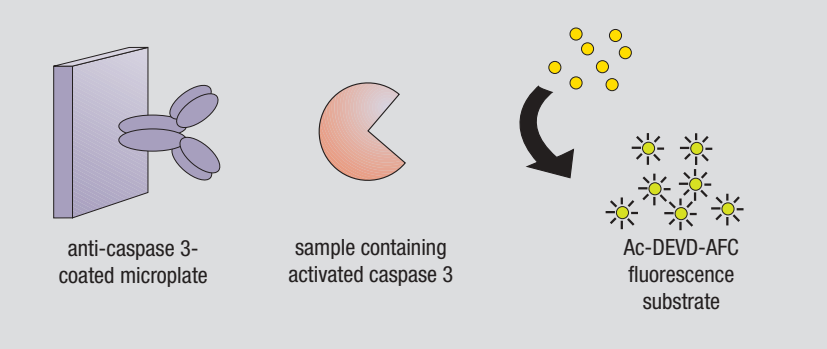
Cat. No. 2 012 952 96 tests

Type	Immunosorbent enzyme assay, fluorometric
Useful for	Specific, quantitative *in vitro* determination of caspase 3 activity
Samples	Cell lysates, recombinant caspase 3 (CPP32)
Method	Cell lysis, followed by capturing of caspase 3 by a specific antibody and fluorometric determination of proteolytic cleavage of the substrate
Time	Approx. 5 h (after induction of apoptosis)

Significance of kit: This kit allows specific, quantitative detection of caspase 3 activity in cellular lysates after induction of apoptosis. Caspase 3 activation seems to play a key role in initiation of cellular events during the apoptotic process. The immunosorbent enzyme assay principle of this kit guarantees high specificity without cross-reactions with other known caspases. The fluorochrome generated by proteolytic cleavage of the caspase substrate is proportional to the concentration of activated caspase 3 in the lysates.

Test principle: The assay uses a fluorometric immunosorbent enzyme assay (FIENA) principle. The procedure (Figure 13 and Flow Chart 4) involves:

1. Inducing apoptosis in cells by desired method (for instance 2 x 10^6 cells). After the induction, the cells are washed and pelleted by centrifugation.
2. Preparing samples by resuspending and incubating cells in lysis buffer. After lysis and following centrifugation, samples can be removed for direct analysis or storage.
3. Coating microplate with anti-caspase 3 solution and blocking of unspecific binding.
4. Transferring a sample to the anti-caspase 3-coated well of a microplate and capturing of caspase 3.
5. Washing the immobilized antibody-caspase 3 complexes three times to remove cell components that are not immunoreactive.
6. Incubating sample with caspase substrate (Ac-DEVD-AFC) that is proteolytically cleaved into free fluorescent AFC.
7. Measuring generated AFC fluorometrically.



▲ **Figure 13:** How the Caspase 3 Activity Assay works.

Sample preparation

Treat sample (2 x 10^6 cells) with apoptosis-inducing agent. Include a negative control without induction. (1–24 h)

Wash treated and control cells with ice-cold PBS and centrifuge (300 x *g*) (5 min, RT)

Incubate cell pellet in lysis buffer (1 min, on ice)

Centrifuge at maximum speed in a tabletop centrifuge (1 min/RT)

Remove 100 µl sample for direct analysis or storage for 1 week at –20°C

Coating microplate

Incubate microplate with anti-caspase 3 coating solution (either at 37°C for 1h or at 4°C over night)

Block unspecific binding by incubation with blocking buffer (30 min, RT)

Remove blocking solution and wash 3 times with incubation buffer (3 x 1 min, RT)

Assaying protease activity

Add sample (100 µl lysate, positive control) into microplate well (1h, 37°C)

Remove sample and wash 3 times with incubation buffer (3 x 1 min, RT)

Add freshly prepared substrate solution (1–3 h, 37°C)

Measure fluorometrically (excitation filter 400 (370–425) nm and emission filter 505 (490–530) nm)

Optional: for calibration, set up a calibration curve with different dilutions of AFC as standard

▲ **Flow Chart 4:** Assay procedure, Caspase 3 Activity Assay.

Sensitivity: In a model system, caspase 3 activity was clearly detectable in lysates of 10^6 cells with 5 % apoptotic cells (Figure 14). However, the lower limit for determination of caspase 3 activity in cellular lysates of dying cells in a particular sample varies with the kinetics of the apoptotic process, the apoptotic agent used, and the number of affected cells within the total cell population.

Specificity: This fluorometric immunosorbent enzyme assay is highly specific for caspase 3 by the use of an anti-caspase 3-specific monoclonal capture antibody in combination with a specific caspase substrate. Enzyme activity of natural and recombinant human caspase 3 is detected by this assay. Cross-reactions with other caspases are not known.

Can be used to assay:

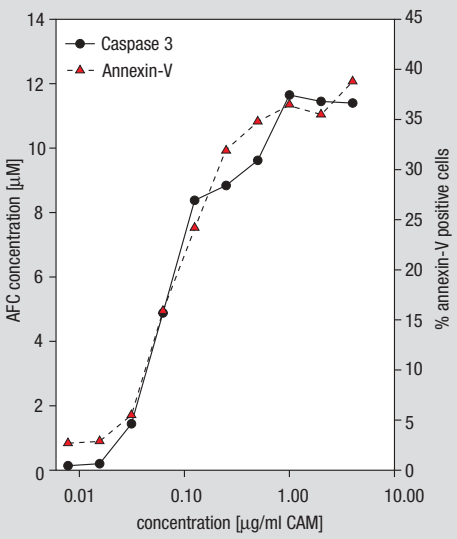
- Lysates of adherent cells, of cells in suspension culture, of cells obtained *ex vivo* or recombinant caspase 3.

Kit contents

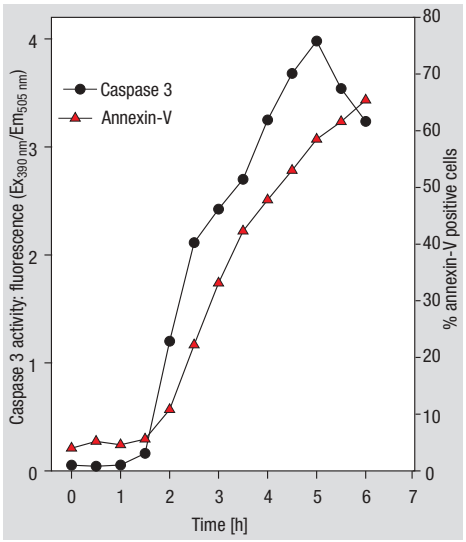
1. Coating buffer, 10x
2. Anti-caspase-3, 20x
3. Blocking buffer, ready-to-use
4. Incubation buffer, 5x
5. DTT, 100x
6. Substrate solution Ac-DEVD-AFC, 20x
7. AFC
8. Positive control, apoptotic U937 cell lysate
9. Microplate modules (12 x 8 wells)
10. Adhesive plate cover

Typical results: see Figures 14–15

The caspase 3 activity assay has been used to detect caspase 3 activation in U937 cells exposed to different concentrations of the apoptosis inducing agent camptothecin (Figure 14, dose response curve). In this model system, the induction of apoptotis in only 5% of U937 cells is sufficient for detection of caspase 3 activation. Caspase 3 activity/fluorochrome development is proportional to the percentage of apoptotic cells.



▲ **Figure 14: Dose-response experiment analyzed by the caspase 3 Activity Assay.** U937 cells were exposed to different concentrations of camptothecin for 4 h at 37°C. Lysates were analyzed for caspase 3 activity and standardized values are plotted versus concentration. Additionally, an aliquot of the same cells was analyzed for annexin-V binding.



▲ **Figure 15: Kinetic study of caspase 3 activation by camptothecin exposure in U937 cells.** U937 cells were exposed to 4 µg/ml camptothecin for different time intervals at 37°C. Lysates were analyzed for caspase 3 activity and fluorescence (minus fluorescence of blank) is plotted versus time. Additionally, an aliqot of the same cells was analyzed for annexin-V binding in parallel.

Homogeneous Caspases Assay

Cat. No. 3 005 372 100 assays (96 well)
Cat. No. 2 236 869 1000 assays (96 well)

Type	One step assay, fluorimetric
Useful for	Specific, quantitative *in vitro* determination of caspases in microplates
Samples	Cell cultures, recombinant caspases
Method	Cell lysis, followed by detection of caspases activity (fluorimeric determination of proteolytic cleavage of the substrate)
Time	Approx. 2 h (after induction of apoptosis)

Significance of kit: The Homogeneous Caspases Assay is a fluorimetric assay for the quantitative *in vitro* determination of caspases activity in microplates, which makes it especially useful for high throughput screening. Apoptotic cells are incubated with DEVD-Rhodamine 110 for 1–24 h. Upon cleavage of the substrate by activated caspases, fluorescence of the released Rhodamine 110 is measured.

Test principle: The kit can be used for the quantification of activated caspases of human as well as animal origin, or screening for caspase inhibitors. It is one step assay, including the cell lysis step.

Specificity: Specifically detects caspases 2, 3 and 7, caspases 6, 8, 9 and 10 to a lesser extent

Can be used to assay: Cell cultures, recombinant caspases

Kit contents
1. Substrate stock solution, 10x
2. Positive control, 10x
3. Rhodamine 110, standard
4. Incubation buffer

Typical results: see Figures 16–17

Dispense double concentrated apoptosis inducing agent into microplate. Include negative control (diluent only). Volume should be 50 µl (96 well plate) or 12.5 µl (384 well plate).

↓

Onto prediluted apoptosis inducing agents, seed cells (4×10^4 per well, volume 50 µl on 96 well plate or 10^4 cells per well, volume 12.5 µl on 384 well plate) and incubate for desired interval for induction of apoptosis.

↓

Add 100 µl (96-wells) or 25 µl (384-well) substrate working solution, freshly prepared. Cover the microplate with a lid and incubate more than 1 h at 37 °C.

↓

Measure with an excitation filter 470–500 nm and emission filter 500–560 nm (maxima λ_{ex} = 499 nm and λ_{em} = 521 nm)

▲ **Flow Chart 5:** Assay procedure, Homogeneous Caspase Assay.

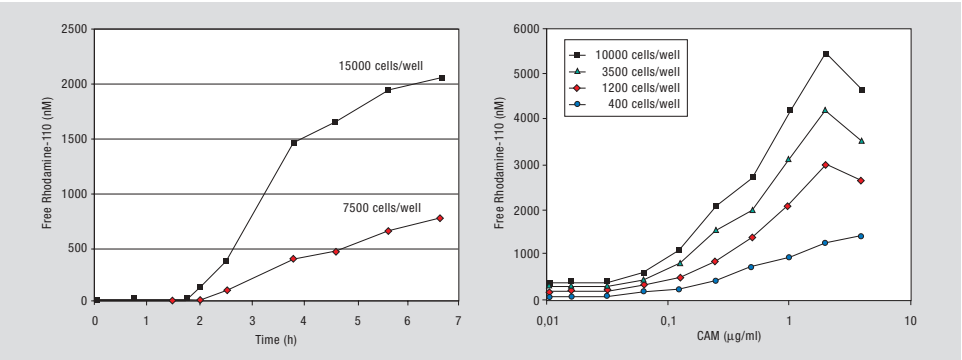


Figure 16: Kinetics of caspase activation in U937 cells by camptothecin (384-well plate). U937 cells were exposed to 4 µg/ml camptothecin for different time intervals at 37 °C, analyzed for caspase activity with the Homogeneous Caspases Assay and fluorescence plotted versus time.

Figure 17: Dose-response curve of U937 cells exposed to different concentrations of camptothecin (384-well plate). U937 cells were exposed to camtothecin for 4 h at 37 °C, analyzed for caspase activity with the Homogeneous Caspases Assay and standardized values plotted versus concentration.

Anti-PARP

Cat. No. 1 835 238 100 µl (50 blots)

Type Polyclonal antiserum, from rabbit

Useful for Detection on Western blots of PARP cleaved by caspases during early stages of apoptosis

Samples Crude cell extracts

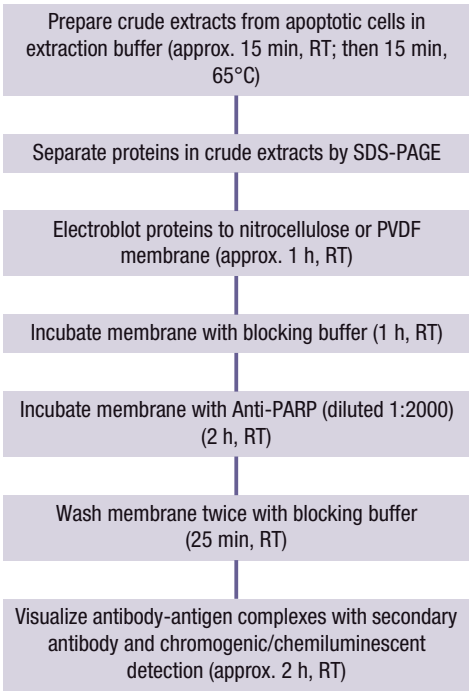
Method Western blot of apoptotic cell extracts, followed by indirect immunodetection of PARP cleavage fragment

Time Approx. 5.5 h (immunodetection only)

Significance of reagent: Anti-PARP recognizes Poly-ADP-Ribose-Polymerase (PARP), a 113 kD protein that binds specifically at DNA strand breaks. PARP is also a substrate for certain caspases (for example, caspase 3 and 7) activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 89 kD and 24 kD. Detection of the 89 kD PARP fragment with Anti-PARP thus serves as an early marker of apoptosis.

Test principle: The Anti-PARP antibody may be used to detect the 89 kD PARP fragment (and intact PARP) from apoptotic cell extracts on a Western blot. The procedure (Flow Chart 6) involves:

1. Preparing crude extracts of apoptotic cells (for instance, by sonication and incubation of 10^5–10^7 cells in the presence of urea, 2-mercaptoethanol, and SDS).
2. Separating proteins in the crude cell extracts on an SDS-polyacrylamide gel.
3. Transferring the separated proteins to a membrane by electroblotting.
4. Detecting PARP fragments (and intact PARP) on the membrane with the Anti-PARP antibody.
5. Visualizing the antibody-protein complexes with an enzyme-conjugated anti-rabbit IgG secondary antibody and a chromogenic or chemiluminescent enzyme substrate (see Table 3).



▲ **Flow Chart 6:** Assay of caspase activity with Anti-PARP.

Antibody supplied as: Polyclonal antiserum from rabbit, stabilized.

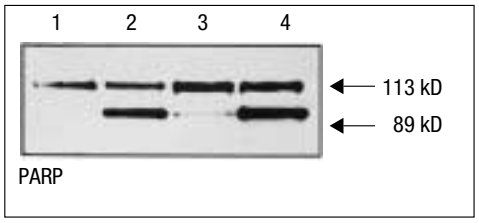
Sensitivity: PARP cleavage fragments from 3 x 10^5 apoptotic cells could be detected on a Western blot (Figure 18).

Specificity: On Western blots, Anti-PARP recognizes intact PARP from primates or rodents, as well as the large PARP fragment generated by caspases. Anti-PARP will immunoprecipitate intact PARP from primates or rodents.

Can be used to assay:

- Crude cell extracts

Typical results: see Figure 18.



▲ **Figure 18: Detection of cleaved PARP in cell extracts of apoptotic CEM T cells.** CEM T cells were incubated with one of three apoptosis-inducing drugs. Cell extracts from 3 x 10^5 treated or untreated cells were fractionated on an 10% polyacrylamide gel in the presence of SDS. After electrophoresis, proteins on the gel were transferred to a PVDF membrane by electroblotting and the blot was blocked with 5% powdered milk. The blocked membrane was incubated with a 1:3000 dilution of Anti-PARP. Subsequent incubations with a peroxidase-conjugated anti-rabbit secondary antibody and a peroxidase substrate revealed the presence of PARP cleavage products on the blot. Note that the antibody recognizes both uncleaved PARP (113 kD) and the larger cleavage fragment (89 kD).
Lane 1: Untreated control cells
Lane 2: Cells treated with 100 ng/ml doxorubicin for 24 h
Lane 3: Cells treated with 1 mg/ml methotrexate for 24 h
Lane 4: Cells treated with 1 mg/ml cytarabin for 24 h.
(Data is courtesy of Dr. Ingrid Herr, German Cancer Research Institute, department of molecular oncology, Heidelberg, Germany)

Product	Cat. No.	Pack Size
BM Chromogenic Western Blotting Kit (Mouse/Rabbit)	1 647 644	for 2000 cm^2 membrane
BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)	1 520 709	for 2000 cm^2 membrane
BM Chemiluminescence Blotting Substrate (POD)	1 500 708 1 500 694	for 1000 cm^2 membrane for 4000 cm^2 membrane
CSPD (chemiluminescent AP substrate), ready-to-use	1 755 633	2 x 50 ml
CDP *Star* (chemiluminescent AP substrate) ready-to-use	1 685 627 1 759 051	1 ml 2 x 1 ml
BM Blue POD Substrate, precipitating	1 442 066	100 ml
BM Purple AP Substrate, precipitating	1 442 074	100 ml

▲ **Table 3:** Related products for visualization of Anti-PARP.

Apoptosis Assay Methods
Summary of methods for studying apoptosis in cell populations

1 Cell Death – Apoptosis and Necrosis

1.2.1.3 Summary of methods for studying apoptosis in cell populations.

Method/ Roche Molecular Biochemicals product	Parameter analyzed	Label	Assay principle	Advantages	Limitations	For product information, see
DNA Fragmentation Assay, radioactive[11, 12]	DNA fragmentation (LMW and HMW DNA)	[^{3}H]-TdR or [^{125}I]- UdR, prelabel	● DNA fragments are released from the cytoplasm of apoptotic cells after lysis with non-ionic detergent. ● The LMW DNA is separated from nuclear HMW DNA by centrifugation. ● The radioactivity in the supernatant and in the pellet is determined by LSC.	● Quantitative measurement over a large range (several orders of magnitude) ● Sensitive (10^3–10^4 cells/test required) ● Suitable for analysis of cell-mediated (cytotoxicity) effects	● Radioactive isotope ● Requires prelabeling and extensive washing of the target cells ● Limited to target cells proliferating *in vitro* ● Increased background due to free [^{3}H]-TdR in the cytoplasm	
DNA Fragmentation Assay, non-radioactive[13] **Cellular DNA Fragmentation ELISA**	DNA fragmentation (LMW DNA)	BrdU, prelabel	● DNA fragments are released from the cytoplasm of apoptotic cells after lysis with a non-ionic detergent. ● The LMW DNA is separated from nuclear HMW DNA by centrifugation. ● The supernatant is analyzed by ELISA.	● Sensitive (10^3–10^4 cells/test required) ● Labeled cells do not have to be washed ● Optimal for microtiter plate format ● Non-radioactive ● Suitable for analysis of cell-mediated (cytotoxicity) effects	● Prelabeling of the target cells required ● Can only assay target cells proliferating *in vitro* ● Narrow range of quantitative measurement (only one order of magnitude)	page 56 of this guide
JAM Test[14]	DNA fragmentation (HMW DNA)	[^{3}H]-TdR, prelabel	● Cells are harvested by vacuum aspiration onto glass fiber filters. While LMW-DNA is washed through the filters, the HMW DNA is retained on these filters. ● The radioactivity retained on the filters is measured by LSC.	● Sensitive (10^3–10^4 cells/test required) ● Only 1 washing step required for the labeled cells ● Low spontaneous release: cytotoxic events causing low cell lysis over prolonged period of time (8–24 h) can be studied ● Optimal for microtiter plate format	● Radioactive isotope ● Prelabeling of the target cells required ● Limited to target cells proliferating *in vitro* ● In apoptotic cells, DNA is only partially lost: viable and damaged cells are separated by only a narrow range of assay values	
Alkaline Elution Analysis[15]	DNA fragmentation (LMW and HMW-DNA)	[^{3}H]-TdR, prelabel	● Cells are loaded onto polycarbonate filters. ● The filters are incubated with three different buffer solutions containing SDS, pH 10, SDS + Proteinase K, pH 7, or SDS, pH 12.3. ● The radioactivity in each fraction (LMW DNA) as well as the radioactivity retained on the filter (HMW DNA) is quantified by LSC.	● Differential elution allows the detection of strand breaks in DNA, DNA-interstrand crosslinks and DNA-protein crosslinks	● Radioactive isotope ● Prelabeling and washing of the target cells required ● Limited to target cells proliferating *in vitro* ● Insensitive (10^6 cells/test required) ● Labor-intensive and time-consuming: only a few tests may be performed simultaneously	
DNA Ladder Assay[16] **(LMW and HMW DNA by size)** **Apoptotic DNA Ladder Kit**	DNA fragmentation	none	● Cellular DNA is isolated by extraction and quickly purified. ● Purified total DNA (LMW and HMW DNA) is analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.	● Hallmark of apoptosis: demonstration of the mono- and oligonucleosomal DNA fragments (180 bp multimers) ● No prelabeling of the cells required: not limited to cells which proliferate *in vitro* ● Non-radioactive	● No quantitative measurement ● Insensitive: More than 10^6 cells/test required ● Labor-intensive and time-consuming: only a few tests may be performed simultaneously	page 11 of this guide
Nucleosome Quantification ELISA[13] **Cell Death Detection ELISA^{PLUS}**	DNA fragmentation (LMW DNA in association with histones)	none	● Histone complexed DNA-fragments (mono- and oligonucleosomes, LMW DNA) are released from the cytoplasm of apoptotic cells after lysis. ● The LMW DNA is separated from nuclear HMW DNA by centrifugation. ● The supernatant is analyzed by ELISA.	● Sensitive (10^2–10^4 cells/test required) ● No prelabeling of the cells required: not restricted to cells which proliferate *in vitro* ● Non-radioactive ● Detection of DNA and histones in one immunoassay demonstrates mono- and oligonucleosomal DNA fragments	● Samples have to be analyzed immediately because storage reduces ELISA signal ● Not recommended for tissue homogenates. Increased background could occur due to activation of nucleases during sample preparation.	page 13 of this guide
DNA Ladder Assay, radioactive[17]	DNA fragmentation (LMW and HMW by size)	γ-[^{32}P]-ATP, postlabel	● Cellular DNA is isolated by extraction and quickly purified. ● Purified total DNA (LMW and HMW DNA) is labeled at the 5'end with γ-[^{32}P]-ATP by T4 Polynucleotide Kinase. ● [^{32}P]-labeled DNA is separated by agarose gel electrophoresis and quantitated in the dried gel by a blot analyzer.	● Definitive marker of apoptosis: demonstration of the mono- and oligonucleosomal DNA fragments (180 bp multimers) ● No prelabeling of the cells required: not limited to cells which proliferate *in vitro* ● Highly sensitive (1000 x more sensitive than ethidium bromide): allows earlier detection of DNA fragmentation after induction of apoptosis	● Labor-intensive and time-consuming: only a few tests may be performed simultaneously ● Radioactive assay (^{32}P) ● End-labeling of purified DNA required	
Protease Activity Assay **Caspase 3 Acitivity Assay**	Activation of caspases (Caspase 3)	none	● Apoptotic process including activation of the caspase cascade is induced in cells by desired method. ● Cells are lysed and cell extracts are prepared. ● Activated caspase 3 is captured out of cellular lysates by an Anti-caspase 3 antibody ● Quantification of fluorochromes cleaved from a caspase specific substrate.	● Quantitative assay, cleavage of substrate is proportional to concentration of activated caspase 3 in samples ● Detection of very early stages of apoptosis ● Highly specific for caspase 3, no cross reactions with other members of the caspase family	● High cell numbers needed ● Fluorescence reader, equipped with special fluorescence filters needed	page 19 of this guide
Protease Activity Assay **Anti-PARP**	Discrete cleavage of DNA repair enzyme (PARP)	none	● Cells are treated with an apoptosis-inducing agent, which leads to induction of caspase 3 and the cleavage of Poly-ADP-Ribose-Polymerase (PARP). ● Cell extracts are prepared with SDS, fractionated by SDS-PAGE, and transferred to a PVDF membrane by western blotting. ● Blot is probed with an antibody to PARP, then with a peroxidase-labeled secondary antibody. ● Cleavage products of PARP (about 85 kD) on the membrane are revealed after an incubation with a peroxidase substrate.	● Flexible, can be used with many different types of cells ● No prelabeling of cells required: not limited to cells which proliferate *in vitro* ● Non-radioactive ● Marker for very early stage of apoptosis	● Insensitive (requires 10^5–10^6 cells/test) ● Labor-intensive and time-consuming: only a few tests may be performed simultaneously	page 24 of this guide

▲ **Table 4:** Methods for studying apoptosis in cell populations.

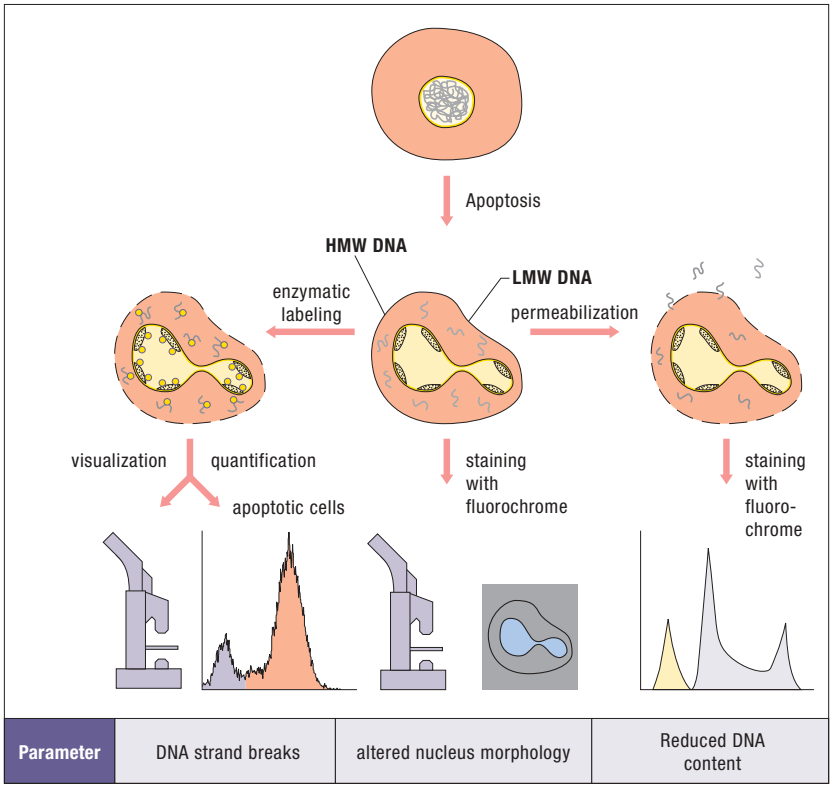
1.2.2 Methods for studying apoptosis in individual cells

A number of methods have now been developed to study apoptosis in individual cells. In the following sections, we will describe details of several of these apoptosis assays.

We focus on two key apoptotic events in the cell:

① DNA fragmentation used to study death in cell populations may also be used to study death in individual cells. As described in Section 1.2.1.1, DNA cleavage is a hallmark for apoptosis, and assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death.

The methods used to assess DNA strand breaks are based on labeling/staining the cellular DNA. The labeled/stained DNA is subsequently analyzed by flow cytometry, fluorescence microscopy or light microscopy (Figure 19).



▲ **Figure 19:** Schematic illustration of the two basic principles for detecting DNA fragmentation in single cells.

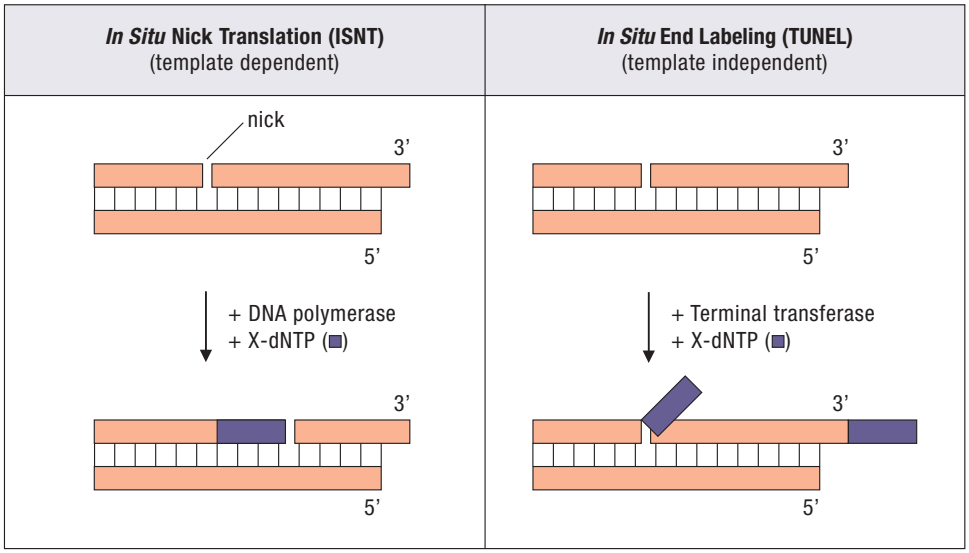
In general, two different labeling methods may be used to identify DNA in apoptotic cells:

- **Enzymatic labeling:** Cellular DNA is labeled with modified nucleotides (e.g., biotin-dUTP, DIG-dUTP, fluorescein-dUTP) using exogenous enzymes (e.g., terminal transferase, DNA polymerase). This labeling detects extensive DNA strand breaks. Enzymatic labeling is discussed in detail below (section 1.2.2.1 of this guide).
- **Staining with fluorochromes:** Cellular DNA is stained with fluorescent DNA-binding dyes (DNA-fluorochromes) capable of intercalating into DNA. Upon binding to DNA these dyes become highly fluorescent. Apoptotic cells are binding less dye molecules, since they characteristically lose DNA during the staining process (described in section 1.2.2.3 of this guide).

② In addition, individual cell death may be studied by assays that measure alterations in plasma membranes (alterations in the asymmetry or permeability of individual cell membranes, which occur as the membrane shrinks and becomes increasingly convoluted.) For instance, during apoptosis, phosphatidylserine translocates from the cytoplasmic side of the membrane to the extracellular side and can be detected with Annexin V (described in section 1.2.2.2 of this guide).

1.2.2.1 The TUNEL enzymatic labeling assay

Extensive DNA degradation is a characteristic event which often occurs in the early 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 20).



◀ **Figure 20:** Schematic illustration of two enzymatic DNA labeling methods nick translation and end labeling.

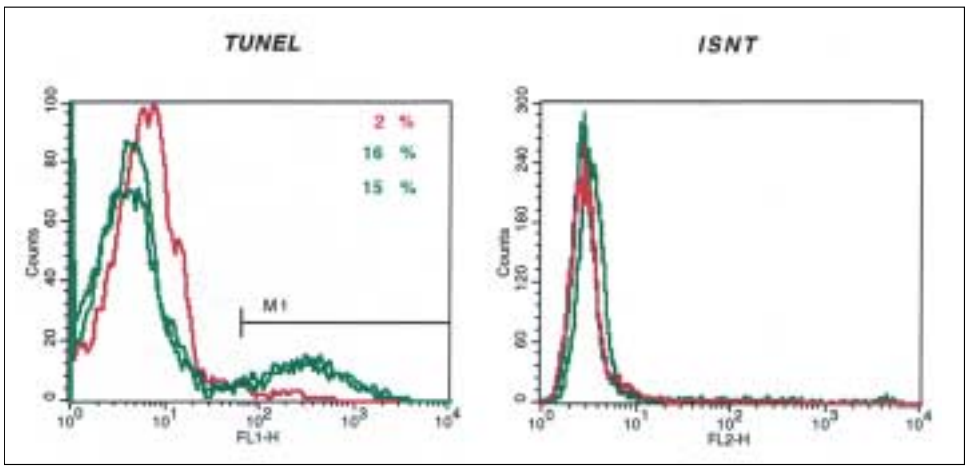
DNA polymerase I catalyzes the template dependent addition of nucleotides when one strand of a double-stranded DNA molecule is nicked. Theoretically, this reaction (*In Situ* Nick Translation, ISNT) should detect not only apoptotic DNA, but also the random fragmentation of DNA by multiple endonucleases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

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. In addition, in early stages 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 necrosis[19].

Note: *For a comparison of results with the TUNEL and ISNT methods, see Figure 21.*

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 cross-links LMW DNA to other cellular constituents and precludes its extraction during the permeabilization step.



▼ **Figure 21: 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 nucleopeptide protein 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 cytometer[20]. Direct labeling with fluorescein-dUTP offers several other advantages. Direct labeling produces less nonspecific background with sensitivity equal to indirect labeling (Figure 22) 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 specific[21].

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 Molecular Biochemicals offers three kits for the detection of DNA strand breaks that occur during cell death. Each is described on the following pages.

Note: *For technical tips on the TUNEL method, see page 105 of the Appendix.*

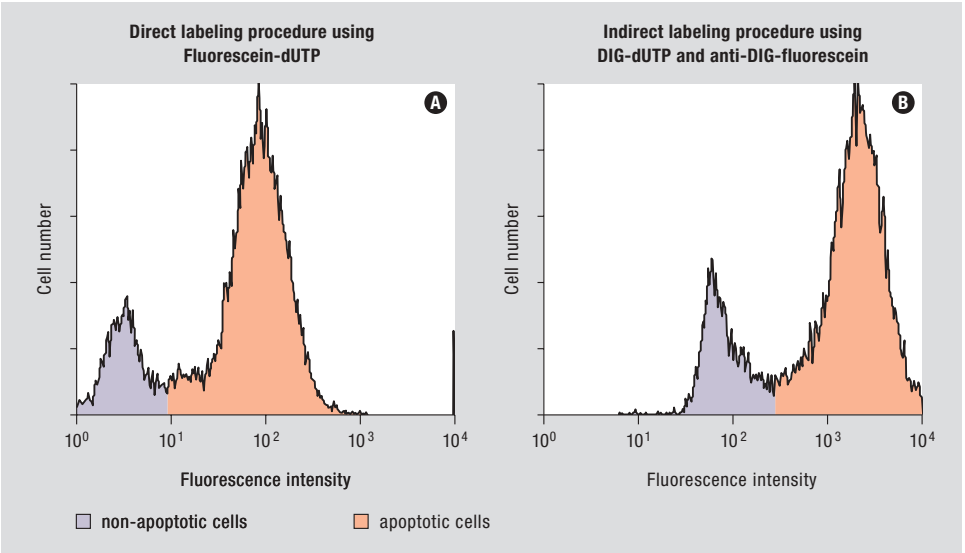


Figure 22: Comparison of direct and indirect labeling of DNA strand breaks in apoptotic cells. PBL were incubated with 1 µM dexamethasone for 24 h at 37°C and then labeled by TUNEL. Recordings were made at the same photomultiplier settings. (Data were kindly provided by R. Sgonc, University of Innsbruck, Austria). **Result:** Direct labeling is as sensitive as indirect labeling, but produces less non-specific background. ▶

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

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

In Situ Cell Death Detection Kit, Fluorescein

Cat. No. 1 684 795 50 tests

In Situ Cell Death Detecion Kit, TMR red

Cat. No. 2 156 792 50 tests

Type	Direct TUNEL labeling assay
Useful for	Detection of DNA strand breaks in apoptotic cells by flow cytometry or fluorescence microscopy
Samples	Cells in suspension, adherent cells, cell smears, frozen or paraffin-embedded tissue sections
Method	End-labeling of DNA with fluorescein-dUTP, followed by direct analysis of fluorescent cells
Time	1–2 h (+ sample preparation, permeabilization, etc.)

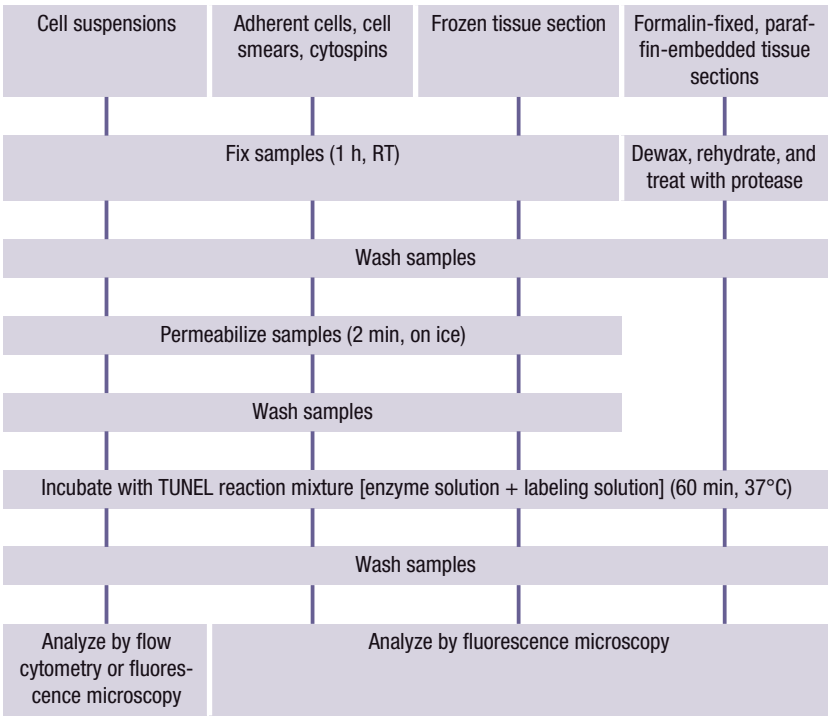
Significance of kit: The *In Situ* Cell Death Detection Kit, Fluorescein measures and quantitates cell death (apoptosis) by labeling and detection of DNA strand breaks in individual cells by flow cytometry or fluorescence microscopy. The kit offers a direct TUNEL detection method, for maximum sensitivity and minimal background.

Test principle: The assay uses an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP. The procedure involves:

1. Fixing and permeabilizing apoptotic cells.
2. Incubating the cells with the TUNEL reaction mixture containing TdT and fluorescein-dUTP. During this incubation step, TdT catalyzes the attachment of fluorescein-dUTP to free 3'OH ends in the DNA.
3. Visualizing the incorporated fluorescein with a flow cytometer and/or a fluorescence microscope.

For a detailed overview of the steps in the procedure, see Flow Chart 7.

Sensitivity: The enzymatic labeling allows the detection of an apoptotic event that occurs, prior to changes in morphology and even before DNA fragments become detectable in the cytoplasm[22].



▲ **Flow Chart 7:** Assay procedure, *In Situ* Cell Death Detection Kit, Fluorescein, TMR red.

Specificity: The amount of DNA strand breaks in apoptotic cells is so large that the degree of cell labeling in these assays is an adequate discriminator between apoptotic and necrotic cells[19].

Can be used to assay:

- Cells in suspension (permanent cell lines, normal and tumor cells *ex vivo*)
- Cytospins, cell smears
- Adherent cells cultured on chamber slides
- Frozen tissue sections
- Formalin-fixed, paraffin-embedded tissue sections

Kit contents

1. Enzyme solution (TdT), 5 tubes
2. Labeling solution (nucleotide mix), 5 tubes

Typical results: See Figures 23–24a.

Technical tips: For more information on the use of the kit for flow cytometric analysis, see page 111 in the Appendix of this guide.

Other applications: For more examples of how the *In Situ* Cell Death Detection Kit can be used in the lab, see Appendix, page 122.

▲ **Figure 24: Detection of apoptotic cells (green) by fluorescence microscopy in a tissue section from rat.** A tissue section from a rat spinal cord was prepared and assayed with the *In Situ* Cell Death Detection Kit, Fluorescein. The treated section was viewed under a fluorescence microscope. (Photomicrograph was kindly provided by R. Gold, University of Würzburg, Germany.) **Result:** A subpopulation of apoptotic cells, scattered throughout the tissue section, are intensely stained (green) by the TUNEL treatment and are easily visible under the microscope.

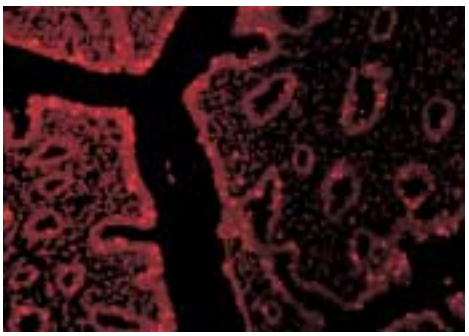
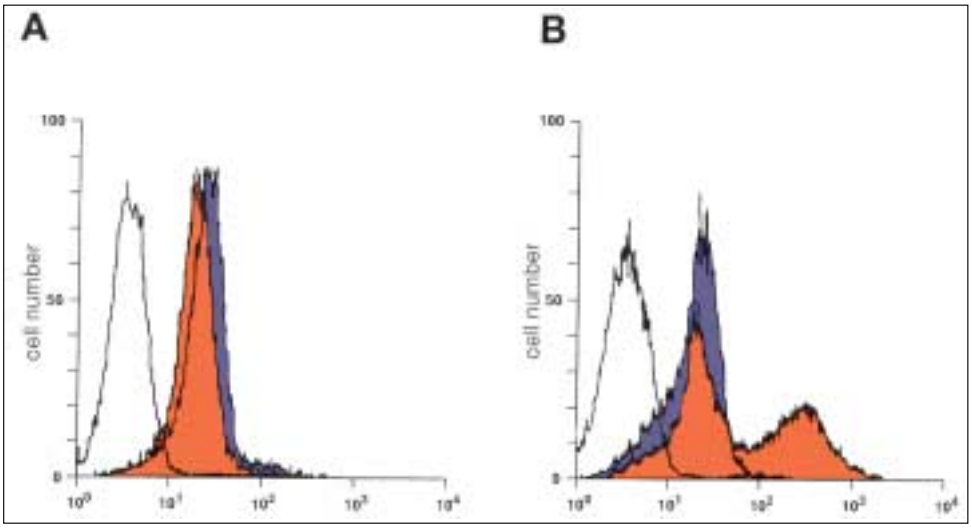


Figure 24a: Rabbit endometrium, stained with the *In Situ* Cell Death Detection Kit, TMR red



▲ **Figure 23: Detection of apoptotic cells by flow cytometry using the *In Situ* Cell Death Detection Kit, Fluorescein.** HL60 cells were cultured in the absence (A) or presence (B) of 2 µg/ml Camptothecin for 3 h at 37°C. Cells were incubated either with TUNEL reaction mixture (■) or label solution (□) or PBS for autofluorescence (■).

In Situ Cell Death Detection Kit, AP

Cat. No. 1 684 809 50 tests

In Situ Cell Death Detection Kit, POD

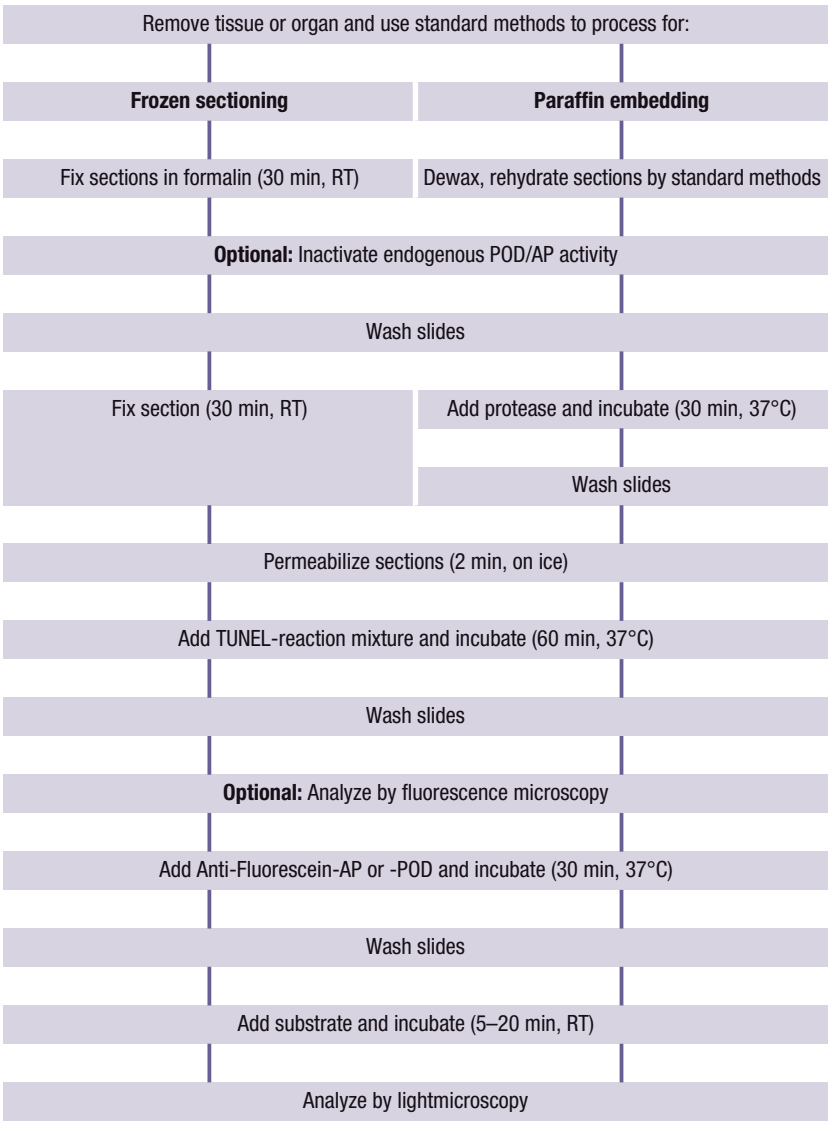
Cat. No. 1 684 817 50 tests

Type	Indirect TUNEL labeling assay
Useful for	Detection of DNA strand breaks in apoptotic cells under a light microscope
Samples	Cell smears, adherent cells, cytospins, frozen or fixed tissue sections
Method	End-labeling of DNA with fluorescein-dUTP, followed by detection of incorporated fluorescein with an antibody and visualization of the antibody
Time	Approx. 3 h (+ sample preparation, permeabilization, etc.)

Significance of kits: These two *In Situ* Cell Death Detection Kits measure cell death (apoptosis) by detecting DNA strand breaks in individual cells by light microscopy. The AP and POD kits offer an indirect TUNEL detection method, which is a fast, sensitive, and specific light microscopic assay.

Test principle: The assay uses an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP. The procedure involves:

1. Fixing and permeabilizing apoptotic cells or tissue sections.
2. Incubating the cells with the TUNEL reaction mixture containing TdT and fluorescein-dUTP. During this incubation step, TdT catalyzes the attachment of fluorescein-dUTP to free 3'OH ends in the DNA.
3. Detecting the incorporated fluorescein with an anti-fluorescein antibody AP conjugate (*In Situ* Cell Death Detection Kit, AP) or an anti-fluorescein antibody POD conjugate (*In Situ* Cell Death Detection Kit, POD).
4. Visualizing the immunocomplexed AP or POD with a substrate reaction.



▲ **Flow Chart 8:** Assay procedure *In Situ* Cell Death Detection Kits (AP or POD).

Sensitivity: The enzymatic labeling of DNA strand breaks allows the detection of an early apoptotic event. This is especially important if apoptosis is studied *in vivo*, e.g. in tissue sections, since apoptotic cells are rapidly and efficiently removed *in vivo*.

Specificity: The amount of DNA strand breaks in apoptotic cells is so large that the degree of cell labeling in these assays is an adequate discriminator between apoptotic and necrotic cells.

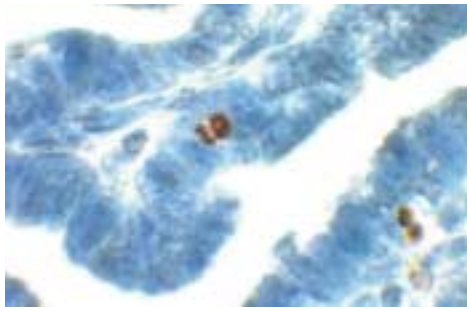
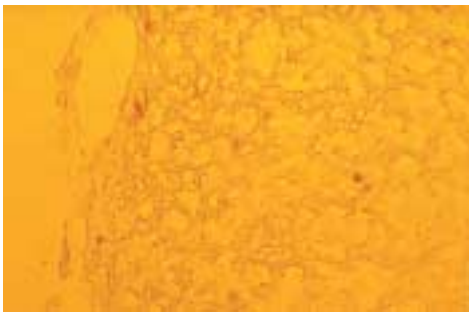


Figure 25: Detection of apoptotic cells by TUNEL and peroxidase staining in rabbit endometrium. A tissue section from rabbit endometrium was prepared and assayed with the *In Situ* Cell Death Detection Kit, POD. Slide was counterstained with hematoxylin and viewed under a light microscope.
Result: A subpopulation of apoptotic cells, scattered throughout the tissue section, are intensely stained (brown) by the TUNEL treatment and subsequent peroxidase immunostaining. ▶



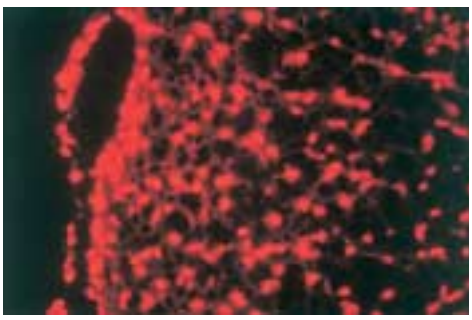


Figure 26: Detection of apoptotic cells by TUNEL and alkaline phosphatase staining in rat spinal cord. A tissue section from rat spinal cord was prepared and assayed with the *In Situ* Cell Death Detection Kit, AP. The slide was viewed under a light microscope (Panel A). After viewing, the same slide was stained with propidium iodide and viewed by fluorescence microscopy (Panel B).
Result: A few apoptotic cells (red) are clearly visible after TUNEL treatment and subsequent alkaline phosphatase immunostaining (Panel A). However, the apoptotic cells are not visible in the same slide after staining with propidium iodide (Panel B). ▶

Can be used to assay:

- Cells smears, adherent cells
- Cytospins
- Tissue sections (frozen or paraffin-embedded).

Kit contents

***In Situ* Cell Death Detection Kit, AP**

1. Enzyme solution (TdT), 5 tubes
2. Labeling solution (nucleotide mix), 5 tubes
3. Anti-Fluorescein-AP conjugate, ready to use

***In Situ* Cell Death Detection Kit, POD**

1. Enzyme solution (TdT), 5 tubes
2. Labeling solution (nucleotide mix), 5 tubes
3. Anti-Fluorescein-POD conjugate, ready to use

Note: *For added flexibility and convenience, the components of these kits, as well as several AP and POD precipitating substrates are also available as single reagents (Table 8).*

Typical results: see Figures 25–26.

Technical tips: For more information on the use of the kits for light microscopic analysis, see page 107 in the Appendix, of this guide.

Other applications: For more examples of how the *In Situ* Cell Death Detection Kits can be used in the lab, see Appendix, page 122 .

For your convenience, we offer a number of additional single reagents to optimize your TUNEL reaction (Table 6).

Product	Cat. No.	Pack Size
TUNEL Label Mix	1 767 291	3 x 550 µl (30 tests)
TUNEL Enzyme	1 767 305	2 x 50 µl (20 tests)
TUNEL POD (Anti-Fluorescein, POD conjugate)	1 772 465	3.5 ml (70 tests)
TUNEL AP (Anti-Fluorescein, AP conjugate)	1 772 457	3.5 ml (70 tests)
TUNEL Dilution Buffer	1 966 006	2 x 10 ml
DAB Substrate, metal enhanced, precipitating (POD substrate)	1 718 096	1 pack
NBT/BCIP Stock Solution (AP substrate)	1 681 451	8 ml
Fast Red Tablets (AP substrate)	1 496 549	20 tablets

▲ **Table 6:** Single reagents available for the TUNEL technique.

1.2.2.2 Assays that measure membrane alterations

In contrast to necrosis, apoptosis occurs without inflammation. In the end stages of apoptosis, apoptotic bodies are engulfed by macrophages and other phagocytic cells[2] *in vivo*. Thus, apoptotic cells are removed from the population without spilling their contents and eliciting an inflammatory response.

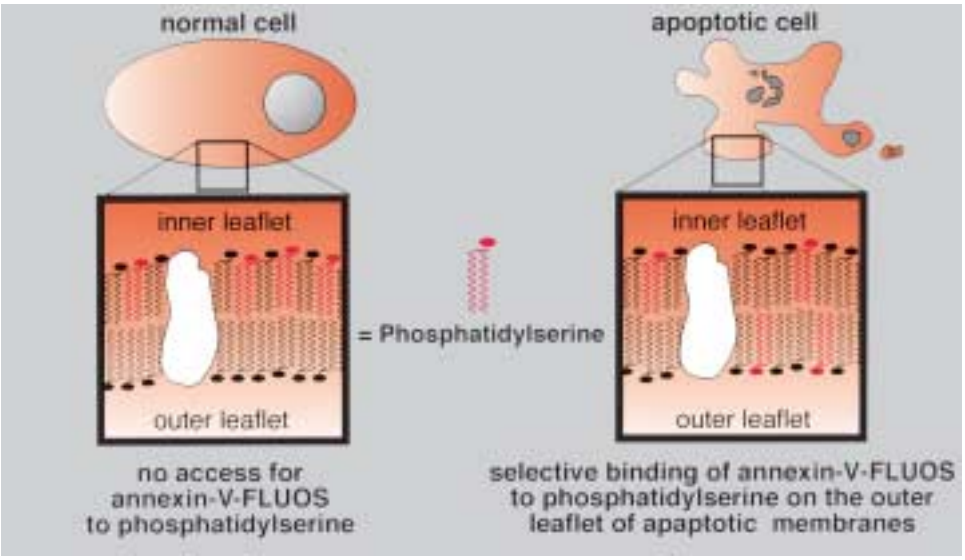
The exact mechanism by which the apoptotic cell becomes a target for phagocytes is unclear. However, it has been shown that a number of changes in cell surface (membrane) markers occur during apoptosis, any one of which may signal "remove now" to the phagocytes. These membrane changes include:

- Loss of terminal sialic acid residues from the side chains of cell surface glycoproteins, exposing new sugar residues[23, 24].
- Emergence of surface glycoproteins that may serve as receptors for macrophage-secreted adhesive molecules such as thrombospondin[25].
- Loss of asymmetry in cell membrane phospholipids, altering both the hydrophobicity and charge of the membrane surface[26].

In theory, any of these membrane changes could provide an assay for apoptotic cells. In fact, one of them has – the alteration in phospholipid distribution.

In normal cells (Figure 27, left diagram), the distribution of phospholipids is asymmetric, with the inner membrane containing anionic phospholipids (such as phosphatidylserine) and the outer membrane having mostly neutral phospholipids. In apoptotic cells (Figure 27, right diagram) however, the amount of phosphatidylserine (PS) on the outer surface of the membrane increases, exposing PS to the surrounding liquid[27].

Annexin V, a calcium-dependent phospholipid-binding protein, has a high affinity for PS[27]. Although it will not bind to normal living cells, Annexin V will bind to the PS exposed on the surface of apoptotic cells (Figure 28, 29). Thus, Annexin V has proved suitable for detecting apoptotic cells[28, 29]. Roche Molecular Biochemicals supplies a number of products for the detection of PS translocation by Annexin V.



▲ **Figure 27: Detection of surface morphology changes during apoptosis.** During apoptosis, the distribution of neutral phospholipids (black symbols) and anionic phospholipids such as phosphatidylserine (red symbols) in the cell membrane changes. Phosphatidylserine is present in the outer membrane of apoptotic cells, but not of normal cells. An exogenously added molecule specific for phosphatidylserine, such as Annexin-V-FLUOS, will bind to phosphatidylserine on the outer membrane of apoptotic cells, but cannot react with the phosphatidylserine of normal cells.

Annexin-V-FLUOS

Cat. No. 1 828 681 250 tests

Annexin-V-FLUOS Staining Kit

Cat. No. 1 858 777 50 tests

Annexin-V-Alexa 568

Cat. No. 1 985 485 250 tests

Type	Direct fluorescence staining for flow cytometric or microscopic analysis
Useful for	Detection of apoptotic cells with membrane alterations (phosphatidylserine translocation); differentiation of apoptotic from necrotic cells
Samples	Cell lines (adherent or suspensions), freshly isolated cells
Method	Simultaneous staining of cell surface phosphatidylserine [with Annexin-V-FLUOS (green dye) or Annexin-V-Alexa 568 (red dye)] and necrotic cells (with propidium iodide)
Time	Approx. 15 min (after induction of apoptosis)

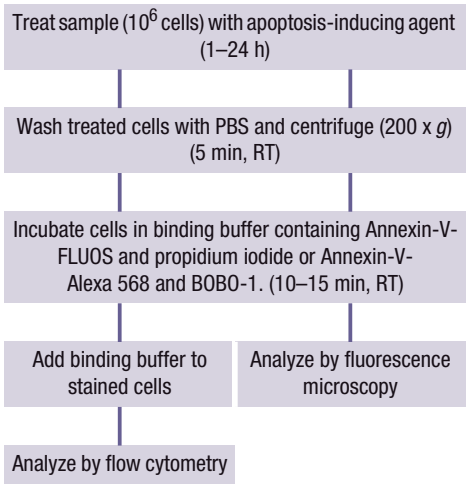
Significance of reagent and kit: Annexin V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). Detection of cell-surface PS with annexin V thus serves as a marker for apoptotic cells. Analysis may be by flow cytometry or by fluorescence microscopy.

Test principle: Annexin-V-FLUOS (green dye) and Annexin-V-Alexa 568 (red dye) serves as a fluorescent probe for apoptotic cells. They will not bind normal, intact cells. However, since necrotic cells are leaky enough to give Annexin-V-FLUOS and Annexin-V-Alexa 568 access to inner membrane PS, apoptotic cells have to be differentiated from necrotic cells. Thus, the assay involves simultaneous staining with both Annexin-V-FLUOS and the DNA stain propidium iodide or Annexin-V-Alexa 568 and BOBO-1 (or propidium iodide). Exclusion of propidium iodide or BOBO-1, coupled with binding of Annexin-V-FLUOS or Annexin-V-Alexa 568, indicates an apoptotic cell (Table 7). The procedure (Flow Chart 9) involves:

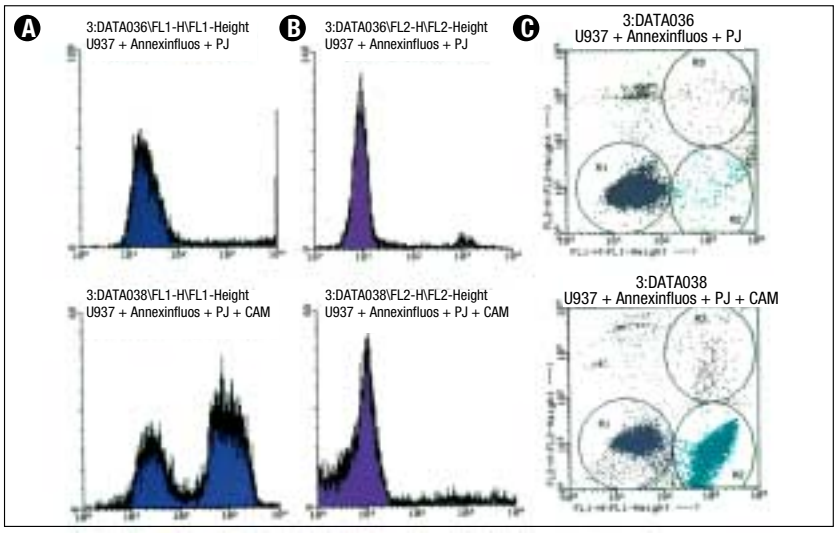
1. Washing suspended cells, then pelleting the cells.
2. Resuspending cells in a staining solution containing Annexin-V-FLUOS and propidium iodide or Annexin-V-Alexa 568 and BOBO-1.
   Note: *Cells may also be labeled with other membrane stains, such as a fluorescein-, phycoerythrin- or TRITC-labeled monoclonal antibody simultaneously.*
3. Analyzing samples in a flow cytometer or under a fluorescence microscope.

	Normal cells	Apoptotic cells	Necrotic cells
Annexin-V staining	–	+	+
Propidium iodide staining	–	–	+

▲ **Table 7:** Distinguishing apoptosis using Annexin-V.



▲ **Flow Chart 9:** Assay procedure, Annexin-V-FLUOS Staining Kit and Annexin-V-Alexa 568.



▲ **Figure 28: Flow cytometric analysis of apoptotic U937 cells stained with Annexin-V-FLUOS and propidium iodide.** U937 cells (a leukemic cell line) were cultivated for 4 h with (bottom row) or without (top row) 4 µg/ml campothecin. Cells were then stained with the components of the Annexin-V-FLUOS Staining Kit and analyzed.
Panels A (upper and lower), single parameter analysis, Annexin-V-FLUOS only;
Panels B, single parameter analysis, propidium iodide (PI) only;
Panels C, dual parameter analysis, Annexin-V-FLUOS and propidium iodide. FL1, Annexin-V-FLUOS; FL2, propidium iodide.
Result: Flow cytometric analysis clearly differentiates normal (living) cells (R1) with low Annexin and low PI staining, apoptotic cells (R2) with high Annexin and low PI staining, and necrotic cells (R3) with high Annexin and high PI staining.

Specificity: Annexin-V-FLUOS and Annexin-V-Alexa 568 bind apoptotic cells and leaky necrotic cells. Propidium iodide and BOBO-1 are excluded from apoptotic and normal cells, but is taken up by necrotic cells.

Can be used to assay:

- Cell lines (adherent or suspensions)
- Freshly isolated cells

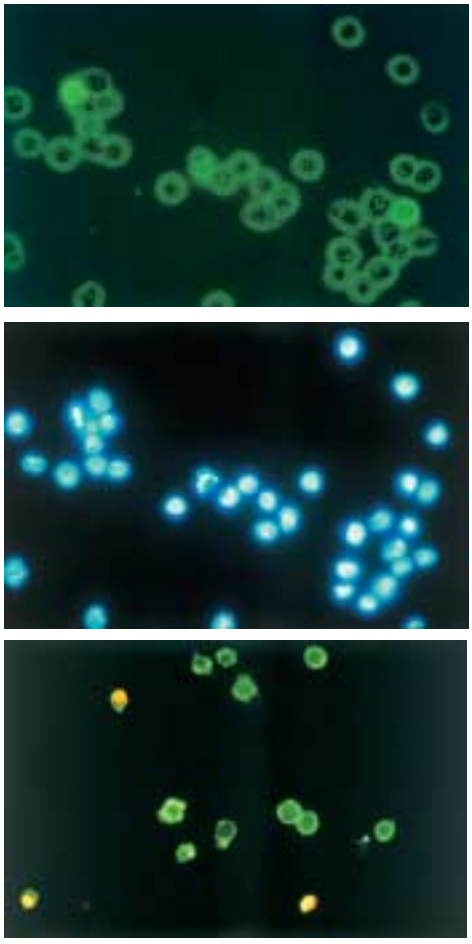
Reagent contents:

- Annexin-V-FLUOS solution, 50 x concentrated
- Annexin-V-Alexa 568, 50 x concentrated

Kit contents
Annexin-V-FLUOS Staining Kit

1. Annexin-V-FLUOS, 50 x concentrated
2. Propidium iodide solution, 50 x concentrated
3. Binding buffer for flow cytometry, ready to use

Typical results: see Figures 28 and 29.



◄ **Figure 29: Fluorescent microscopic analysis of apoptotic SKW6.4 cells stained with Annexin-V-FLUOS, DAPI, and propidium iodide.** SKW6.4 cells were treated with anti-fas antibody, then stained with a series of fluorescent dyes.
Panel A: Cells stained with Annexin-V-FLUOS (green)
Panel B: Same slide, stained with DAPI (blue)
Panel C: Another slide, stained with propidium iodide (orange) and Annexin-V-FLUOS (green).
Result: A few apoptotic cells are visible in panel A (bright green stain) and can be differentiated from necrotic cells by the propidium iodide staining in panel C. (Necrotic cells take up propidium iodide and stain orange/green, while apoptotic cells stain green only). An additional stain, DAPI (panel B) shows that the apoptotic cells have characteristic condensed nuclei .
Note: A and B are identical cells, C is different.

Annexin-V-Biotin

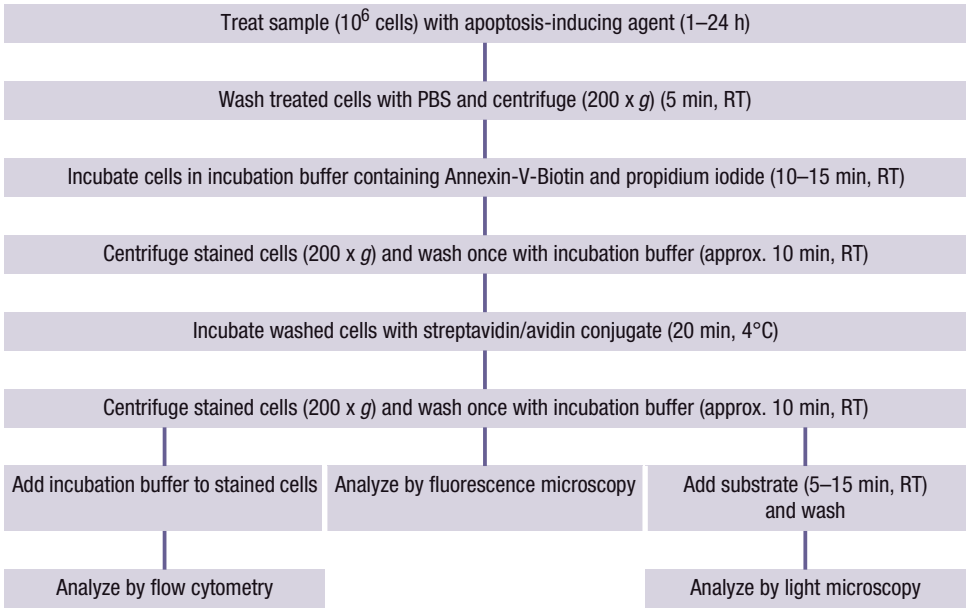
Cat. No. 1 828 690 250 tests

Type	Indirect fluorescence staining for flow cytometric, fluorescence or light microscopic analysis
Useful for	Detection of apoptotic cells with membrane alterations (phosphatidylserine translocation); differentiation of apoptotic from necrotic cells
Samples	Cell lines (adherent and suspensions), freshly isolated cells
Method	Simultaneous staining of cell surface phosphatidylserine (with Annexin-V-Biotin) and necrotic cells (with propidium iodide), followed by detection of biotin (with streptavidin/avidin conjugate)
Time	Approx. 75 min (after induction of apoptosis)

Significance of reagent: Annexin V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). During apoptosis, PS translocates to the outer surface of apoptotic cells. Detection of cell-surface PS with annexin V thus serves as a marker for apoptotic cells. Labeling of cells with the Biotin-conjugate of Annexin-V allows fixation after Annexin-V binding for further analysis of additional cellular parameters in combination with detection of apoptosis (van Engeland, M., Ramaekers FCS, Schutte, B & Reutelingsperger, CPM (1996): A Novel Assay to Measure Loss of Plasma Membrane Asymmetry During Apoptosis of Adherent Cells in Culture. *Cytometry* **24**: 131–139). For distinguishing apoptosis using Annexin-V, see Table 7, page 36.

Test principle: Annexin-V-Biotin serves as a probe for apoptotic cells. It will not bind normal, intact cells. However, since necrotic cells are leaky enough to give Annexin-V-Biotin access to inner membrane PS, apoptotic cells have to be differentiated from necrotic cells. Thus, the assay involves simultaneous staining with both Annexin-V-Biotin and propidium iodide. Exclusion of propidium iodide, coupled with binding of Annexin-V-Biotin, indicates an apoptotic cell. Annexin-V-Biotin is visualized with a streptavidin conjugate. Analysis may be by flow cytometry, by fluorescence microscopy, or by light microscopy. The procedure (Flow Chart 10) involves:

1. Washing suspended cells, then pelleting the cells.
2. Resuspending cells in a staining solution containing Annexin-V-Biotin and propidium iodide.
   Note: *Cells may also be labeled with other membrane stains, such as a fluorescein-, phycoerythrin- or TRITC-labeled monoclonal antibody simultaneously.*
3. Washing labeled cells.
4. Incubating cells with a streptavidin (SA)/avidin (A) conjugate (Table 8).
5. Analyzing samples in a flow cytometer, under a fluorescence microscope, or under a light microscope (depending on the SA conjugate).



◀ **Flow Chart 10:** Assay procedure, Annexin-V-Biotin.

Specificity: Annexin-V-Biotin binds apoptotic cells and leaky necrotic cells.

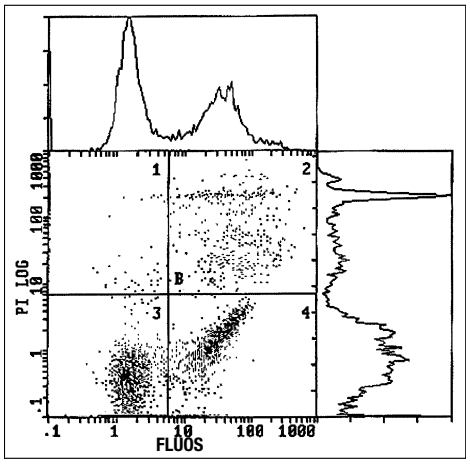
Can be used to assay:

- Cell lines (adherent/suspensions)
- Freshly isolated cells

Reagent contents:

- Annexin-V-Biotin solution, 50 x concentrated.

Typical results: see Figure 30.



◀ **Figure 30: Flow cytometric analysis of apoptotic U937 cells stained with Annexin-V-Biotin, Streptavidin-FLUOS and propidium iodide.** U937 cells (a leukemic cell line) were cultivated for 4 h with 4 µg/ml campothecin. Cells were stained with Annexin-V-Biotin and propidium iodide, then incubated with Streptavidin-fluorescein (SA-FLUOS) and analyzed. Single parameter histograms are shown at the top (Annexin-V-Biotin/SA-FLUOS) and on the right side (PI) of the diagram. Two parameter histograms are shown in quadrants 1–4. PI, propidium iodide; FLUOS, fluorescein.
Result: Flow cytometric analysis clearly differentiates normal cells (quadrant 3) with low FLUOS and low PI staining, apoptotic cells (quadrant 4) with high FLUOS and low PI staining, and necrotic cells (quadrant 2) with high FLUOS and high PI staining.

Product	Application	Cat. No.	Pack Size
Avidin-Fluorescein	fluorescence microscopy, flow cytometry	1 975 595	1 mg
Avidin-Rhodamine	fluorescence microscopy, flow cytometry	1 975 609	1 mg
SA-Peroxidase	light microscopy	1 089 153	500 U (1 ml)
SA-Alkaline Phosphatase	light microscopy	1 089 161	1000 U (1 ml)
SA-β-Galactosidase	light microscopy	1 112 481	500 U

▲ **Table 8:** Streptavidin (SA) conjugates available for the indirect assay of apoptotic cells with Annexin-V-Biotin.

Note: Additional substrates can be found in Table 6.

1.2.2.3 Assays that use DNA stains

One can differentiate between three methods for studying cell death that use DNA stains: dye exclusion method, profile of DNA content, morphological changes.

Dye exclusion method

Viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be discriminated by differential staining. Cells with disturbed plasma membrane permeability are stained, whereas undamaged (viable) cells are not stained with dyes that do not penetrate the plasma membrane ("exclusion dyes"). The most frequently used dye for exclusion tests is trypan blue. In addition, the fluorescent dye, propidium iodide (PI) which becomes highly fluorescent after binding to DNA, can be used in the same manner. The stained and unstained cells are counted with a standard light microscope (trypan blue), or flow cytometer (PI) (Table 9).

Profile of DNA content

If cells are permeabilized, the LMW DNA inside the cytoplasm of apoptotic cells leaks out during the subsequent rinse and staining procedure. The lower DNA content of these cells means they contain less DNA stained by the fluorochrome. Thus, cells with lower DNA staining than that of G_1 cells (the so-called "sub-G_1 peaks", "A_0" cells) have been considered apoptotic. The reduction in staining/DNA content of these cells is measured by flow cytometry (Figure 31). The major disadvantage of this technique is that apoptotic G_2-Phase cells exhibit a reduced DNA content, which could represent the DNA content of a G_1-cell. Therefore it may not be detected as apoptotic. This would result in an underestimation of the apoptotic population.

DNA-binding dyes (Fluorochromes)	Dye enters		Dye stains	
	Viable cells	Non viable cells	Nucleus (DNA)	Cytoplasm (RNA)
Acridine orange	Yes	Yes	Green	Red-orange
Hoechst 33342	Yes	Yes	Blue	No
Hoechst 33258	No	Yes	Blue	No
DAPI	No	Yes	Bright blue	No
Ethidium bromide	No	Yes	Orange	Slightly red
Propidium iodide	No	Yes	Red	No

▲ **Table 9:** Common fluorochromes used to stain the genomic DNA of viable and/or non-viable cells.

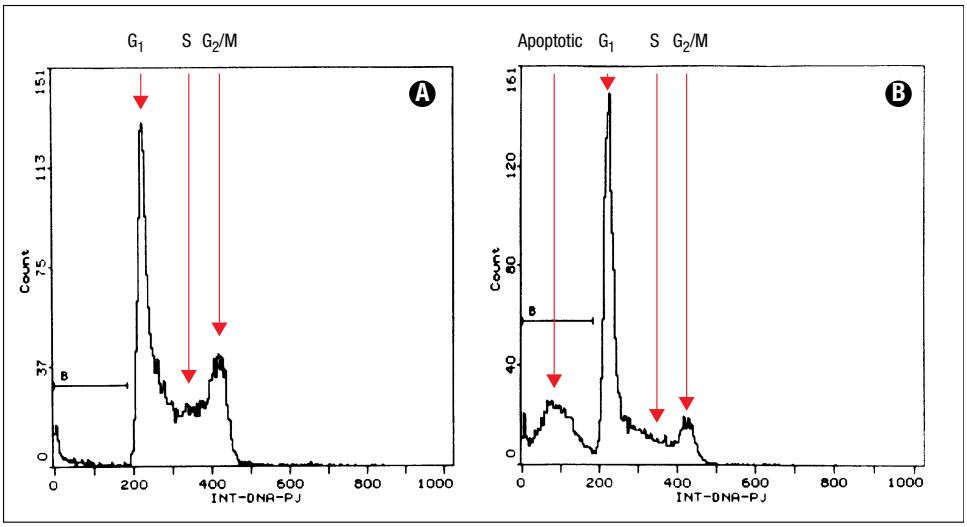


Figure 31: Typical flow cytometric profile of the DNA content in normal (A) and apoptotic cells (B), stained with PI. **Result:** A prominent "sub-G_1" peak (earliest peak) appears in apoptotic cells, but not in normal cells. ▶

Morphological changes

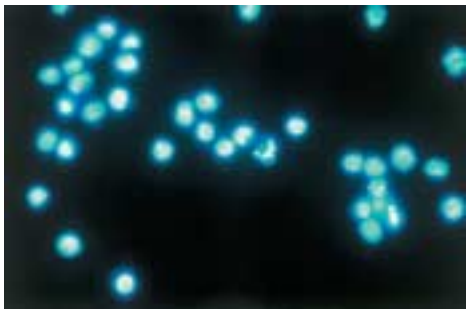
On the other hand, the bisbenzimidazole dye, Hoechst 33342 (and also acridine orange), penetrates the plasma membrane and stains DNA in cells; without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. They are also visible in permeabilized apoptotic cells stained with other DNA binding dyes like DAPI (Figure 32).

Dive et al.[30] have reported that during a short exposure to Hoechst 33342, apoptotic cells have stronger blue fluorescence compared to non-apoptotic cells. Co-staining of the cells with PI allows the discrimination of dead cells from apoptotic cells. If 7-amino-actinomycin is used instead of PI, cell surface antigens immunostained with fluorescein and phycoerythrin may be quantitated simultaneously[31].

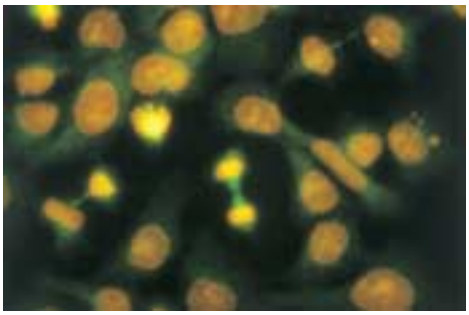
One drawback of using any vital staining method for measuring apoptosis is the variability of active dye uptake in different cells and its possible change during certain treatments. Therefore, the ability of Hoechst 33342 to discriminate apoptotic cells from normal cells by increased uptake of dye has to be tested for each new cell system[31].

Reagent	Cat. No.	Pack size	Fluorescence	Typical results
Propidium iodide*	1 348 639	20 ml	red orange	See Table 9 and Figure 24
DAPI 4',6-Diamidine-2'-phenylindole dihydrochloride	236 276	10 mg	blue	See Table 9 and Figure 27

▲ **Table 10:** Fluorescent dyes that stain double-stranded DNA
*Only sold in the US



▲ **Figure 32: Fluorescent microscopic analysis of apoptotic cells stained with DAPI.** DAPI stains the nuclei of all cells (blue).
Result: The characteristic condensed nuclei of apoptotic cells are clearly visible here.



▲ **Figure 33: Fluorescent microscopic analysis of mitotic cells stained with ethidium bromide.** DNA was stained with ethidium bromide (orange). Mitotic spindles were stained with anti-tubulin antibody (green).
Result: Mitotic cells (with condensed DNA) are brightly stained. Without the double stain, mitotic cells could be mistaken for apoptotic cells, since both have condensed DNA.

Apoptosis Assay Methods
Summary of methods for studying apoptosis in individual cells
1 Cell Death – Apoptosis and Necrosis

1.2.2.4 Summary of methods for studying apoptosis in individual cells.

Method/ Roche Molecular Biochemicals product	Parameter analyzed	Assay principle	Advantages	Limitations	For product information, see
Staining of chromosomal DNA after permeabilization (DNA content)[22, 32] DAPI, propidium iodide*	DNA fragmentation (HMW DNA, DNA content)	● Apoptotic cells are permeabilized with ethanol or detergent. During this procedure the LMW DNA inside the apoptotic cell leaks out and is removed during the subsequent washing steps. ● The HWM DNA retained in the cells is stained with a DNA binding dye such as propidium iodide. ● The amount of HMW DNA is quantified by flow cytometry ("sub G_1" or "A_0" peak).	● Quick and cheap ● Minimal overlap between the peak representing apoptotic ("sub G_1") and normal G_1 cells ● Estimation of position in cell cycle allows cell cycle specificity of apoptosis to be studied ● Applicability to any DNA fluorochrome and instrument	● Degree of extraction of LMW DNA during the washing and staining procedure not always reproducible ● Not specific for apoptosis: Sub G_1 peak can also represent mechanically damaged cells, cells with different chromatin structure, or normal cells with lower DNA content in heterogeneous cell populations ● May not detect cells induced to apoptosis in G_2 ● No discrimination of apoptotic cells from dead cells which have lost their membrane integrity	page 40 of this guide
Staining of chromosomal DNA	Chromatin morphology	● Apoptotic cells are stained by the addition of DNA fluorochromes which are able to cross the intact plasma membrane, such as acridine orange. Can be done with DAPI on fixed cells. ● The stained DNA allows the altered morphology of the nuclear chromatin to be visualized by fluorescence microscopy.	● Quick and cheap ● Discrimination between viable and dead cells when counterstained with propidium iodide using vital dyes (acridine orange, Hoechst 33342) ● Simultaneous staining of cell surface antigens with standard fluorescein and phycoerythrin conjugates possible if Hoechst 33342 is combined with 7-amino-actinomycin D	● No quantitative measurement ● Subjective: no clear cut-off point between normal and apoptotic cells ● Clear morphologically distinct apoptotic nuclei appear late during apoptosis: May lead to an underestimation of apoptotic cells	
Active labeling of cells by nick translation (ISNT)[34]	DNA strand breaks (nicks) and DNA fragmentation (staggered DNA ends)	● Apoptotic cells are fixed with formaldehyde and subsequently permeabilized. ● DNA strand breaks are labeled with modified nucleotides using exogenous DNA polymerase (nick translation). ● The incorporated nucleotides are visualized with a secondary detection system which has a reporter molecule (e.g. fluorescein, AP, POD).	● Counterstaining with DNA fluorochrome (profile of DNA content) allows cell cycle specificity of apoptosis to be studied (only by flow cytometry) ● Identification of apoptosis at a molecular level (DNA strand breaks) ● Suitable for tissue sections	● Labor-intensive and time-consuming; only a few tests may be performed simultaneously ● Undefined cell loss during fixation procedure (loss of specific cell population?) ● Many cells (2–5 x 10^6/test) required	
Active labeling of cells by end labeling (TUNEL)[20, 35] *In Situ* Cell Death Kit, Fluorescein, TMR, AP, POD	DNA strand breaks (nicks) and DNA fragmentation (staggered DNA ends)	● Apoptotic cells are fixed with formaldehyde and subsequently permeabilized. ● DNA strand breaks are labeled with modified nucleotides using exogenous terminal transferase (end labeling). ● The incorporated nucleotides are visualized directly (e.g. Fluorescein-dUTP) or with a secondary detection system which has a reporter molecule (e.g. fluorescein, AP, POD).	● Advantages like *in situ* nick translation; in addition: ● More sensitive: maximum intensity of labeling (cell staining) of apoptotic cells is higher compared to ISNT ● Fast kinetics of dUTP incorporation in comparison with the DNA polymerase method: 30 min is sufficient for the labeling reaction ● Higher sensitivity for apoptosis: TUNEL preferentially labels apoptosis in comparison to necrosis ● Direct detection possible by the use of Fluorescein-dUTP (without secondary detection system) for maximum sensitivity and minimal background ● With the direct TUNEL labeling assay less working steps are required than with the indirect assay.	● Labor-intensive and time-consuming; only a few tests may be performed simultaneously ● Many cells (2–5 x 10^6/test) required ● Undefined cell loss during fixation procedure (loss of specific cell population?)	pages 31, 33 of this guide
Detection of translocated membrane component[27] Annexin V-FLUOS Annexin V-Biotin Annexin V-Staining Kit Annexin V-Alexa 568	Detection of phosphatidylserine on surface of apoptotic cells	● Apoptotic cells are incubated with an assay protein (e.g. annexin V) conjugated to a reporter molecule. ● Assay protein binds a membrane component (e.g. phosphatidylserine) found on the outer surface of apoptotic cells only. ● A DNA strain is added to distinguish necrotic cells (permeable) from apoptotic cells (impermeable). ● Apoptotic cells are made visible by assay of reporter molecule in flow cytometer or under a microscope.	● Unique marker for apoptosis related plasma membrane changes ● Allows analysis by flow cytometry fluorescence microscopy, or light microscopy ● Allows simultaneous labeling of other cell surface antigens ● Annexin-V-Biotin allows fixation following Annexin-V binding for further analysis of additional cellular parameters	● Not specific for apoptosis: Annexin V can stain inner membrane of ruptured cells; must distinguish apoptotic from necrotic cells with an additional DNA stain ● Many cells (10^6/test) required ● Cannot be used on tissue sections or any fixed samples	pages 36, 38 of this guide
Trypan Blue Exclusion Assay	Damage/leakage of plasma membrane	● Cells are incubated with dye. ● Dead cells take up dye; living cells do not. ● Stained (dead or damaged) cells are determined under a light microscope. ● Dye binds to intracellular proteins of leaky cells.	● The classical standard method to distinguish viable from dead cells by light microscopy ● Quick and cheap ● Only a small fraction of total cells from a cell population is required	● Each individual sample has to be counted: only a few tests may be performed simultaneously ● Subjective evaluation ● Not suitable for detection of apoptosis ● Stains only necrotic cells or very late apoptotic cells (secondary necrosis)	
Propidium Iodide Exclusion Assay Propidium iodide solution*	Damage/leakage of plasma membrane	● Cells are incubated with fluorescent dye. ● Dead cells take up dye; living cells do not. ● Stained (dead or damaged) cells are determined under a microscope or by flow cytometry. ● Dye binds to DNA of leaky cells.	● The standard method to distinguish viable from dead cells by fluorescence microscopy and flow cytometry ● Double labeling procedures possible: simultaneous detection of e.g. surface antigens ● Quick and cheap ● Only a small fraction of total cells from a cell population is required	● Each individual sample has to be counted: only a few tests may be performed simultaneously ● Not specific for apoptosis	page 40 of this guide

*Sold only in the US

▲ **Table 11:** Methods for studying apoptosis in individual cells.

1.2.3 Detection of apoptosis-related proteins

There are a number of genes that regulate apoptosis. That is, the products of these genes interfere with apoptotic pathways. Assays to detect the proteins encoded by these genes can complement the assays described in the previous sections.

The study of apoptosis-regulating genes and gene products is still evolving. The picture so far is complex (as summarized in Section 1.1.3 of this guide). For instance, in some cases, the same gene has an effect on both the survival of normal cells and the development of cancers by preventing apoptosis[36]. A detailed discussion of the field is beyond the scope of this guide, but is covered in a number of reviews[36, 37]. As an introduction to the field, we discuss the characteristics of a few of these apoptosis-regulating proteins.

The relationship of the ced (**c**aenorhabditis **e**legans cell **d**eath) genes to apoptosis in the nematode *Caenorhabditis elegans* has been extensively studied[38]. Of these, the ced-3 and ced-4 genes[39] encode proteins that must be active to initiate apoptosis. In contrast, the ced-9 gene product protects cells from apoptotic cell death, ensuring their survival[40]. In other words, apoptosis is more likely when levels of ced-3 and ced-4 protein are high or when levels of ced-9 protein are low.

In mammalian systems, the Bcl-2 proto-oncogene serves much the same function as ced-9, blocking the induction of cell death[41]. The Bcl-2 oncoprotein also protects against the cytotoxic effects of certain drugs[42].

The Bcl-2 protein can dimerize with itself or with the product of the bax gene[43]. The presence of the bax protein seems to counteract the anti-apoptotic activity of Bcl-2. In summary, apoptosis is more likely when bax protein levels are high or when Bcl-2 protein levels are low.

Another mammalian gene product, p53, is a tumor suppressor because it induces apoptosis in potentially malignant cells[44]. Absence or mutation of the p53 gene product led to malignant transformation and immortalization of the cell.

Increases in expression of a growth stimulating gene, the c-myc proto-oncogene, actually induces apoptosis in the absence of other growth factors[45, 46]. High levels of the Bcl-2 protein can counteract the effect of the c-myc protein.

For the analysis of apoptosis-regulating proteins, Roche Molecular Biochemicals offers a set of antibodies to p53 (and an ELISA kit for the detection of p53 in fluids or extracts), and an antibody to the Bcl-2 oncoprotein.

Cell surface receptor genes (APO-1/Fas/CD95), other growth-stimulating genes (e.g., Ras), and other tumor-suppressing genes (e.g., Rb) have also been implicated in the regulation of apoptosis[2, 37]. The Fas (CD95/APO-1) molecule has originally been identified as a cell surface receptor that could mediate apoptotic cell death of transformed cells and cause regression of experimental tumors growing in nude mice. The function of Fas was assessed by establishment of mouse cell transformants that constitutively expressed human Fas. When the transformed cells were treated with the antibody to human Fas, the cells died by apoptosis within 5 hours, which indicated that Fas can transduce an apoptotic signal and that anti-Fas works as an agonist. The subsequent purification of human APO-1 antigen and molecular cloning of its cDNA established the identity of APO-1 and Fas. Meanwhile, numerous reports have shown a pivotal role of Fas in various physiological and pathological processes. The Anti-Fas provided by Roche Molecular Biochemicals is suitable for the induction of apoptosis as well as for the detection of the Fas receptor.

Anti-Fas (CD95/Apo-1)

Cat. No. 1 922 432 100 µg

Type	Monoclonal antibody, clone 2R2, IgG3, mouse
Useful for	Apoptosis induction in Fas expressing cells
Samples	Cell suspensions, adherent cells
Method	Direct induction of apoptosis by adding antibody to cell cultures
Time	Approx. 3–5 h (induction of apoptosis)

Significance of reagent: The antibody may be used for the induction of apoptosis in cell cultures through Fas by imitating the Fas-ligand. The Fas (CD95/Apo-1) molecule has been identified as a cell surface receptor that could induce apoptotic cell death of transformed cells upon activation by its ligand and cause regression of experimental tumors in mice.

Test principle: The antibody may be used for induction of apoptosis:

1. Add antibody (1 µg/ml) into culture medium of Fas-bearing cells
2. Incubation for 3–5 hours
3. Detection of apoptosis by various assays

Antibody supplied as: Mouse monoclonal antibody (clone 2R2, IgG3) in cell culture supernatant; sterile filtered.

Sensitivity: The antibody is suitable for induction of apoptosis at 0.5 µg/ml in SKW6.4 and Jurkat cells. If secondary cross-linking with an anti-mouse IgG is used, the apoptosis inducing concentration could be reduced to 100 ng/ml. In Fas transfectants apoptosis is induced without cross-linking at 100 ng/ml. It has to be mentioned, that some Jurkat subclones do not or only in high doses respond to Anti-Fas induction of apoptosis.

Specificity: The antibody was generated by immunizing mice with transformed murine L-cells bearing recombinant human Fas receptor. On Western blots, Anti-Fas binds the human Fas/Apo-1 (CD95).

Can be used for:

- Induction of apoptosis through the Fas-receptor

Anti-Bcl-2 oncoprotein, human

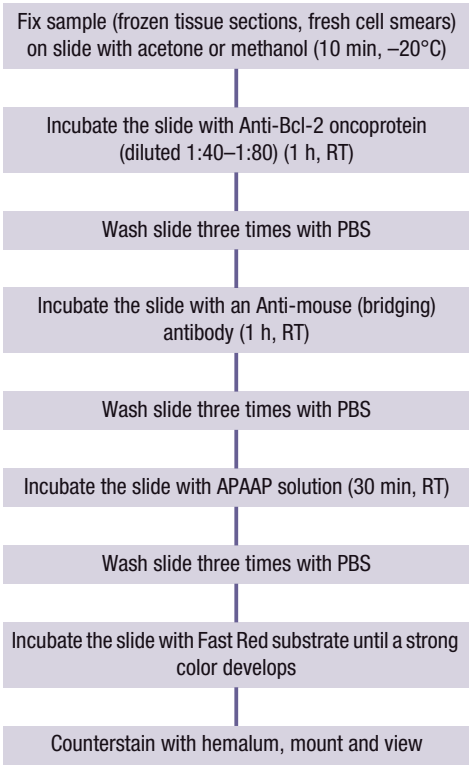
Cat. No. 1 624 989 1 ml

Type	Monoclonal antibody, from mouse
Useful for	Detection of the apoptosis-suppressing Bcl-2 protein on slides or Western blots
Samples	Cytospins, cell smears, frozen or fixed paraffin-embedded tissue sections; crude cell extracts
Method	Preparation of sample, followed by indirect immunodetection of Bcl-2 protein
Time	Approx. 3.5 h (immunodetection only)

Significance of assay: The antibody may be used to detect Bcl-2 in tissue or cells (by immunohistochemistry or immunocytochemistry) or in crude cell extracts (on Western blots). The product of the Bcl-2 proto-oncogene is thought to be a negative regulator (suppressor) of apoptosis in mammalian cells. The 26 kD Bcl-2 protein is found principally in lymphoid tissue and cells (e.g., lymph node, spleen, thymus, peripheral blood lymphocytes).

Test principle: The antibody may be used to detect Bcl-2 *in situ* by immunohistochemistry or immunocytochemistry. The staining process (Flow Chart 11) involves:

1. Fixing the sample (frozen sections, fresh cells) on a slide.
2. Detecting Bcl-2 protein in the sample with Anti-Bcl-2 antibody solution.
3. Detecting the immobilized Anti-Bcl-2 antibody with an anti-mouse (bridging) antibody.
4. Binding the bridging antibody with alkaline-phosphatase-anti-alkaline phosphatase (APAAP) solution.
5. Visualizing the antibody-antigen complexes with chromogenic alkaline phosphatase substrate (Fast Red tablets, Cat. No. 1 496 549).



▲ **Flow Chart 11:** Assay procedure, immunohistochemistry/immunocytochemistry with Anti-Bcl-2.

Antibody supplied as: Mouse monoclonal antibody (clone 124) in cell culture supernatant.

Specificity: On Western blots, Anti-Bcl-2 binds the 26 kD product of the Bcl-2 proto-oncogene. In tissue, the antibody reacts strongly with amino acids 41–54 of the Bcl-2 protein on cytospins, cell smears, and frozen lymphoid tissue sections. In routinely fixed, paraffin-embedded tissue, the antibody reacts with a lower percentage of cells, since the epitope is not always preserved under these conditions.

Can be used to assay:

- Cytospins
- Cell smears
- Frozen tissue, some paraffin-embedded tissue
- Crude cell extracts (on Western blots)

Anti-p53-Protein, mutant

Monoclonal antibody

Cat. No. 1 696 823 100 µg

Anti-p53-Protein pan

Monoclonal antibody

Cat. No. 1 413 147 100 µg

Anti-p53 pan

Polyclonal antibody

Cat. No. 1 810 928 200 µg

Anti-p53, Biotin labeled

Polyclonal antibody

Cat. No. 1 810 936 150 µg

Anti-p53, Peroxidase labeled

Polyclonal antibody

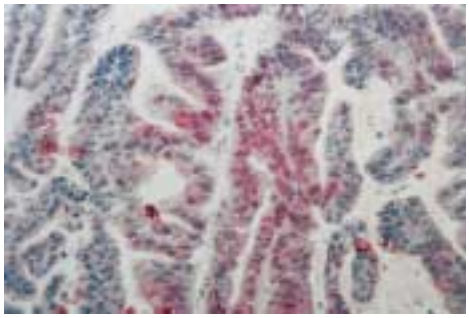
Cat. No. 1 810 944 50 U

Significance of reagents: The antibodies are suitable for the detection of the p53 protein in immunohistochemistry and Western blot.

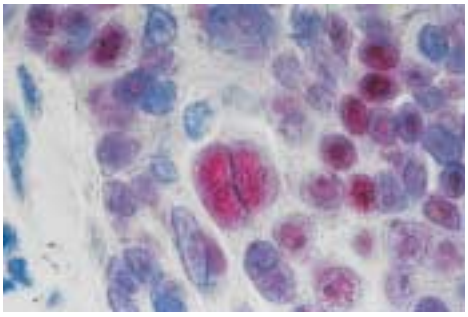
Antibody	Recognizes p53 from	Clone	Ig Class	Typical results
Anti-p53-Protein, mutant	human, mouse, rat, hamster, monkey, cow, chicken	PAb240	mouse IgG1	
Anti-p53-Protein pan	human, mouse, rat, hamster, monkey	PAb122	mouse IgG2b	See Figure 34*
Anti-p53 pan	human, mouse, rat, hamster, monkey, cow, chicken	polyclonal serum	sheep IgG	See Figure 35
Anti-p53, Biotin labeled	human, mouse, rat, hamster, monkey, cow, chicken	polyclonal serum	sheep IgG	
Anti-p53, Peroxidase labeled	human, mouse, rat, hamster, monkey, cow, chicken	polyclonal serum	sheep IgG	

▲ **Table 12:** Antibodies to p53

*For another example of how this antibody can be used in the laboratory, see Del Bufalo, D. et al. (1996) *J. Clin. Invest.* **98**, 1165–1173.



▲ **Figure 34: Detection of p53 in a human ovarian adenocarcinoma with a monoclonal antibody.**
A cryostat section was prepared from a partially differentiated, papillary, advanced ovarian carcinoma. The section was stained with 5 fg/ml of mouse monoclonal anti-p53 pan (Cat. No. 1 413 147). A biotinylated rabbit anti-mouse antibody was used as secondary antibody. The slide was counterstained with hemalum (Slide kindly provided by H. Merz).
Result: The section showed a strong p53 signal, even with a very low concentration of staining antibody.



▲ **Figure 35: Detection of p53 in a human ovarian adenocarcinoma with a polyclonal antibody.**
A paraffin-fixed section was prepared from a partly solid, partly papillary, advanced ovarian carcinoma. The section was stained with polyclonal sheep anti-p53 (Cat. No. 1 810 928). A biotinylated donkey anti-sheep antibody (1:300 dilution, Immunotech) was used as secondary antibody. The slide was counterstained with hemalum (Slide kindly provided by H. Merz).
Result: The nuclei of the cells were strongly stained for p53.

p53 pan ELISA

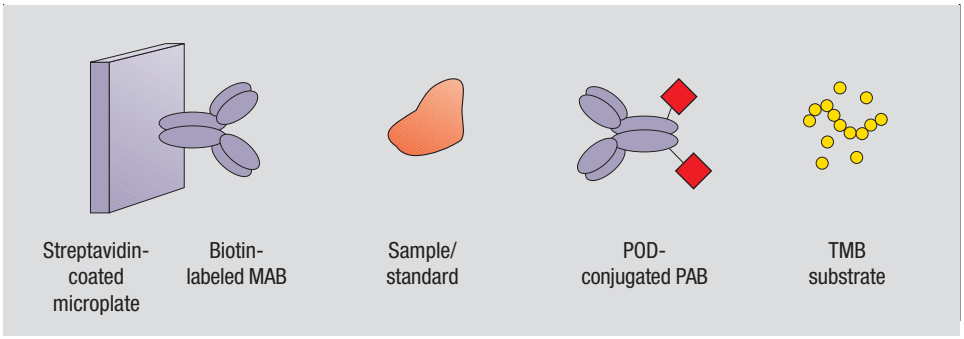
Cat. No. 1 828 789 96 tests

Type	One-step sandwich ELISA, colorimetric
Useful for	Quantitation of p53 protein, both wild-type and mutant forms
Samples	Tissue homogenates, cell lysates, serum, or plasma
Method	Lysis/homogenization of sample, followed by immunochemical determination of p53 in a microplate
Time	Approx. 2.5 h

Significance of kit: This kit uses antibodies to quantitate the levels of human, mouse, or rat p53 in smear/plasma, in tumor tissue, or in tumor cell lines. Wild type p53 is a protein that suppresses malignant transformation by inducing apoptosis. Mutations of the p53 gene which increases its stability allow transformation (immortalization) of the cell and are quite commonly found in malignancies. The p53 protein seems also to be involved in cell death induced by cytotoxic drugs.

Test principle: The assay uses a one-step sandwich immunoassay (Figure 36) to detect wild-type and mutant p53. The procedure (Flow Chart 12) involves:

1. Preparing sample (e.g., detergent lysis of cells, homogenization of tissue), followed by centrifugation.
2. Transferring an aliquot of the sample supernatant to streptavidin-coated well of a microplate.
3. Binding p53 in the supernatant with two antibodies, a biotin-labeled monoclonal anti-p53 (capture antibody), which is pre-bound to the streptavidin-coated plate, and a peroxidase-labeled polyclonal anti-p53 (detection antibody).
4. Washing the immobilized antibody-p53 complexes five times to remove sample components that are not immunoreactive.
5. Incubating sample with peroxidase substrate (tetramethylbenzidine, TMB).
6. Determining the amount of colored product (and thus, of immobilized antibody-p53 complexes) spectrophotometrically.



▲ **Figure 36:** How the p53 pan ELISA works.

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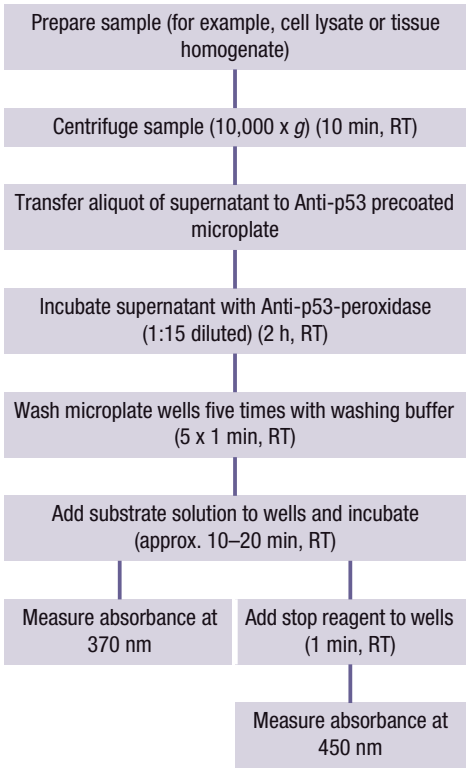


▲ **Flow Chart 12:** Assay procedure, p53 pan ELISA.

Sensitivity: In 4 independent assays, the lower limit of detection for the assay was determined to be 9 pg/ml.

Specificity: The biotin-labeled capture antibody from mouse recognizes a conserved, pantropic, denaturation stable antigenic determinant of the p53 protein (human, mouse, rat). The peroxidase-labeled detection antibody is highly specific for wild-type and mutant p53 from different species.

Can be used to assay:

- Tissue homogenates
- Cell lysates
- Serum or plasma

Kit contents

1. Anti-human-p53 pan, polyclonal, peroxidase-labeled
2. Human p53 standards (six)
3. Incubation buffer/Sample diluent, ready-to-use
4. Washing buffer, 10 x concentrated
5. TMB substrate solution
6. TMB stop solution
7. Streptavidin-coated microplate, precoated with monoclonal Anti-p53 (biotin-labeled)
8. Adhesive plate cover foils

Note: *The peroxidase-labeled antibody in this kit, as well as other antibodies to p53 are available as separate reagents* *see page 48.*

1.3 Cytotoxicity Assay Methods

1.3.1 Relationship between cytotoxicity, apoptosis and necrosis

As discussed in Section 1.1.1 of this guide, there are two experimentally distinguishable mechanisms of cell death: necrosis, the "accidental" cell death that occurs when cells are exposed to a serious physical or chemical insult, and apoptosis, the "normal" cell death that removes unwanted or useless cells.

In contrast to these two cell death processes, cytotoxicity does not define a specific cellular death mechanism. Rather, cytotoxicity is simply the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (such as a cytotoxic T cell), independent from the mechanisms of death.

Note: *Cytotoxicity may also be used, as it is in this guide, to denote a laboratory method for detecting dead cells, regardless of the mechanism of their death.*

Example of cytotoxicity
A common example of cytotoxicity is cell-mediated cytotoxicity. Cells of the immune system [such as cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated (LAK) cells] can recognize and destroy damaged, infected and mutated target cells. Although the recognition machinery used by these cells is very different, their mechanism of target cell destruction may be very similar.

Two possible cytocidal mechanisms have been proposed for cell-mediated cytotoxicity: (i) the apoptotic mechanism, in which the effector cell triggers an autolytic cascade in the target cell and the genomic DNA fragments before cell lysis; and (ii) the lytic mechanism, in which lytic molecules, notably perforin, are secreted by the effector cell into the intercellular space and polymerize to form pores in the target cell membrane, leading to cell lysis[3, 47]. These two mechanisms are not mutually exclusive and, quite possibly, are complementary.

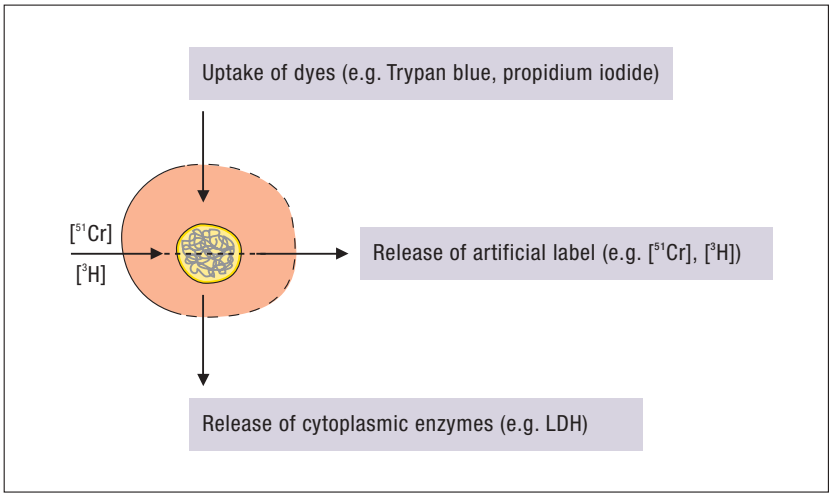
1.3.2 Methods for studying cytotoxicity

Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release (leakage) of components into the supernatant or the uptake of dyes, normally excluded by viable cells (Figure 37) (see also 1.2.2.3, on page 40 "dye exclusion method"). A serious disadvantage of such permeability assays is that the initial sites of damage of many, if not most cytotoxic agents are intracellular. Therefore, cells may be irreversibly damaged and committed to die and the plasma membrane is still intact. Thus, these assays tend to underestimate cellular damage when compared to other methods. Despite this fact, some permeability assays have been widely accepted for the measurement of cytotoxicity.

Alternatively, dead cells are unable to metabolize various tetrazolium salts (see also Section 2.2.1.1). This allows the use of the colorimetric assays MTT, XTT, or WST-1 to measure cell survival. Apoptosis, however, is an active mode of cell death requiring the metabolism of cells. Thus, like the permeability assays mentioned above, the colorimetric assays may underestimate cellular damage and detect cell death only at the later stages of apoptosis when the metabolic activity of the cells is reduced.

Regardless of this disadvantage, the colorimetric assays are very useful for quantitating factor-induced cytotoxicity within a 24 to 96 h period of cell culture. However, these colorimetric assays are of limited value for measuring cell mediated cytotoxici-

Figure 37: Schematic illustration of the three basic principles to assess plasma membrane leakage.



ty, since most effector cells become activated upon binding to the target cells. This activation results in an increased formazan production by the effector cell, which tends to mask the decreased formazan production resulting from target cell death.

Note: *Assays for cytotoxicity can be, and frequently are, used to measure cell necrosis.*

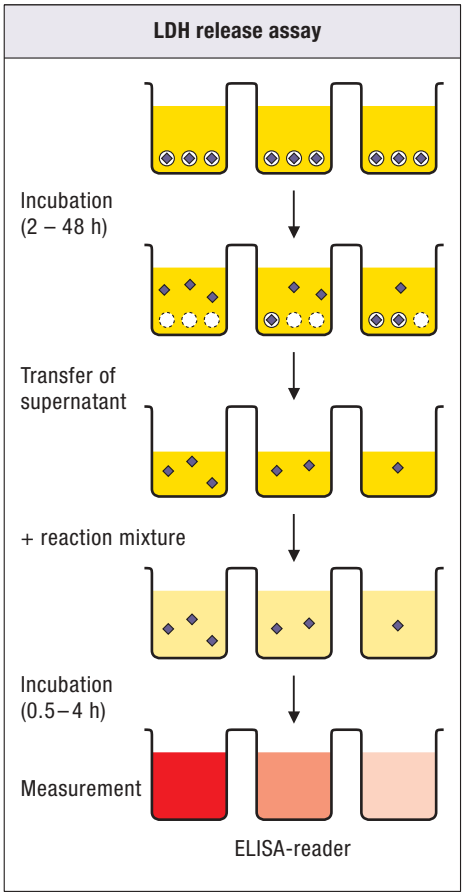
1.3.2.1 Assays that measure plasma membrane leakage

Widely used standard methods for measuring plasma membrane leakage are based on the uptake or exclusion of molecules with special light-absorbing or fluorescent properties. Viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be discriminated by differential staining. Because dyes stain individual cells, each sample has to be analyzed by flow cytometry or microscopy. This kind of single cell analysis is not suitable if many different samples have to be measured. In contrast, assays which quantitate plasma membrane disintegration in cell populations allow many different samples to be handled simultaneously in a single MTP.

One group of standard assays performed in a MTP is based on the release of radioactive isotopes ([^{51}Cr], [^{3}H]-thymidine, [^{3}H]-proline, [^{35}S]- or [^{75}Se]-methionine, 5-[^{125}I]-2-deoxy-uridine) or fluorescent dyes (bis-carboxyethyl-carboxyfluorescein (BCECF) or calcein-AM) from prelabeled target cells[48, 49, 50]. The disadvantages of such assays however, are (i) the use of radioactive isotopes in most of them, (ii) the necessity for prelabeling of the target cells, and (iii) the high spontaneous release of most labels from the prelabeled target cells.

Another group of assays is based on the measurement of cytoplasmic enzymes released by damaged cells. The amount of enzyme activity detected in the culture supernatant corresponds to the proportion of lysed cells[51, 52]. Enzyme release assays have been described for alkaline and acid phosphatase, for glutamate-oxalacetate transaminase, for glutamate pyruvate transaminase, and for argininosuccinate lyase. However, their use has been hampered by the low amount of those enzymes present in many cells and by the elaborate kinetic assays required to quantitate most enzyme activities.

In contrast to the above mentioned cytoplasmic enzymes, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. With the Cytotoxicity Detection Kit (see page 54), LDH activity can easily be measured in culture supernatants by a single point assay. The use of a spectrophotometric microplate reader (ELISA plate reader) allows the simultaneous measurement of multiple probes and thereby guarantees the easy processing of a large number of samples (Figure 38).



▲ **Figure 38:** Measurement of LDH activity (◆) using the microplate format (see also Flow Chart 13).

Cytotoxicity Detection Kit (LDH)

Cat. No. 1 644 793 2000 tests

Type	Colorimetric assay, microplate format
Useful for	Quantitation of LDH activity released from damaged/dying cells
Samples	Cell-free supernatants from cells in culture
Method	Preparation of cell-free supernatant, followed by incubation of supernatant with INT to form colored formazan, a product which may be quantitated colorimetrically
Time	0.5–1 h (+ induction of cell death)

Significance of kit: The Cytotoxicity Detection Kit measures cytotoxicity and cell lysis by detecting LDH activity released from damaged cells. The assay is performed in a 96-well microplate. The kit can be used in many different *in vitro* cell systems where damage of the plasma membrane occurs. Examples are:

- Detection and quantification of cell mediated cytotoxicity.
- Determination of mediator-induced cytolysis.
- Determination of the cytotoxic potential of compounds in environmental and medical research, and in the food, cosmetic, and pharmaceutical industries.
- Determination of cell death in bioreactors.

Test principle: The assay is based on a the cleavage of a tetrazolium salt when LDH is present in the culture supernatant. The procedure involves:

1. Incubating the cells in culture to allow cell death to occur. An increase in the amount of dead or plasma membrane-damaged cells during the assay results in an increase of LDH in the culture supernatant.
2. Collecting the cell-free culture supernatant.
3. Adding the substrate mixture from the kit to the culture supernatant. Any LDH released into the supernatant during Step 1 will reduce the tetrazolium salt INT to formazan by a coupled enzymatic reaction. Thus, release of LDH into the supernatant directly correlates to the amount of formazan formed in this step.
4. Quantitating the formazan dye formed in an ELISA plate reader. The formazan dye formed is water-soluble and shows a broad absorption maximum at about 500 nm.

For a detailed overview of the steps involved in the procedure, see Figures 38 and 39 and Flow Chart 13.

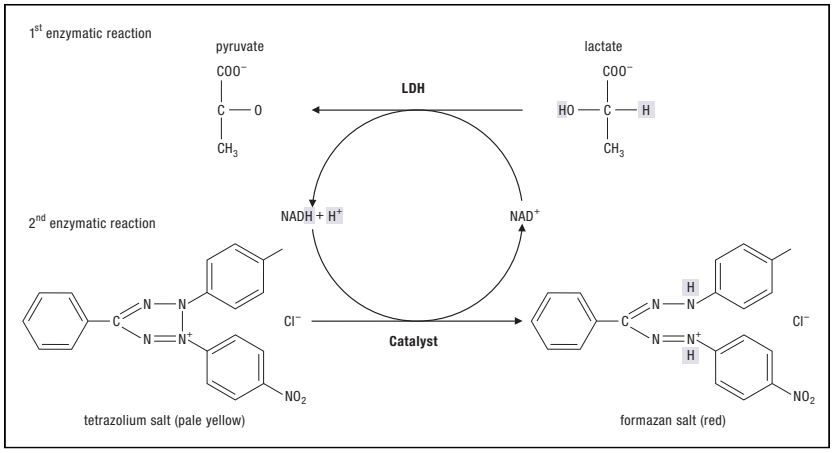


Figure 39: Biochemistry of the Cytotoxicity Detection Kit (LDH): In the first enzymatic reaction LDH reduces NAD^+ to NADH + H^+ by oxidation of lactate to pyruvate; in the second enzymatic reaction the catalyst (diaphorase) transfers H/H^+ from NADH + H^+ to the tetrazolium salt INT.

Incubate cells in a microplate or batch culture for a certain period of time (optional: induction of death by treatment with compounds or test substances)

↓

Centrifuge cells

↓

Remove 100 ml supernatant and transfer it to another microplate

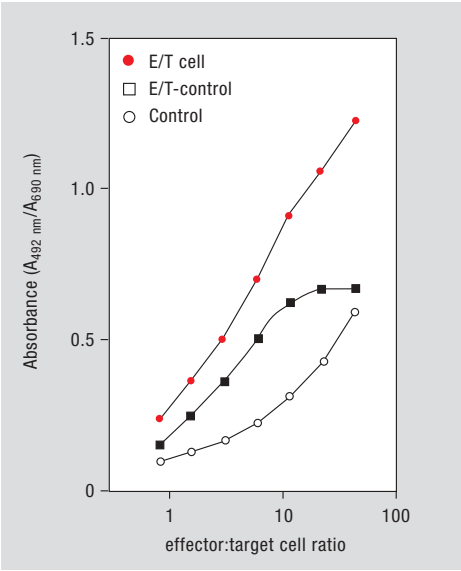
↓

Add 100 µl/well reaction mixture to each supernatant and incubate for 0.5 h

↓

Measure absorbance using an ELISA plate reader

▲ **Flow Chart 13:** Assay procedure, Cytotoxicity Detection Kit (LDH).



◀ **Figure 40. Determination of the cytolytic activity of allogen-stimulated cytotoxic T lymphocytes (CTLs) using the Cytotoxicity Detection Kit (LDH).** Spleen cells of C57/Bl 6 mice (H-2b) were stimulated *in vitro* with P815 cells (H-2d). Viable CTLs were purified and titrated in the microplate as described in the package insert. Target cells (1 x 10^4 cells/well) were incubated in the presence or absence (effector cell controls) of effector cells for 4 hours. Culture supernatant samples (100 µl/well) for effector controls and the effector-target cell mix were assayed for LDH activity. The middle curve is generated when the background control values are subtracted from the effector-target cell values.

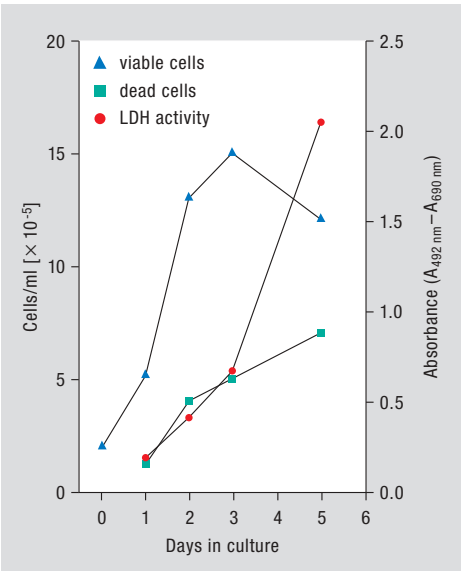
Can be used to assay:

- Cell-free supernatants obtained from cells cultured in 96-well microplates or batch cultures.

Kit contents

1. Catalyst (Diaphorase/NAD + mixture)
2. Dye solution (INT and sodium lactate)
   Note: *To prepare the reaction mixture, mix catalyst with dye solution prior to use. Purified LDH (Cat. No. 107 077) may be used as a positive control.*

Typical results: see Figures 40 and 41.



◀ **Figure 41: Correlation of cell death (defined by increased plasma membrane permeability) and LDH release.** Ag8 cells (starting cell concentration: 2 x 10^5 /ml) were cultured and after 1, 2, 3 and 5 days, aliquots were removed. The amount of viable (▲) and dead (■) cells was determined by trypan blue exclusion. LDH activity in cell free culture supernatant was determined using the Cytotoxicity Detection Kit (●).
Result: Increased LDH release clearly correlated with the increase of dead cells.

Other applications: For more examples of how the Cytotoxicity Detection Kit (LDH) can be used in the lab, see Appendix, page 122.

Cellular DNA Fragmentation ELISA

Cat. No. 1 585 045 500 tests

Type	Sandwich ELISA, colorimetric
Useful for	Quantitation of BrdU-labeled DNA fragments either released from cells during necrosis or cell-mediated cytotoxicity, or within the cytoplasm of apoptotic cells
Samples	Cell-free supernatants from cultured cells or cytoplasmic lysates of cells, prelabeled with BrdU
Method	Prelabeling of cells with BrdU, followed by immunodetection of BrdU-labeled DNA fragments in sample
Time	4.5–5.5 h (+ BrdU labeling and induction of cell death)

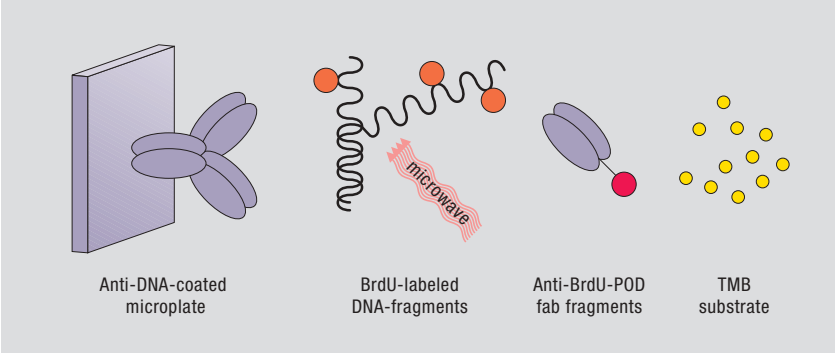
Significance of kit: The Cellular DNA Fragmentation ELISA measures apoptosis, necrosis, or cell mediated cytotoxicity by quantitating the fragmentation and/or release of BrdU-labeled DNA. The kit detects DNA fragments:

- In the cytoplasm of apoptotic cells, thus providing a non-radioactive alternative to the [^{3}H]-thymidine-based DNA fragmentation assay.
- Released into the culture supernatant during cell mediated cytotoxicity, thus providing a non-radioactive alternative to the [^{3}H]-thymidine- and [^{51}Cr]-release assays.

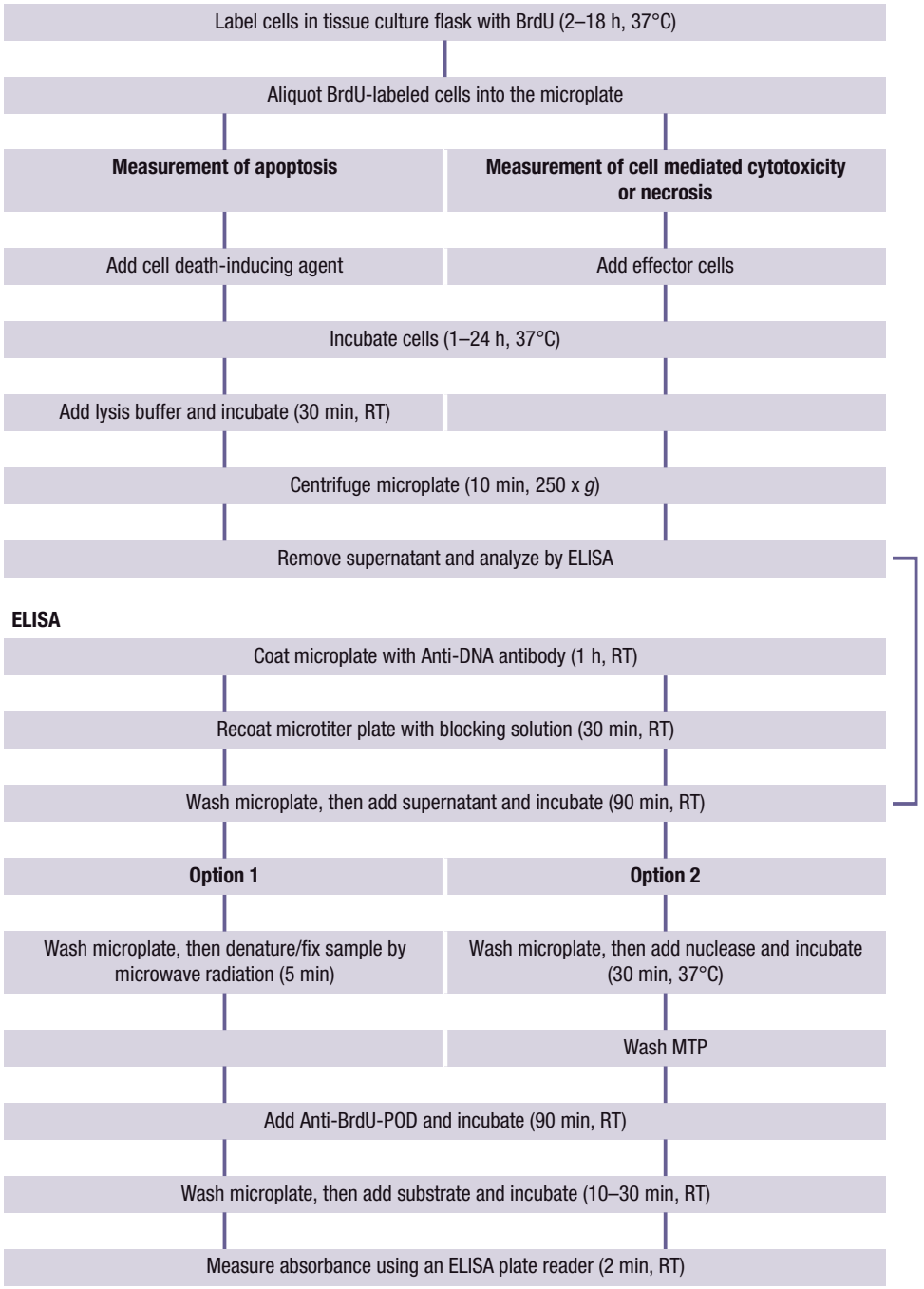
Test principle: The assay is a sandwich enzyme-linked immunosorbent assay (ELISA). It uses two mouse monoclonal antibodies: one directed against DNA the other against BrdU (Figure 42). The procedure (Flow Chart 14) involves:

1. Prelabeling of cells with BrdU.
2. Incubating the labeled cells in the presence of either an apoptosis inducing agent or effector cells (for cell mediated cytotoxicity). At the end of the incubation, cells are centrifuged and either supernatant is analyzed (for cell mediated cytotoxicity or necrosis) or cellular lysate is analyzed for apoptosis. The supernatant, containing LMW-DNA is used for the assay. If desired, both sample types can be prepared and assayed (See Flow Chart 14).
3. Adsorbing the Anti-DNA antibody onto the wells of a microplate.
4. Adding the supernatant of Step 2 to the microplate. BrdU-labeled DNA fragments in the sample bind to the immobilized Anti-DNA antibody.
5. Denaturing the immunocomplexed BrdU-labeled DNA-fragments by microwave irradiation or nuclease treatment. This procedure is necessary for the accessibility of the BrdU antigen.
6. Reacting Anti-BrdU antibody peroxidase conjugate (Anti-BrdU-POD) with the BrdU-labeled DNA to form an immunocomplex.
7. Quantitating the bound Anti-BrdU-POD in the immunocomplex with a peroxidase substrate (TMB).

Figure 42: How the Cellular DNA Fragmentation ELISA works.

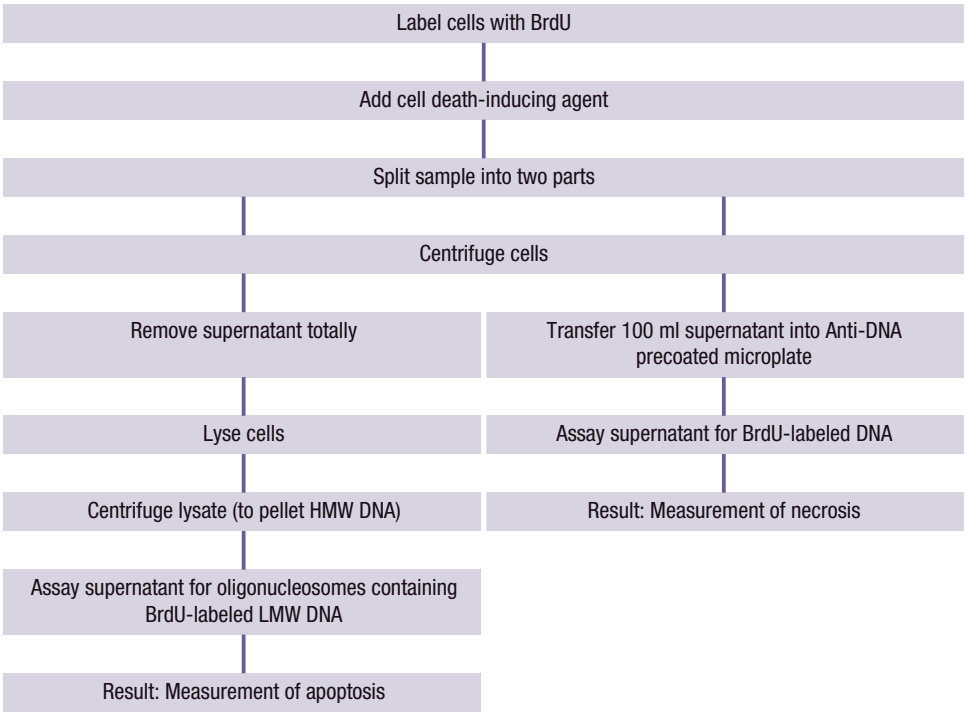


Sample preparation:



◀ **Flow Chart 14:** Assay procedure, Cellular DNA Fragmentation ELISA.

Flow Chart 15: Simultaneous analysis of apoptosis and necrosis in the same sample with the Cellular DNA Fragmentation ELISA. ▶



Sensitivity

Apoptosis: When HL60/CAM is used as a model system for apoptosis, the ELISA can detect BrdU-labeled DNA fragments in the cytoplasm of 1 x 10^3 cells/well (Figure 43).

Cell mediated cytotoxicity: When allogeneic-stimulated cytotoxic T cells are used as effector cells to lyse P815 target cells in a cell mediated cytotoxicity assay, the ELISA can detect BrdU-labeled DNA fragments from 2 x 10^3 target cells/well.

Note: *The ability to detect a minimum number of dying/dead cells in a particular sample strongly depends on the kinetics of cell death, the cytotoxic agent or the effector cells used to induce cell death, and the amount of BrdU incorporated into the target cells.*

Specificity

- ▶ The Anti-DNA antibody binds to single-and double-stranded DNA. It shows no cross-reactivity with BrdU.
- ▶ The conjugated antibody (Anti-BrdU-POD, Fab fragments) will bind to BrdU-labeled DNA after the DNA is partially denatured. The antibody specifically recognizes 5-bromo-2'-deoxyuridine. The antibody conjugate shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.
- ▶ The ELISA specifically detects BrdU-labeled DNA fragments in culture supernatant and cytoplasm. The ELISA can detect BrdU-labeled DNA from any species, so the assay is not species-restricted.

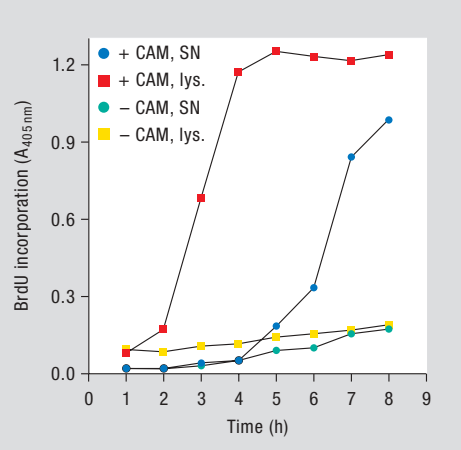
Can be used to assay:

- Culture supernatant and cytoplasmic fractions (lysates) of cells whose DNA have been metabolically prelabeled with BrdU (e.g. cell lines and other *in vitro* proliferating cells). Thus, only cells which proliferate *in vitro* can be used.

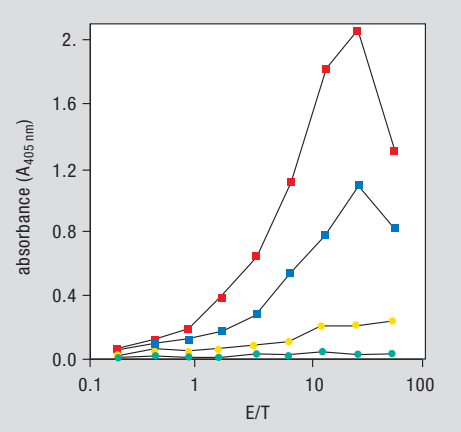
Kit contents

1. Anti-DNA antibody (clone M-CA-33)
2. Anti-BrdU-POD, Fab fragments (clone BMG 6H8)
3. Coating buffer
4. Washing buffer
5. Incubation buffer
6. Substrate solution
7. BrdU labeling reagent
8. Adhesive cover foils

Typical results: see Figures 43 and 44.



▲ **Figure 43: Kinetics of camptothecin (CAM) induced cell death in HL60 cells.** Cells were prelabeled with BrdU overnight. Then, cells (1 x 10^4/well) were incubated either in the presence of 200 ng/ml CAM (●, ■) or without CAM (●, ■) for 1–8 h. Supernatant (100 µl/well) was removed, then cells were lysed and both supernatant (●, ●) and lysate (■, ■) were analyzed by Cellular DNA Fragmentation ELISA.
Result: Apoptosis clearly occurs after 3–4 h incubation. After 6–8 h, secondary necrosis begins to be seen.



▲ **Figure 44: Kinetics of cytotoxic T lymphocyte-mediated cytotoxicity in P815 target cells quantified with the Cellular DNA Fragmentation ELISA.** 2 x 10^4 BrdU-labeled target cells/well were incubated with CTLs at different effector-to-target ratios (E/T) for varying times. After incubation, culture supernatant samples (100 µl/well) were assayed for DNA fragments. 1 h (●), 2 h (●), 4 h (■), and 6 h (■).

Other applications: For more examples of how the Cellular DNA Fragmentation ELISA can be used in the lab, see Appendix, page 120.

1.3.2.2 Assays that measure metabolic activity

Living (metabolically active) cells reduce tetrazolium salts to colored formazan compounds; dead cells do not. Thus, tetrazolium salt-based colorimetric assays detect viable cells exclusively. Because they are sensitive, these assays can readily be performed in a microplate with relatively few cells.

Since a cytotoxic factor will reduce the rate of tetrazolium salt cleavage by a population of cells, these metabolic activity assays are frequently used to measure factor-induced cytotoxicity or cell necrosis[53, 54]. Applications include:

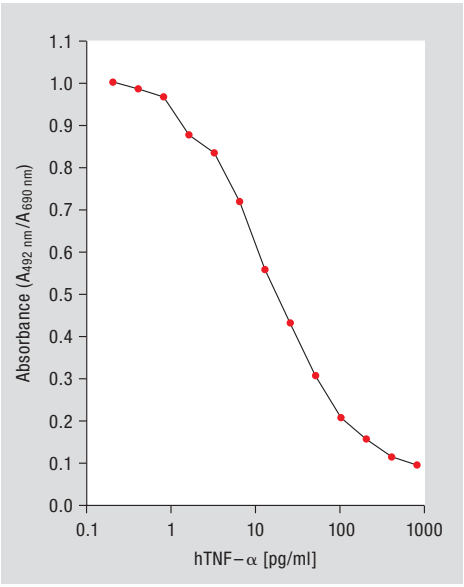
- Assessment of growth-inhibitory or cytotoxic effects of physiological mediators (Figure 45).
- Analysis of the cytotoxic and cytostatic effects of potential anti-cancer and other drugs (Figure 46).
- Analysis of cytopathic effects of viruses and screening of compounds with potential anti-viral activity.
- Screening of antibodies for growth-inhibiting potential.

Roche Molecular Biochemicals offers three microplate-based metabolic activity assays. All may be used to assay factor-induced cytotoxicity or necrosis. They are:

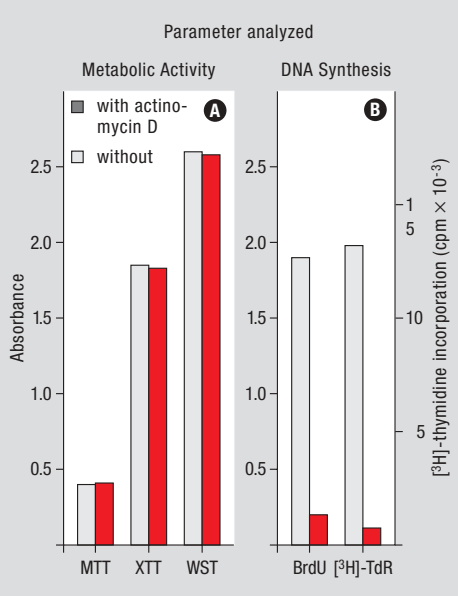
- **Cell Proliferation Kit I (MTT)**, Cat. No. 1 465 007, in which metabolically active cells cleave the tetrazolium salt MTT to a water-insoluble formazan that can be solubilized and quantitated with an ELISA plate reader (for a more detailed description of this kit, see page 75 in this guide).
- **Cell Proliferation Kit II (XTT)**, Cat. No. 1 465 015, in which metabolically active cells cleave the modified tetrazolium salt XTT to a water-soluble formazan, which may be directly quantitated with an ELISA plate reader (for a more detailed description of this kit, see page 76 in this guide).
- **Cell Proliferation Reagent WST-1**, Cat. No. 1 644 807, a modified tetrazolium salt that can be cleaved by metabolically active cells to a water-soluble formazan, which may be directly quantitated with an ELISA plate reader (for a more detailed description of this reagent, see page 77 in this guide).

Note: *Since proliferating cells are metabolically more active than non-proliferating (resting) cells, these tetrazolium salt-based assays are also frequently used to measure cell activation and proliferation. For a full discussion of this application, see Section 2.2.1.1 on page 72 of this guide.*

For a more complete discussion of the principles behind these metabolic assays, see the topic, "Biochemical and cellular basis of cell proliferation assays that use tetrazolium salts" (Appendix, page 113) in this guide.



▲ **Figure 45: Measurement of the cytotoxic effects of human tumor necrosis factor alpha (hTNF-α) on the mouse fibrosarcoma cell line WEHI-164.** Cells in culture (10^6 cells/ml) were preincubated with actinomycin C (1 µg/ml) for 3 h. Aliquots of these pretreated cells were transferred to a microtiter plate (5 x 10^4 cells/well) and incubated with actinomycin C and various amounts of hTNF-alpha for 24 h. Cellular response to TNF was measured with the Roche Molecular Biochemicals Cell Proliferation Kit II (XTT) and plotted against TNF concentration.
Result: Under the assay conditions, 50% of the WEHI-164 cells were killed by a TNF concentration of 35–40 pg/ml.



◀ **Figure 46: Differentiation of cytotoxic and cytostatic effects of actinomycin D.** A549 cells were added to a microtiter plate (10^4 cells/well) and incubated with (■) or without (□) actinomycin D (10 ng/ml) for 20 h.

Graph A: Some aliquots of actinomycin-treated cells were assayed for cytotoxic effects (changes in metabolic activity). These cells were assayed with either the Cell Proliferation Kit I (MTT), Cell Proliferation Kit II (XTT), or Cell Proliferation Reagent WST-1 (WST). Cells were incubated with each tetrazolium salt for 4 h, then analyzed on an ELISA plate reader.

Graph B: Other aliquots of actinomycin-treated cells were assayed for cytostatic effects (suppression of DNA synthesis). These cells were incubated with either non-radioactive bromodeoxyuridine (BrdU) or tritiated thymidine ([^{3}H]-TdR). Incorporation of BrdU into DNA was determined with the Cell Proliferation ELISA, BrdU (colorimetric). Incorporation of [^{3}H]-TdR into DNA was determined by liquid scintillation counting.

Result: Although actinomycin D is not significantly cytotoxic (as indicated by graph A) under these conditions, it does have a profound cytostatic (proliferation-inhibiting) effect (as indicated by graph B).

1.3.2.3 Summary of methods for studying cytotoxicity.

Method/ Roche Molecular Biochemicals product	Label	Parameter analyzed	Assay principle	Advantages	Limitations	For product information, see
[^{51}Cr] Release Assay[55]	[^{51}Cr] prelabel	Damage/ leakage of plasma membrane	● Viable cells are incubated with $Na_2[^{51}Cr]O_4$, which binds tightly to most intracellular proteins (prelabeling). ● After washing, cells are incubated with cytotoxic agent. During this period, labeled proteins are released into the culture supernatant (SN) due to plasma membrane damage. ● The radioactivity in the SN is determined with a gamma-counter.	● Labeling of proteins by [^{51}Cr] generally independent of the rate of protein synthesis ● Generally not restricted to target cell type: non-proliferating or slow turn-over populations can be studied ● Quantitative measurement over a large logarithmic range ● Measurement of cell death in mixed cell populations; may be used to quantitate cell-mediated cytotoxicity	● Radioactive isotope ● Requires prelabeling and extensive washing of the target cells ● High spontaneous release: assay limited to cytotoxic events causing high cell lysis over short period of time (2–5 h) ● For proper intracellular binding $[^{51}Cr]^{6+}$ has to be converted to $[^{51}Cr]^{3+}$: cells with low metabolic activity may not label sufficiently	
[^{3}H]-Thymidine ([^{3}H]-TdR) Release Assay[56]	[^{3}H]-TdR prelabel	Damage/ leakage of plasma membrane and DNA fragmentation	● Cells proliferating *in vitro* are incubated with [^{3}H] TdR, which is incorporated into the genomic DNA. ● After they are washed, cells are incubated with a cytotoxic agent. During this period, [^{3}H] labeled DNA is released into the culture SN due to plasma membrane damage. ● The radioactivity in the SN and in the pellet is determined with a scintillation β-counter.	● Quantitative measurement over a large logarithmic range ● Low spontaneous release: cytotoxic events causing low cell lysis over a prolonged period of time (8–24 h) can be studied ● Measurement of cell death in mixed cell populations; may be used to quantitate cell-mediated cytotoxicity	● Radioactive isotope ● Requires prelabeling and extensive washing of the target cells ● Limited to target cells proliferating *in vitro*	
DNA Release Assay, nonradioactive **Cellular DNA Fragmentation ELISA**	BrdU prelabel	Damage/leakage of plasma membrane and DNA fragmentation	● Cells proliferating *in vitro* are incubated with BrdU, which is incorporated into the genomic DNA. ● Cells are incubated with a cytotoxic agent or non-labeled effector cells (for cell-mediated cytotoxicity). ● During this period, BrdU-labeled DNA is released into the cytoplasm of apoptotic cells or into the culture SN due to plasma membrane leakage of damaged cells. ● The BrdU-labeled DNA in the SN or cytoplasm is determined with an enzyme linked immunosorbent assay.	● Sensitive (10^3–10^4 cells/test required) ● Labeled cells do not have to be washed ● Optimal for microplate format ● Non-radioactive ● Measurement of cell death in mixed cell populations; may be used to quantitate cell-mediated cytotoxicity ● Possible to measure apoptosis and necrosis in parallel	● Prelabeling of the target cells required ● Can only assay target cells proliferating *in vitro* ● Narrow range of quantitative measurement (only one order of magnitude)	page 56 of this guide
LDH Release Assay[51] **Cytotoxicity Detection Kit, LDH**	none	Damage/leakage of plasma membrane	● Target cells are incubated with cytotoxic agent. During this period, cytoplasmic LDH is released into the culture SN due to plasma membrane damage. ● The LDH activity in the culture SN is measured by a substrate reaction and quantitated with an ELISA plate reader.	● Constitutively expressed ubiquitous protein: assay generally not restricted by target cell type ● Does not require prelabeling and extensive washing of the target cells	● LDH activity in serum: special assay medium (reduced serum concentrations or BSA instead of serum) required ● Spontaneous release of LDH by target cells and effector cells: assay limited to cytotoxic events causing high cell lysis over short period of time (2–8 h)	page 54 of this guide
Metabolic activity assays **Cell Proliferation Kit I (MTT), Kit II (XTT), Reagent (WST-1)**	BrdU	Reduced metabolic activity	● Dye solution is added to cells cultured in MTP and cells are incubated. Viable (metabolically active) cells cleave tetrazolium salts to colored formazan compounds; dead cells do not. ● Amount of formazan is quantitated with an ELISA plate reader.	● No cell type restriction ● Does not require prelabeling and washing of the cells ● Optimal for microplate format	● Increased metabolic activity of effector cells may mask target cell death during cell-mediated cytotoxicity ● No changes in the metabolic activity during the early phases of apoptosis	pages 75–77 of this guide

▲ **Table 13:** Methods for studying cytotoxicity.

Apoptosis Assay Methods
Summary of methods for studying cytotoxicity
1 Cell Death – Apoptosis and Necrosis

2 Cell Proliferation and Viability

2.1 Introduction

Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations involving *in vitro* and *in vivo* studies. Examples of where determination of cell number is useful include the analysis of growth factor activity, serum batch testing, drug screening, and the determination of the cytostatic potential of anticancer compounds in toxicology testing. In such toxicological studies, *in vitro* testing techniques are very useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells.

2.1.1 Terminology of cell proliferation and viability

Usually, one of two parameters is used to measure the health of cells: cell viability or cell proliferation. In almost all cases, these parameters are measured by assaying for "vital functions" that are characteristic of healthy cells.

Cell Viability
Cell viability can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or are quiescent is not distinguished. Cell viability assays are often useful when non-dividing cells (such as primary cells) are isolated and maintained in culture to determine optimal culture conditions for cell populations.

The most straightforward method for determining viable cell number is a direct counting of the cells in a hemocytometer. Sometimes viable cells are scored based on morphology alone; however, it is more helpful to stain the cells with a dye such as trypan blue. In this case, viability is measured by the ability of cells with uncompromised membrane integrity to exclude the dye.

Alternatively, metabolic activity can be assayed as an indication of cell viability. Usually metabolic activity is measured in populations of cells by incubating the cells with a tetrazolium salt (MTT, XTT, WST-1) that is cleaved into a colored formazan product by metabolic activity.

Cell Proliferation
Cell proliferation is the measurement of the number of cells that are dividing in a culture. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto the appropriate matrix and the number of colonies that are formed after a period of growth are enumerated. Drawbacks to this type of technique are that it is tedious and it is not practical for large numbers of samples. In addition, if cells divide only a few times and then become quiescent, colonies may be too small to be counted and the number of dividing cells may be underestimated. Alternatively, growth curves could be established, which is also time-consuming and laborious.

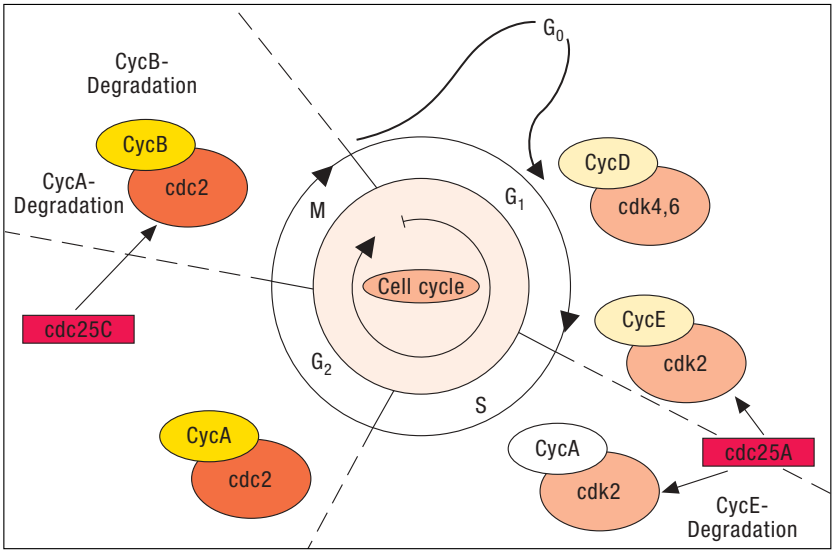
Another way to analyze cell proliferation is the measurement of DNA synthesis as a marker for proliferation. In these assays, labeled DNA precursors (^{3}H-thymidine or bromodeoxyuridine) are added to cells and their incorporation into DNA is quantified after incubation. The amount of labeled precursor incorporated into DNA is quantified either by measuring the total amount of labeled DNA in a population, or by detecting the labeled nuclei microscopically. Incorporation of the labeled precursor into DNA is directly proportional to the amount of cell division occurring in the culture.

Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the cell cycle are measured either by their activity (e.g. CDK kinase assays) or by quantifying their amounts (e.g. Western blots, ELISA, or immunohistochemistry).

2.1.2 Cell Cycle

In an organism, the rate of cell division is a tightly regulated process that is intimately associated with growth, differentiation and tissue turnover. Generally, cells do not undergo division unless they receive signals that instruct them to enter the active segments of the cell cycle. Resting cells are said to be in the G_0 phase (quiescence) of the cell cycle (Figure 47). The signals that induce cells to divide are diverse and trigger a large number of signal transduction cascades.

Figure 47: Cell cycle: A schematic overview. ▶



A thorough discussion of the types of signals and the variety of responses they can elicit are beyond the scope of this guide (Table 14). Generally, signals that direct cells to enter the cell cycle are called growth factors, cytokines, or mitogens.

Abbreviation	Description	Reference
RTK	Receptor Tyrosine Kinase	Marshall, (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**: 179–185.
RAS	GTP exchange protein	White, M. A. et al. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell* **80**: 533–541.
RAF	MAP kinase kinase kinase	Avruch, J. et al. (1994) Raf meets Ras-Completing the framework of a signal transduction pathway. *Trends Biochem. Sci.* **19**: 279–283.
MEK	MAP kinase kinase or MAPk/Erk kinase	Marshall, C. J. (1994) MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Curr. Opin. Genet. Dev.* **4**: 82–89.
MAPK	Mitogen activated protein kinase or Erk	Marshall, C. J. (1994) MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Curr. Opin. Genet. Dev.* **4**: 82–89.
PKC	Protein Kinase C	Blobe, G. et al. (1996) Protein Kinase C isoenzymes: regulation and function. *Cancer Surveys* **27**: 213–248.
JAK	Just Another Kinase or Janus Kinase	Ihle, J. N. et al. (1994) Signaling by the cytokine receptor superfamily: Jaks and STATs. *TIBS* **19**: 222–227.
STAT	Signal Transducers and Activators of Transcription	Ihle, J. N. et al. (1994) Signaling by the cytokine receptor superfamily: Jaks and STATs. *TIBS* **19**: 222–227.
Cyclins		Marx, J. (1994) How cells cycle toward cancer. *Science* **263**: 319–321.
CDK	Cyclin Dependent Kinase	MacLachlan, T. K., Sang, N., and Giordano, A. (1995) Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. *Crit. Rev. Eukaryot. Gene Expr.* **5**: 127–156.
CDC2	Cell division cycle mutant	MacLachlan, T. K., Sang, N., and Giordano, A. (1995) Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. *Crit. Rev. Eukaryot. Gene Expr.* **5**: 127–156.
CAK	CDK Activating Kinase	Morgan, D. O. (1995) Principles of CDK Regulation. *Nature* **374**: 131–134.

▲ **Table 14:** Published sources that contain more information about cell proliferation.

Signal Transduction Pathways

Three major types of signal transduction pathways are activated in cells in response to growth factors or mitogenic stimuli. The response to these stimuli varies from cell type to cell type and the pathways continue to grow more and more complex. These types of pathways continue to be the focus of a great deal of research and, considering the importance of cell cycle regulation in biology, the pathways will continue to grow in complexity for some time to come.

- The MAP kinase (MAPK) type of pathways are triggered through a cascade of phosphorylation events that begins with a growth factor binding to a tyrosine kinase receptor at the cell surface. This causes dimerization of the receptor and an intermolecular cross-phosphorylation of the two receptor molecules. The phosphorylated receptors then interact with adaptor molecules that trigger downstream events in the cascade. The cascade works through the GTP ex-

change protein RAS, the protein kinase RAF (MAPKKK), the protein kinase MEK (MAPKK), and MAP kinase (Erk). MAPK then phosphorylates a variety of substrates that control transcription, the cell cycle, or rearrangements of the cytoskeleton.

- The protein kinase C (PKC) pathways consist of a family of phospholipid dependent protein kinases. PKC is regulated by a large variety of metabolic pathways involving phospholipids and calcium levels within a cell. The main regulator of the pathway is diacylglycerol (DAG) which appears to recruit PKC to the plasma membrane and cause its activation. The activity of DAG is mimicked by the phorbol-ester tumor promoters. Once activated, PKC can phosphorylate a wide variety of cellular substrates that regulate cell proliferation and differentiation. Responses to PKC appear to vary with the types of PKCs expressed and the types of substrates available within a cell. Some evidence shows that the PKC pathway may interact with and exert effects through the MAPK pathway.
- The JAK/STAT pathway is activated by cytokine interaction with a family of receptors called the cytokine receptor superfamily. These receptors do not contain a protein kinase domain themselves, but they associate with and activate a family of protein kinases called the JAK (Just Another Kinase or JAnus Kinase) family. JAK family members are recruited to receptor complexes that are formed as a result of ligand binding. The high concentration of JAK in the complex leads to a cross-phosphorylation of JAK and thus activation. JAK then phosphorylates members of another protein family called STAT (signal transducers and activators of transcription). These proteins then translocate to the nucleus and directly modulate transcription.

Control of the Cell Cycle

Once the cell is instructed to divide, it enters the active phase of the cell cycle, which can be broken down into four segments:

- During G_1 (G = gap), the cell prepares to synthesize DNA. In the latter stages of G_1, the cell passes through a restriction point (R) and is then committed to complete the cycle.
- During S phase the cell undergoes DNA synthesis and replicates its genome.
- During G_2 the cell prepares to undergo division and checks its replication using DNA repair enzymes.
- During M phase, the cell undergoes division by mitosis or meiosis and then re-enters G_1 or G_0.

In most instances, the decision for a cell to undergo division is regulated by the passage of a cell from G_1 to S phase. Progression through the cell cycle is controlled by a group of kinases called cyclin-dependent kinases (CDKs), (see Figure 42). CDKs are thought to phosphorylate cellular substrates, such as the retinoblastoma gene, that are responsible for progression into each of the phases of the cell cycle. CDKs are activated by associating with proteins whose levels of expression change during different phases of the cell cycle. These proteins are called cyclins. Once associated with cyclins, CDKs are activated by phosphorylation via CDK-activating kinase (CAKs) or by dephosphorylation via a phosphatase called CDC25.

D-types cyclins are the primary cyclins that respond to external cellular factors. Their levels start off low during G_1 and increase towards the G_1/S boundary. Cyclin D regulates CDK4 and CDK6. Cyclin E is expressed transiently during the G_1/S transition and is rapidly degraded once the cell enters S. Cyclin E regulates CDK2 and perhaps CDK3. When S phase begins, levels of cyclin A increase and activate CDK2. The cyclin A/CDK2 complex is thought to have a direct role in DNA replication. The progression through mitosis is regulated by the presence of cyclin B. Cyclin B associates with CDC2 and forms the primary kinase present during mitosis (MPF = "M-phase/maturation promoting factor"). During anaphase cyclin B is degraded. This degradation of cyclin B appears to regulate the cell's progression out of mitosis and into G_1.

2.2 Cell proliferation/viability assay methods

A variety of methods have been devised that measure the viability or proliferation of cells *in vitro* and *in vivo*. These can be subdivided into four groups:

1. Reproductive assays can be used to determine the number of cells in a culture that are capable of forming colonies *in vitro*. In these types of experiments, cells are plated at low densities and the number of colonies is scored after a growth period. These clonogenic assays are the most reliable methods for assessing viable cell number[57, 58, 59]. These methods, however, are very time-consuming and become impractical when many samples have to be analyzed.

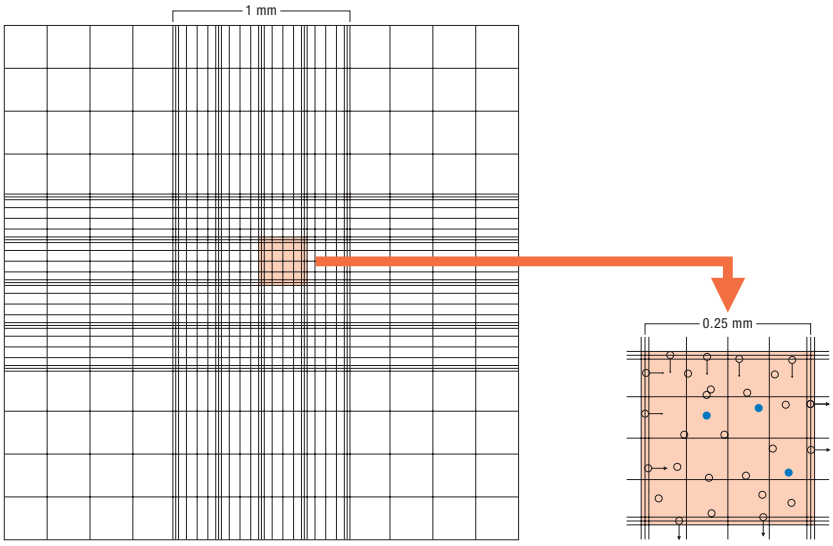
2. Permeability assays involve staining damaged (leaky) cells with a dye and counting viable cells that exclude the dye. Counts can either be performed manually using a hemocytometer and for example trypan blue. (Figure 48). This method is quick, inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to determine the cell concentration (cell number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment[60].

Or counts can be performed mechanically using for example a flow cytometer and propidium iodide. Alternatively, membrane integrity can be assayed by quantifying the release of substances from cells when membrane integrity is lost, e. g. Lactate dehydrogenase (LDH) or ^{51}Cr (described in section 1.3.2.1 starting on page 53 of this guide.)

3. Metabolic activity can be measured by adding tetrazolium salts to cells. These salts are converted by viable cells to colored formazan dyes that are measured spectrophotometrically.

4. Direct proliferation assays use DNA synthesis as an indicator of cell growth. These assays are performed using either radioactive or nonradioactive nucleotide analogs. Their incorporation into DNA is then measured.

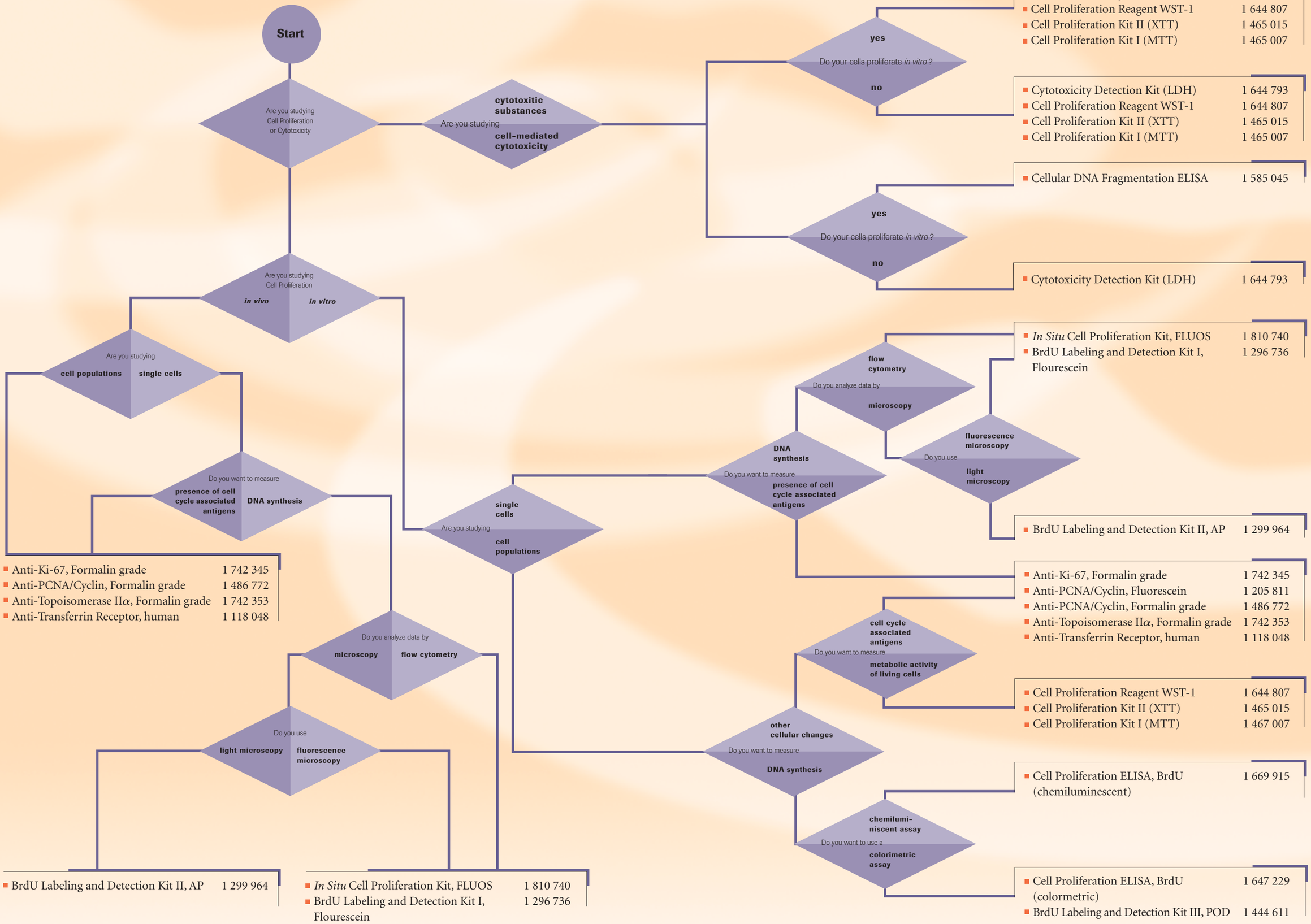
Figure 48: Measurement of proliferation by counting the cells with a hemocytometer. The addition of trypan blue helps to distinguish viable, unstained cells (○) from non-viable, blue-stained cells (●). ▼



The first section describes those assays designed to study cell proliferation in whole populations of cells, followed by a section covering proliferation assays designed to measure proliferation in individual cells (*in situ*).

For a discussion of the advantages and limitations of all types of cell proliferation assays, read Sections 2.2.1.3 and 2.2.2.3 of this guide.

For discussions of particular assays, turn to the pages indicated in the following method selection guide:


Start
Are you studying Cell Proliferation or Cytotoxicity
cytotoxic substances
Are you studying
cell-mediated cytotoxicity
yes
Do your cells proliferate *in vitro*?
no
Cellular DNA Fragmentation ELISA 1 585 045
Cell Proliferation Reagent WST-1 1 644 807
Cell Proliferation Kit II (XTT) 1 465 015
Cell Proliferation Kit I (MTT) 1 465 007
Cytotoxicity Detection Kit (LDH) 1 644 793
Cell Proliferation Reagent WST-1 1 644 807
Cell Proliferation Kit II (XTT) 1 465 015
Cell Proliferation Kit I (MTT) 1 465 007
Cellular DNA Fragmentation ELISA 1 585 045
yes
Do your cells proliferate *in vitro*?
no
Cytotoxicity Detection Kit (LDH) 1 644 793
Are you studying Cell Proliferation
in vivo
in vitro
Are you studying
cell populations
single cells
Do you want to measure
presence of cell cycle associated antigens
DNA synthesis
Anti-Ki-67, Formalin grade 1 742 345
Anti-PCNA/Cyclin, Formalin grade 1 486 772
Anti-Topoisomerase IIα, Formalin grade 1 742 353
Anti-Transferrin Receptor, human 1 118 048
Do you analyze data by
microscopy
flow cytometry
Do you use
light microscopy
fluorescence microscopy
BrdU Labeling and Detection Kit II, AP 1 299 964
In Situ Cell Proliferation Kit, FLUOS 1 810 740
BrdU Labeling and Detection Kit I, Flourescein 1 296 736
Are you studying
single cells
cell populations
flow cytometry
Do you analyze data by
microscopy
In Situ Cell Proliferation Kit, FLUOS 1 810 740
BrdU Labeling and Detection Kit I, Flourescein 1 296 736
fluorescence microscopy
Do you use
light microscopy
BrdU Labeling and Detection Kit II, AP 1 299 964
DNA synthesis
Do you want to measure
presence of cell cycle associated antigens
Anti-Ki-67, Formalin grade 1 742 345
Anti-PCNA/Cyclin, Fluorescein 1 205 811
Anti-PCNA/Cyclin, Formalin grade 1 486 772
Anti-Topoisomerase IIα, Formalin grade 1 742 353
Anti-Transferrin Receptor, human 1 118 048
other cellular changes
Do you want to measure
DNA synthesis
cell cycle associated antigens
Do you want to measure
metabolic activity of living cells
Cell Proliferation Reagent WST-1 1 644 807
Cell Proliferation Kit II (XTT) 1 465 015
Cell Proliferation Kit I (MTT) 1 467 007
chemilumi-niscent assay
Do you want to use a
colorimetric assay
Cell Proliferation ELISA, BrdU (chemiluminescent) 1 669 915
Cell Proliferation ELISA, BrdU (colormetric) 1 647 229
BrdU Labeling and Detection Kit III, POD 1 444 611

2.2.1 Methods for studying cell proliferation and viability in cell populations

A number of methods have been developed to study cell viability and proliferation in cell populations. The most convenient modern assays have been developed in a microplate format (96-well plates). This miniaturization allows many samples to be analyzed rapidly and simultaneously. The microplate format also reduces the amount of culture medium and cells required as well as cost of plasticware. Colorimetric assays allow samples to be measured directly in the microplate with an ELISA plate reader.

Microplate assays have been developed based on different parameters associated with cell viability and cell proliferation. The most important parameters used are metabolic activity and DNA synthesis for microplate format.

- Cellular damage will inevitably result in loss of the ability of the cell to maintain and provide energy for metabolic cell function and growth. Metabolic activity assays are based on this premise. Usually they measure mitochondrial activity. The cells are incubated with a colorometric substrate (MTT, XTT, WST-1) (described on pages 75–77 of this guide.
- As outlined above, during the S phase the cell undergoes DNA synthesis and replicates its genome. If labeled DNA precursors, in our case BrdU, are added to the cell culture, cells that are about to divide incorporate BrdU into their DNA. The incorporated BrdU can then be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies against BrdU (described on page 83 of this guide).

In the following sections we will describe details of each of these cell viability and proliferation assays.

Besides microplate assays, cell populations can be analyzed for expression of cell cycle associated antigens by Western blotting, described in section 2.2.2.2 on page 97 of this guide.

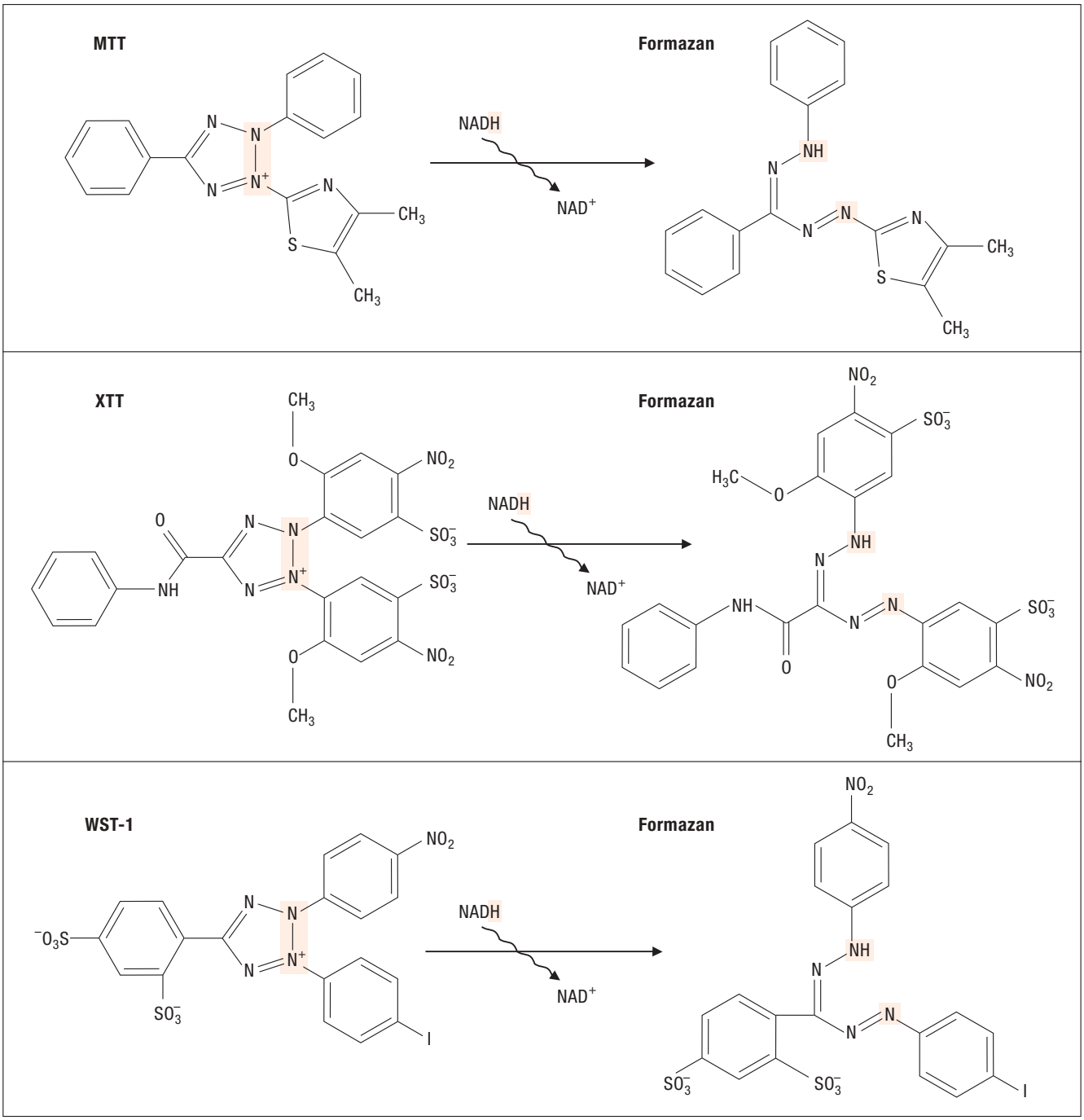
2.2.1.1 Assays that measure metabolic activity

One parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. For example, a microtiter plate assay which uses the tetrazolium salt MTT is now widely used to quantitate cell proliferation and cytotoxicity[53, 65].

Because tetrazolium salts are reduced to a colored formazan only by metabolically active cells, these assays detect viable cells exclusively. For instance, in the MTT assay, MTT is reduced by viable cells to a colored, water-insoluble formazan salt. After it is solubilized, the formazan formed can easily and rapidly be quantitated in a conventional ELISA plate reader at 570 nm (maximum absorbance).

*[**Author's note:** MTT is cleaved to formazan by the "succinate-tetrazolium reductase" system (EC 1.3.99.1) which belongs to the mitochondrial respiratory chain and is active only in viable cells. Interestingly however, recent evidence suggests that mitochondrial electron transport may play a minor role in the cellular reduction of MTT. Since most cellular reduction occurs in the cytoplasm and probably involves the pyridine nucleotide cofactors NADH and NADPH, the MTT assay can no longer be considered strictly a mitochondrial assay.]*

More recently, modified tetrazolium salts like XTT[62, 67], MTT[68], and WST-1 (Figure 50) have become available. The major advantage of these new compounds is that viable cells convert them to a water-soluble formazan. Thus, a metabolic assay with any of these compounds requires one less step (solubilization of product) than an assay with MTT. In addition, WST-1 is stable enough to be packaged as a ready-to-use solution.



▲ **Figure 50:** Molecular structure of MTT, XTT, WST-1 and their corresponding reaction products.

Since proliferating cells are metabolically more active than non-proliferating (resting) cells, the assays are suitable not only for the determination of cell viability and factor-mediated cytotoxicity (see Section 1.3.2.2.) but also for the determination of cell activation and proliferation. However, one has to keep in mind that under non-ideal cell culture conditions (such as the pH and D-glucose concentration in culture medium), the MTT response may vary greatly in viable cells due to the metabolic state of the cells (e.g., cellular concentration of pyridine nucleotides)[65, 69].

These colorimetric assays are very rapid and convenient. Because this technique needs no washing or harvesting of the cells,

the complete assay from the start of the microculture to data analysis in an ELISA plate reader is performed in the same microplate. In addition, an ELISA plate reader linked with an on-line computer allows rapid and automated data processing (Figure 51).

Roche Molecular Biochemicals offers three microplate-based assays similar to the ones described in this section. All three assays are suitable for measurement of cell proliferation in response to growth factors, cytokines, mitogens and nutrients.

One of these assays uses MTT, which forms an **insoluble** formazan product; the other two use tetrazolium salts (XTT and WST-1) that form **soluble** formazan products. All three assays are described on the following pages.

Note: *For a more detailed discussion of the principles behind these metabolic assays, see the topic, "Biochemical and cellular basis of cell proliferation assays that use tetrazolium salts" (Appendix, page 113) in this guide.*

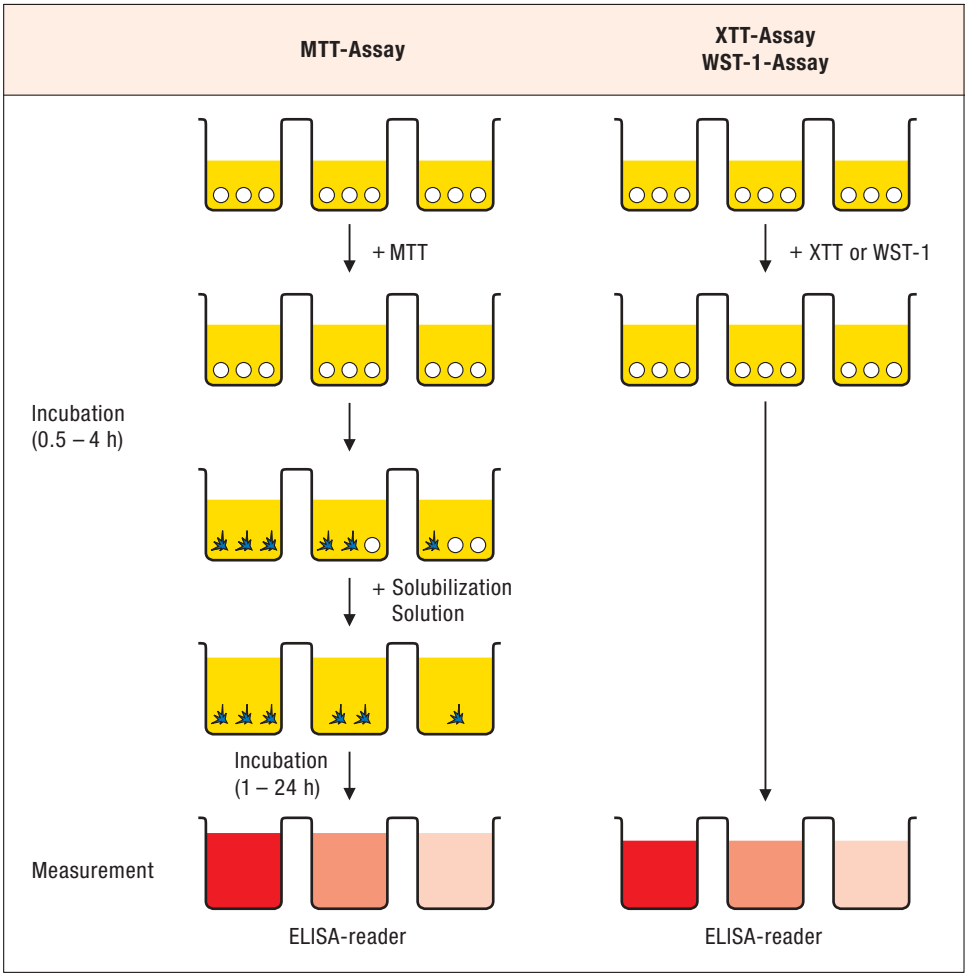


Figure 51: Measurement of metabolic activity using the tetrazolium salts MTT, XTT and WST-1. ▶

Cell Proliferation Kit I (MTT)

Cat. No. 1 465 007 2500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability and proliferation as well as cytotoxicity
Samples	Adherent or suspension cell cultures
Method	Incubation of cells with MTT, followed by solubilization and spectrophotometric assay of colored product
Time	5–28 h

Significance of kit: The Cell Proliferation Kit I (MTT) measures the metabolic activity of viable cells. The assay is nonradioactive and can be performed entirely in a microplate. It is suitable for measuring cell proliferation, cell viability or cytotoxicity (see section 1.3 on page 52 of this guide).

Test principle: The assay is based on the reduction of the tetrazolium salt MTT by viable cells. The reaction produces a water-insoluble formazan salt which must be solubilized. The procedure involves:

1. Culturing the cells in a 96-well microplate, then incubating them with MTT solution for approx. 4 h. During this incubation period, viable cells convert MTT to a water-insoluble formazan dye.
2. Solubilizing the formazan dye in the microplate.
3. Quantitating the dye with an ELISA plate reader. The absorbance directly correlates with the cell number.

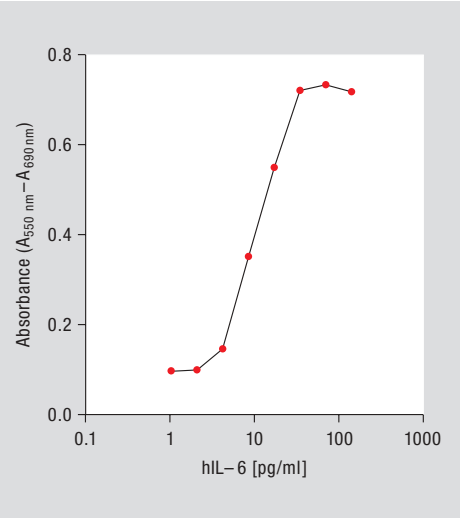
Can be used to assay:

- Adherent and suspension cells cultured in microplate.

Kit contents

1. MTT labeling reagent
2. Solubilization solution

Typical results: see Figures 52 and 54.



▲ **Figure 52: Measurement of human Interleukin 6 (hIL-6) activity on the mouse hybridoma cell line 7TD1.** Cells (2 x 10^3/well) were incubated in the presence of various amounts of hIL-6. After 4 days incubation, cell proliferation was analyzed by Cell Proliferation Kit I (MTT).

Other applications: For more examples of how the Cell Proliferation Kit I (MTT) can be used in the lab, see Appendix, page 125.

Cell Proliferation Kit II (XTT)

Cat. No. 1 465 015 2500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability, proliferation, or cytotoxicity
Samples	Adherent or suspension cell cultures
Method	Incubation of cells with XTT, followed by spectrophotometric assay of colored product
Time	4 h

Significance of kit: The Cell Proliferation Kit II (XTT) measures the metabolic activity of viable cells. The assay is nonradioactive and can be performed entirely in a microplate. It is suitable for measuring cell proliferation, cell viability, or cytotoxicity (see section 1.3 on page 52 of this guide).

Test principle: The assay is based on the reduction of the tetrazolium salt XTT by viable cells in the presence of an electron coupling reagent. The reaction produces a soluble formazan salt. The procedure involves:

1. Culturing the cells in a 96-well microplate, then incubating them with XTT solution for approx. 4 h. During this incubation period, viable cells convert XTT to a water-soluble formazan dye.
2. Quantitating the formazan dye in the microplate with an ELISA plate reader. The absorbance directly correlates with the cell number.

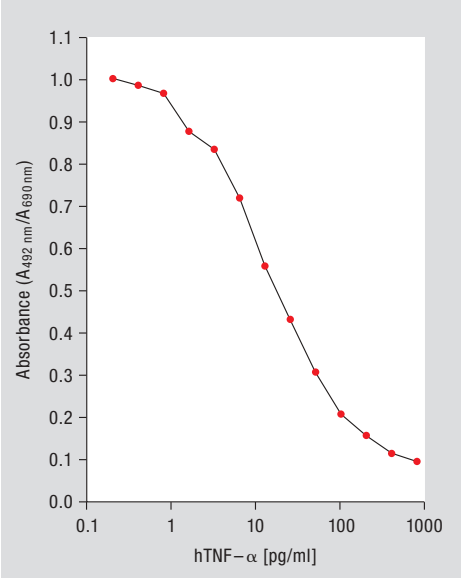
Can be used to assay:

- Adherent and suspension cells cultured in microplate.

Kit contents
1. XTT Labeling reagent
2. Electron-coupling reagent

Note: *To prepare XTT labeling mixture, mix XTT labeling reagent with electron-coupling reagent prior to use.*

Typical results: see Figure 53.



▲ **Figure 53: Measurement of human tumor necrosis factor α (hTNFα) activity on the mouse fibrosarcoma cell line WEHI-164.** After preincubation of the cells (1 x 10^6/ml) with actinomycin C (1 µg/ml) for 3 h, cells (5 x 10^4/well) were incubated in the presence of actinomycin C and various amounts of hTNFα for 24 h. The cellular response was analyzed by Cell Proliferation Kit II (XTT).

Other applications: For more examples of how the Cell Proliferation Kit II (XTT) can be used in the lab, see Appendix, page 125.

Cell Proliferation Reagent WST-1

Cat. No. 1 644 807 2500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability, proliferation, or cytotoxicity
Samples	Adherent or suspension cell cultures
Method	Incubation of cells with WST-1, followed by spectrophotometric assay of colored product
Time	0.5–4 h

Significance of reagent: The Cell Proliferation Reagent WST-1 is a ready-to-use substrate which measures the metabolic activity of viable cells. The WST-1 assay is nonradioactive and can be performed entirely in a microplate. It is suitable for measuring cell proliferation, cell viability or cytotoxicity (see section 1.3 on page 52 of this guide).

Test principle: The assay is based on the reduction of WST-1 by viable cells. The reaction produces a soluble formazan salt. The procedure involves:

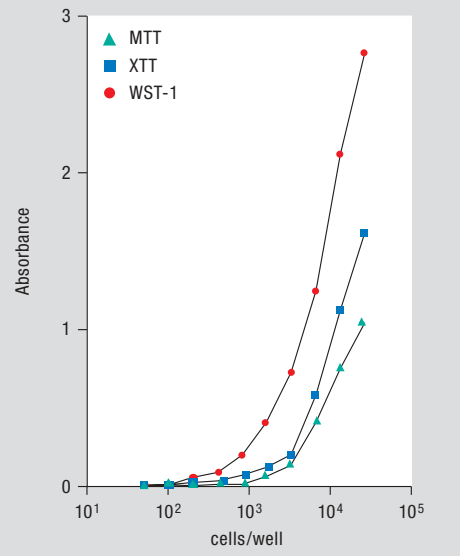
1. Culturing the cells in a 96-well microplate, then incubating them with WST-1 for approx. 0.5–4 h. During this incubation period, viable cells convert WST-1 to a water-soluble formazan dye.
2. Quantitating the formazan dye in the microplate with an ELISA plate reader. The absorbance directly correlates with the cell number.

For a detailed comparison of the WST-1 assay procedure with the MTT and XTT assays, see Flow Chart 16.

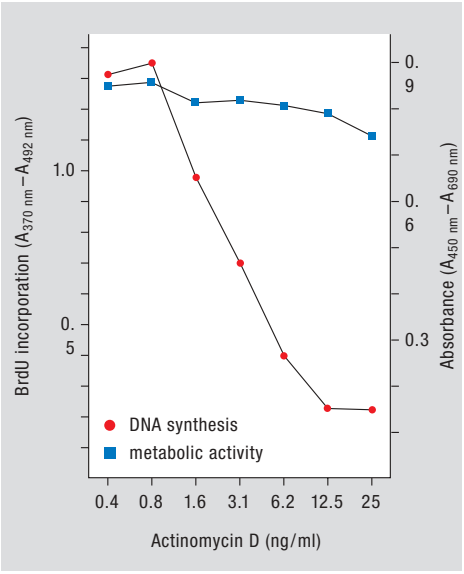
Can be used to assay:

- Adherent and suspension cells cultured in microplate.

Typical results: see Figures 54–55.



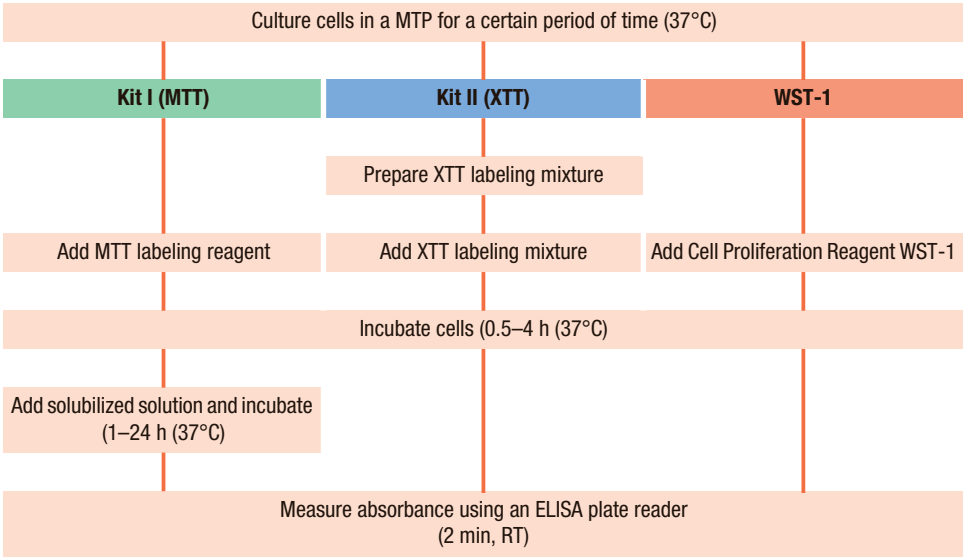
▲ **Figure 54: Comparison of the sensitivity of various tetrazolium salts.** P815 cells were preincubated at various concentrations for 20 h before MTT (▲), XTT (■) or Cell Proliferation Reagent WST-1 (●) was added. After 4 h substrate reaction, the absorbance was determined at the respective wavelength with an ELISA plate reader.



▲ **Figure 55: Combined use of the Cell Proliferation Reagent WST-1 and the Cell Proliferation ELISA, BrdU (colorimetric) for the simultaneous measurement of cell viability and cell proliferation.** A549 cells (1 x 10^4/well in 100 µl) were incubated in the presence of various amounts of actinomycin D for 20 h. After labeling the cells with BrdU for 2 h, additionally Cell Proliferation Reagent WST-1 was added and cells were reincubated for another 2 h. Thereafter, the formazan formed was quantitated at 450 nm with an ELISA plate reader (■). Subsequently, BrdU incorporation was determined using the Cell Proliferation ELISA, BrdU (colorimetric) (●).
Result: Actinomycin D inhibits DNA synthesis (●), but it does not inhibit the metabolic activity of the cell (■). Thus, actinomycin D is cytostatic (inhibition of DNA synthesis) but not cytotoxic (no inhibition of metabolic activity).

Other applications: For more examples of how the Cell Proliferation WST-1 can be used in the lab, see Appendix, page 125.

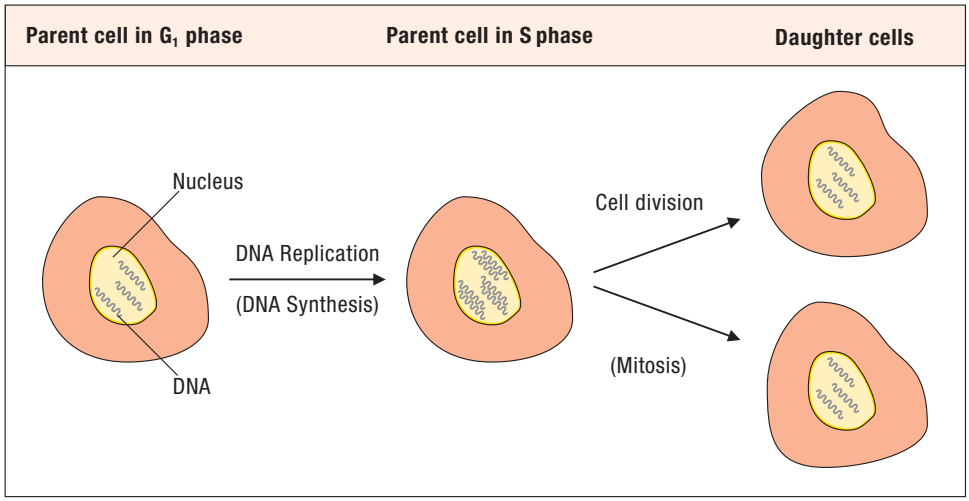
Flow Chart 16: Assay procedures, Cell Proliferation Kit I (MTT), Cell Proliferation Kit II (XTT), and Cell Proliferation Reagent WST-1. ▶



2.2.1.2 Assays that measure DNA synthesis

During cell proliferation the DNA has to be replicated before the cell is devided into two daughter cells.

This close association between DNA synthesis and cell doubling (Figure 56) makes the measurement of DNA synthesis very attractive for assessing cell proliferation. If labeled DNA precursors are added to the cell culture, cells that are about to divide incorporate the labeled nucleotide into their DNA. Traditionally, those assays involve the use of radiolabeled nucleosides, particularly tritiated thymidine ([^{3}H]-TdR). The amount of [^{3}H]-TdR incorporated into the cellular DNA is quantitated by liquid scintillation counting (LSC)[63, 70].

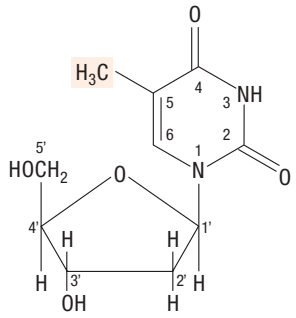
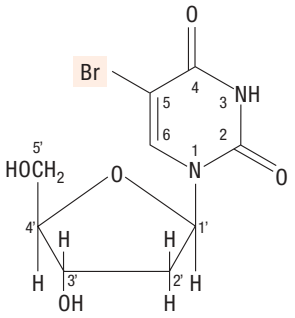


▲ **Figure 56:** Cell proliferation, a close association between DNA synthesis and cell doubling.

Experiments have shown that the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) is incorporated into cellular DNA like thymidine (Figure 57). The incorporated BrdU could be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies directed against BrdU[64]. The use of BrdU for such proliferation assays circumvents the disadvantages associated with the radioactive compound [^{3}H]-TdR.

The first report of this technique involved the extraction and partial purification of DNA from BrdU-labeled proliferating cells, followed by an enzyme immunoassay in a separate assay[71]. Because this method was relatively laborious, the entire BrdU-based procedure was adapted to a 96 well microplate[72]. This adaptation required no harvesting of the cells; the complete assay from the start of the microculture to data analysis by an ELISA plate reader was performed in the same microplate (Figure 58).

Figure 57: Molecular structure of thymidine and BrdU. ▼

Thymidine (5-methyluracil-2'-deoxyribose)	**5-Bromo-2'-deoxyuridine** (5-Bromouracil-2'-deoxyribose)
	

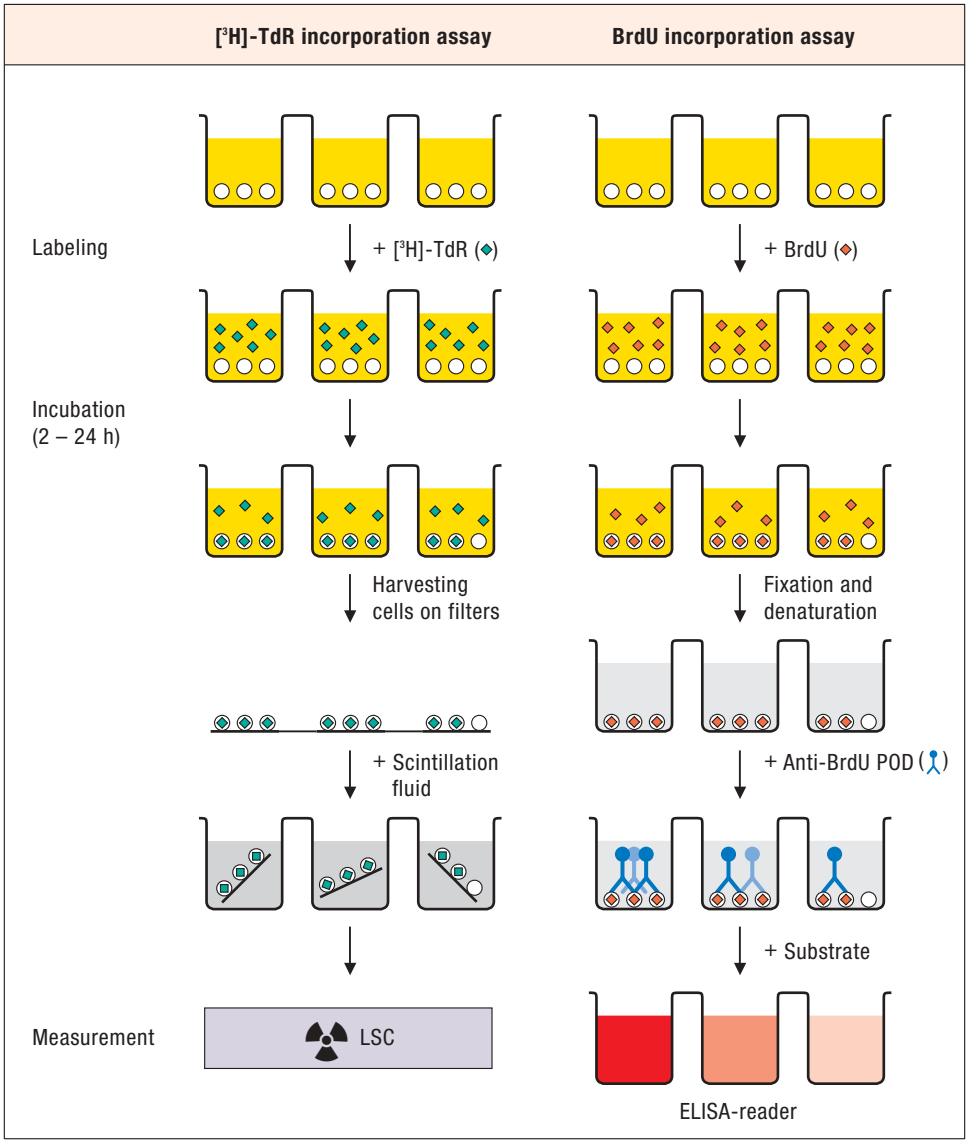


Figure 58: Measurement of DNA synthesis using modified nucleotides [^{3}H]-TdR and BrdU.

Roche Molecular Biochemicals offers three kits that use the convenient BrdU-based assay and the microplate format. The BrdU Labeling and Detection Kit III is a first generation assay. The colorimetric and chemiluminescence Cell Proliferation ELISAs, are second generation assays that offer fewer steps, a faster assay, and greater sensitivity than the first generation assay (Table 15). These three kits are described on the following pages.

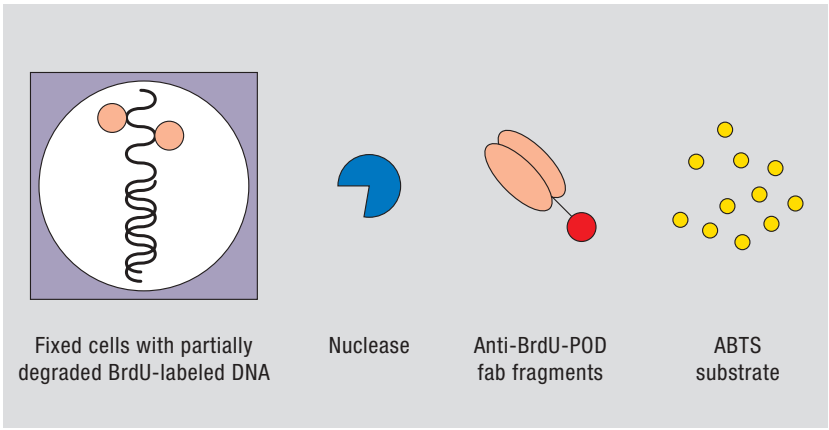
BrdU Labeling and Detection Kit III

Cat. No. 1 444 611 1000 tests

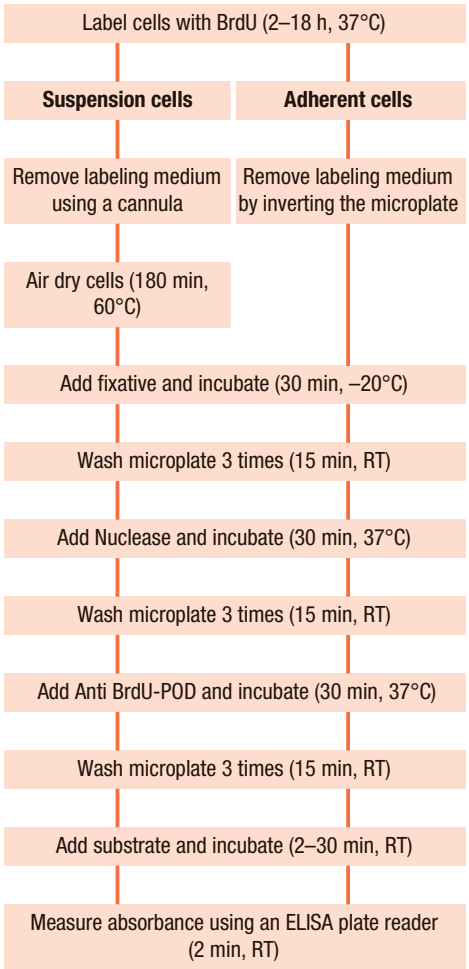
Type	1st generation ELISA with colorimetric detection
Useful for	Quantitation of DNA synthesis during cell activation and proliferation
Sample	Adherent or suspension cell cultures
Method	Incubation of cells with BrdU, followed by partial digestion of DNA and immunodetection of incorporated BrdU label
Time	2.5–6 h (+ cell labeling)

Significance of kit: The BrdU Labeling and Detection Kit III measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells. It offers a nonradioactive alternative to the [^{3}H]-thymidine-based cell proliferation assay.

Test principle: The assay is a cellular immunoassay which uses a mouse monoclonal antibody directed against BrdU (Figure 59 and Flow Chart 17).



▲ **Figure 59:** How the BrdU Labeling and Detection Kit III works.



◀ **Flow Chart 17:** Assay procedure, BrdU Labeling and Detection Kit III.

Note: *This kit belongs to the first generation of kits used to measure DNA synthesis. The same assay procedure has been optimized and improved in the second generation Cell Proliferation ELISA, BrdU (colorimetric) kit (see Table 17).*

Sensitivity: The BrdU Labeling and Detection Kit III is almost as sensitive as the [^{3}H]-thymidine-based cell proliferation assay. The ability to detect a minimum number of proliferating cells in a certain sample strongly depends on the amount of BrdU incorporated into the cells and thus on the labeling period. In most cases, detection requires a labeling period of 2 to 4 h.

Specificity: The antibody conjugate (Anti-BrdU-POD, Fab fragments) will bind to BrdU-labeled DNA after the DNA is denatured. The antibody specifically recognizes 5-bromo-2'-deoxyuridine; it shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.

Can be used to assay:

- Adherent cells as well as cells cultured in suspension in 96-well microplates (e.g. cell lines, activated peripheral blood lymphocytes and other *in vitro* proliferating cells).

Other applications: For examples of how the BrdU Labeling and Detection Kit III can be used in the lab, see Appendix, page 125.

Kit contents

1. BrdU labeling reagent (1000 x), sterile
2. Anti-BrdU-POD Fab fragments
3. Incubation buffer (ready-to-use)
4. Washing buffer (10 x)
5. Nucleases
6. Substrate buffer
7. ABTS substrate tablets
8. Substrate enhancer

Table 15: Improvements of the assay procedure used by the Cell Proliferation ELISA, BrdU (colorimetric) and Cell Proliferation ELISA, BrdU (chemiluminescence) described on the following pages. ▶

Parameter	BrdU Labeling and Detection Kit III	Cell Proliferation ELISA BrdU (colorimetric) Cell Proliferation ELISA, BrdU (chemiluminescence)
Incubation steps	3	2
Washing steps	3–4	1
Working solutions	6 (4 included in the kit)	4 (all included in the kit)
Assay time	2.5–6 h	1.5–2.5 h
Incubation temperatures	–20°C: Fixation RT: Substrate reaction 37°C: Nuclease treatment 60°C: Air drying	For Cell Proliferation ELISA, BrdU (colorimetric) each step at RT
Measuring range	Absorbance: 0.1–2.5 U (factor 25)	Same as BrdU Kit III For Cell Proliferation ELISA, BrdU (chemiluminescence): rlu/s: 10^3–10^6 (factor 1000)
Sensitivity	Almost as sensitive as [^{3}H]-TdR	As sensitive as [^{3}H]-TdR

Cell Proliferation ELISA, BrdU (colorimetric)

Cat. No. 1 647 229 1000 tests

Cell Proliferation ELISA, BrdU (chemiluminescence)

Cat. No. 1 669 915 1000 tests

Type	2nd generation ELISAs with colorimetric or chemiluminescent detection
Useful for	Quantitation of DNA synthesis during cell activation and proliferation
Samples	Adherent or suspension cell cultures
Method	Incubation of cells with BrdU, followed by immunodetection of incorporated BrdU label
Time	1.5–2.5 h (+ cell labeling)

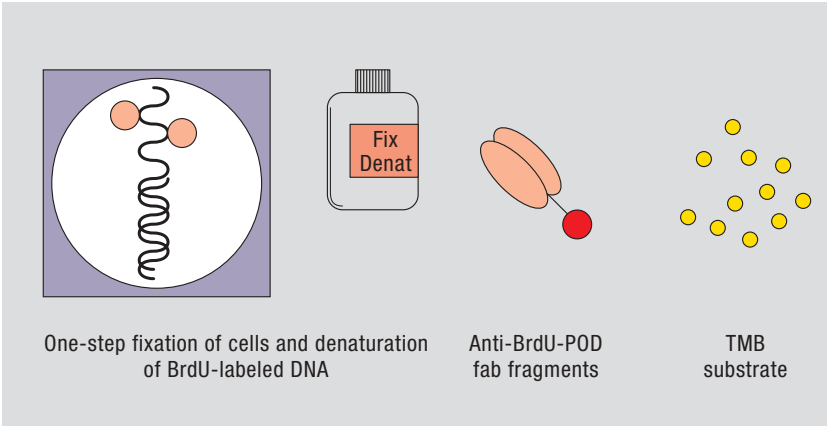
Note: *These two kits belong to the second, improved generation of kits for measuring DNA synthesis (see Table 16).*

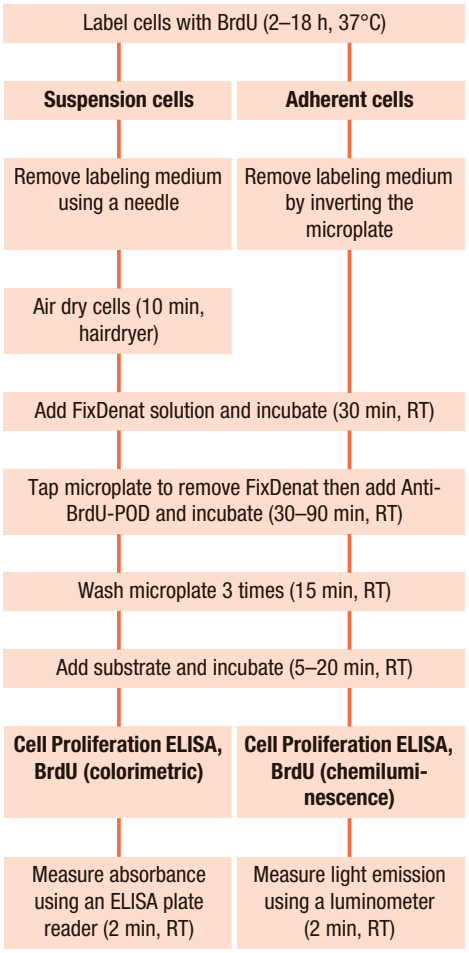
Significance of the kits: The two Cell Proliferation ELISAs measure cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells. They offer a nonradioactive alternative to the [^{3}H]-thymidine-based cell proliferation assay with comparable sensitivity.

Test principle: The assay is a cellular immunoassay which uses a mouse monoclonal antibody directed against BrdU. The procedure (Figure 60, Flow Chart 18) involves:

1. Culturing the cells in a 96-well microtiterplate and pulse-labeling them with BrdU. Only proliferating cells incorporate BrdU into their DNA.
2. Fixing the cells with FixDenat solution. This FixDenat solution also denatures the genomic DNA, exposing the incorporated BrdU to immunodetection.
3. Locating the BrdU label in the DNA with a peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD).
4. Quantitating the bound anti-BrdU-POD with a peroxidase substrate. TMB is used as a substrate in the Cell Proliferation, BrdU (colorimetric). Luminol/4-iodophenol is used as a substrate in the Cell Proliferation, BrdU (chemiluminescence).

Figure 60: How the Cell Proliferation ELISA, BrdU (colorimetric) works. ▼





▲ **Flow Chart 18:** Assay procedures, Cell Proliferation ELISA, BrdU (colorimetric) and Cell Proliferation ELISA, BrdU (chemiluminescence).

Sensitivity: The Cell Proliferation ELISA BrdU (colorimetric) and Cell Proliferation ELISA, BrdU (chemiluminescence) are as sensitive as the [^{3}H]-thymidine-based cell proliferation assay.

Note: *The ability to detect a minimum number of proliferating cells in a certain sample depends on the amount of BrdU incorporated into the cells and thus on the labeling period. In most cases, detection requires a labeling period of 2 to 24 h.*

The use of a chemiluminescence substrate allows the measurement of cell proliferation over a broad range. This range is directly comparable to the measuring range of the [^{3}H]-thymidine-based cell proliferation assay.

Specificity: The antibody conjugate (anti-BrdU-POD, Fab fragments) will bind to BrdU-labeled DNA after the DNA is denatured. The antibody specifically recognizes 5-bromo-2'-deoxyuridine; it shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.

Can be used to assay:

- Adherent cells as well as cells in suspension cultured in 96-well microplates (e.g. cell lines, activated peripheral blood lymphocytes and other *in vitro* proliferating cells).

Kit contents
Cell Proliferation ELISA, BrdU (colorimetric):

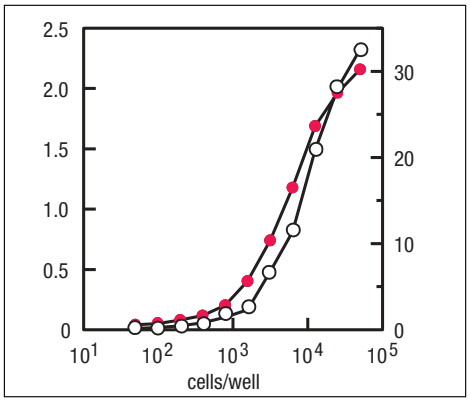
1. BrdU labeling reagent (1000 x), sterile
2. Anti-BrdU-POD Fab fragments
3. Antibody dilution solution (ready-to-use)
4. Washing buffer (10 x)
5. FixDenat (ready-to-use)
6. TMB-substrate solution (ready-to-use)

Cell Proliferation ELISA, BrdU (chemiluminescence):

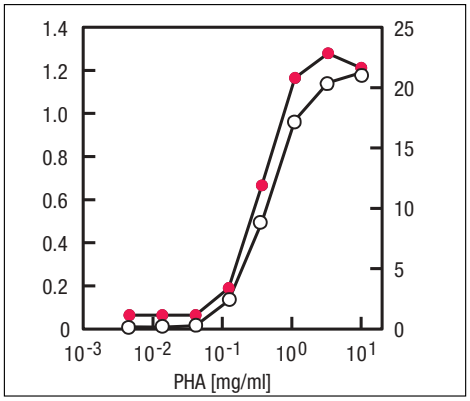
1. BrdU labeling reagent (1000 x), sterile
2. Anti-BrdU-POD Fab fragments
3. Antibody dilution solution (ready-to-use)
4. Washing buffer (10 x)
5. FixDenat (ready-to-use)
6. Substrate component A (luminol/4-iodophenol)
7. Substrate component B (peroxide)

Note: *The FixDenat solution (included in the kits) is also available as a separate reagent (Cat. No. 1 758 764, 4 x 100 ml [enough for 2000 tests]). This ready-to-use solution simplifies detection of BrdU-labeled DNA in ELISA applications, since it simultaneously fixes cells and denatures DNA to expose BrdU epitopes.*

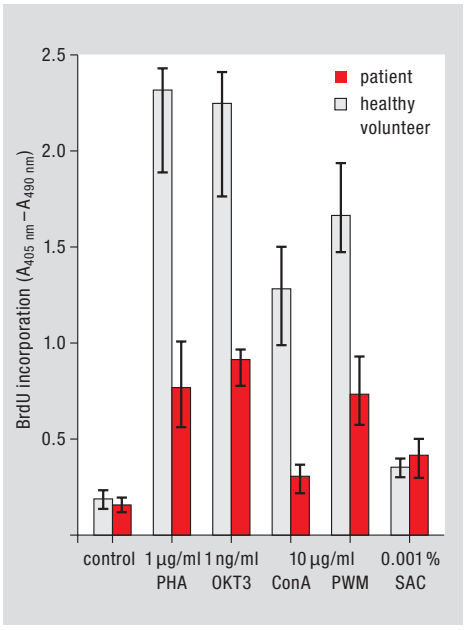
Typical results: see Figures 61–64.



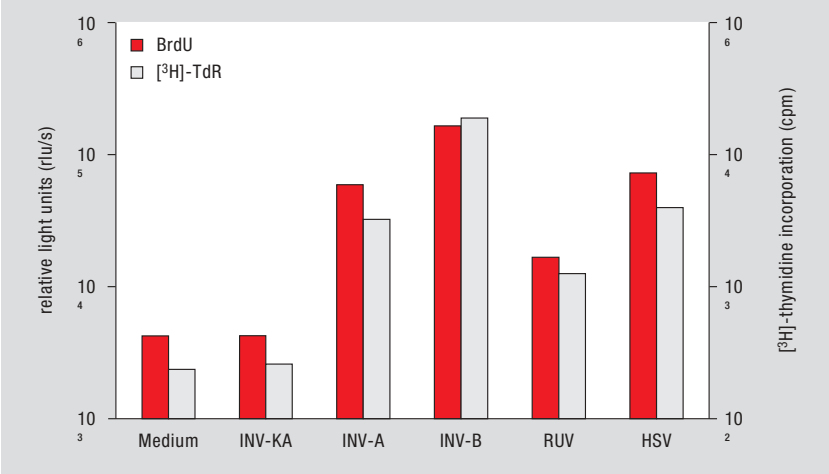
▲ **Figure 61: Comparison of the sensitivity of the Cell Proliferation ELISA, BrdU (colorimetric) and the radioactive thymidine incorporation assay for measuring proliferation in various concentrations of cells.** Various concentrations of L929 cells were cultured in the wells of a microtiter plate. Duplicate cultures of each cell concentration were labeled for 4 h with either bromodeoxyuridine (BrdU) or tritiated thymidine ([^{3}H]-TdR). The cells were assayed for cell proliferation with either the Cell Proliferation ELISA, BrdU (BrdU labeling, ●) or a standard filtration/liquid scintillation counting protocol ([^{3}H]-TdR labeling, ○).
Result: The Cell Proliferation ELISA, BrdU (colorimetric) measures proliferation with a sensitivity comparable to the radioactive thymidine assay at all cell concentrations.



▲ **Figure 62: Comparison of the Cell Proliferation ELISA, BrdU (colorimetric) and the radioactive thymidine incorporation assay for measuring stimulation of various concentrations of mitogen.** Human peripheral blood lymphocytes were cultured in the presence of varying concentrations of phytohemagglutinin (PHA) in the wells of a microtiter plate. Duplicate cultures from each PHA concentration were labeled for 4 h with either bromodeoxyuridine (BrdU) or tritiated thymidine ([^{3}H]-TdR). The cells were assayed for cell proliferation with either the Cell Proliferation ELISA, BrdU (BrdU labeling, ●) or a standard filtration/liquid scintillation counting protocol ([^{3}H]-TdR labeling, ○).
Result: The Cell Proliferation ELISA, BrdU (colorimetric) is able to detect mitogen-stimulation with a sensitivity comparable to the radioactive thymidine assay.



◀ **Figure 63: Reduced PBL proliferation of an immunosuppressed patient in response to various mitogens.** Cells (1 x 10^5/well) from a healthy volunteer (□) or an immunosuppressed patient (■) were incubated in the presence of various mitogens for 56 h. Cells were labeled with BrdU for 16 h, then cell proliferation was analyzed by Cell Proliferation ELISA, BrdU (colorimetric). The error bars indicate the maximum and minimum values of triplicate microcultures (data from T. Brüning, [1994] *Klin. Lab.* **40**, 917–927, Figure 3). Mitogens used were: PHA (phytohemagglutinin), OKT3 (anti-CD3 monoclonal antibody), Con A (concanavalin A), PWM (pokeweed mitogen), and SAC (Staphylococcus aureas Cowan I).
Result: The BrdU ELISA clearly detected the difference in response between the healthy and immunosuppressed subjects.



▲ **Figure 64: Measurement of the proliferation of antigen-activated PBL.** Cells (1 x 10^5/well) were incubated in the presence of various viral antigens on culture medium alone for 5 days. After labeling with BrdU (■) or [^{3}H]-TdR (□) for 16 h, cell proliferation was analyzed by Cell Proliferation ELISA BrdU (chemiluminescence) (■) or LSC (□). Antigens used were: INV-KA (influenza control antigen), INV-A (influenza virus A), INV-B, (nfluenza virus B), RUV (Rubella virus), and HSV (herpes simplex virus type I).
Result: The Cell Proliferation ELISA, BrdU (chemiluminescence) detected antigen stimulation with a sensitivity comparable to the radioactive thymidine assay.

Other applications: For more example of how the Cell Proliferation ELISAs can be used in the laboratory, see Appendix, page 125.

Methods for studying cell proliferation and viability in cell populations
Summary of methods for studying cell proliferation and cell viability in cell populations

2.2.1.3 Summary of methods for studying cell proliferation and cell viability in cell populations

DNA Synthesis

Method/ Roche Molecular Biochemicals product	Label	Assay Principle	Advantages	Limitations	For product information, see
[^{3}H]-TdR Proliferation Assay	[^{3}H]-TdR	● [^{3}H]-TdR is added to cells cultured in MTP and the cells are incubated (usually for 2–24 h). During this labeling period, [^{3}H]-TdR is incorporated into the DNA of proliferating cells. ● Cells are harvested by vacuum aspiration onto glass fiber filters. While free [^{3}H]-TdR is washed through the filters, the [^{3}H]-TdR incorporated in the DNA is retained. ● The radioactivity retained on the filters is measured by liquid scintillation counting (LSC).	● Sensitive (10^3–10^4 cells/test required) ● Linear measurement of cell proliferation over a broad, logarithmic range ● Low background	● Radioactive isotope handling and storage problems ● Long half life ● Radioactive waste disposal costs	
BrdU incorporation assay **BrdU Labeling and Detection Kit III**	BrdU	● BrdU is added to cells cultured in MTP and the cells are incubated (usually for 2–24 h). During this labeling period BrdU is incorporated into the DNA of proliferating cells. ● After the culture supernatant is removed, the cells are fixed and then incubated with an anti-BrdU antibody conjugated with peroxidase (anti-BrdU-POD). ● This antibody binds to BrdU which has been incorporated into the DNA. ● Bound anti-BrdU-POD is detected by a substrate reaction and quantified by an ELISA plate reader.	● No transfer of the cells; the entire assay is performed in a single MTP ● Non-radioactive	● Assay is not linear over a broad logarithmic range of cell proliferation (limitation of the ELISA plate reader) ● 3 washing and incubation steps ● Longer assay time	page 81 of this guide
BrdU incorporation assay **Cell Proliferation ELISA, BrdU (colorimetric)**	BrdU	See above (BrdU incorporation assay)	● No transfer of the cells; the entire assay is performed in a single MTP ● 1 washing and 2 incubation steps only ● Short assay time ● Robust system: low standard deviation ● Sensitive (10^3–10^4 cells/test required)	● Assay is not linear over a broad logarithmic range of cell proliferation (limitation of the ELISA plate reader)	page 83 of this guide
BrdU incorporation assay **Cell Proliferation ELISA, BrdU (chemiluminescence)**	BrdU	See above (BrdU incorporation assay)	● [see also Cell Proliferation ELISA, BrdU (colorimetric)] ● Linear measurement of cell proliferation over a broad, logarithmic range	● For chemiluminescence measurement special MTP (Black with clear, flat bottom) required	page 83 of this guide

▲ **Table 16:** Summary of methods to study DNA synthesis in cell populations.

Metabolic activity

Method/ Roche Molecular Biochemicals product	Label	Assay Principle	Advantages	Limitations	For product information, see
MTT Assay[61] **Cell Proliferation Kit I (MTT)**	Non-isotopic	● MTT solution is added to cells cultured in MTP and the cells are incubated (usually for 4 h). During this period, MTT is converted into a colored, water-insoluble formazan salt by the metabolic activity of viable cells. ● The insoluble formazan is solubilized. ● The amount of formazan is quantified by an ELISA plate reader at 550–600 nm.	● No transfer of the cells; the entire assay is performed in a single MTP ● MTT is metabolized by all cells; the assay can be used with all cell types ● Inexpensive	● Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader ● Insoluble reaction product; resolubilization of the reaction product required ● Connot take multiple time points in a single assay ● Cells with low metabolic activity (e.g., lymphocytes) must be used in high numbers	page 75 of this guide
XTT Assay[62] **Cell Proliferation Kit II (XTT)**	Non-isotopic	● XTT solution is added to cells cultured in MTP and the cells are incubated (usually for 2–4 h). During this period, XTT is converted into a colored, soluble formazan salt by the metabolic activity of viable cells. ● The amount of formazan is quantified by an ELISA plate reader at 450–500 nm.	● No transfer of the cells; the entire assay is performed in a single MTP ● Soluble reaction product ● Can take multiple time points in a single assay	● Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader ● XTT working solution has to be prepared shortly before use ● XTT is not metabolized by all cell types	page 76 of this guide
WST-1 Assay **Cell Proliferation Reagent (WST-1)**	Non-isotopic	● WST-1 solution is added to cells cultured in MTP and the cells are incubated (usually for 0.5–2 h). During this period, WST-1 is converted into a colored, soluble formazan salt by the metabolic activity of viable cells. ● The amount of formazan is quantified by an ELISA plate reader at 420–480 nm.	● No transfer of the cells; the entire assay is performed in a single MTP ● Soluble reaction product ● Repeated measurement of the assay ● Ready-to-use solution	● Assay is not linear over a broad logarithmic range of cell proliferation due to the ELISA plate reader ● WST-1 is not metabolized by all cell types	page 77 of this guide

▲ **Table 17:** Summary of methods to study metabolic activity in cell populations.

2.2.1.4 Single reagents for the measurement of DNA synthesis

Product	Cat. No.	Pack Size
FixDenat	1 758 764	4 x 100 ml (2000 tests)
Anti-Bromodeoxyuridine-Peroxidase, Fab fragments, formalin grade	1 585 860	15 U

◀ **Table 18:** Single reagents available for detection of DNA fragmentation.

2 Cell Proliferation and Viability

2.2.2 Methods for studying cell proliferation and viability in individual cells

As mentioned in Section 2.1, the viability as well as proliferation of individual cells can be assessed by standard microscopic methods. For instance, cells may be treated with a vital stain or exclusion dye and counted directly in a hemocytometer. The same cell parameters may be determined by flow cytometry if the cells are differentially stained with fluorescent dyes that bind DNA (DNA fluorochromes), see also section 1.2.2.3 on page 40 of this guide.

In the following sections we will describe details of the following proliferation assays:

- Assays that measure DNA synthesis: As outlined above, if labeled DNA precursors are added to the cell culture, cells that are about to divide incorporate this precursor into their DNA (described on the following pages of this guide)
- Asssays that monitor expression of cell cycle-associated antigens: Molecules that regulate the cell cycle are measured either by their activity (e.g. CDK kinase assays) or by quantifying their amounts (e.g. Western blots, ELISA, or immunohistochemistry) (described on page 97 of this guide).

In the following sections we will describe details of several of these proliferation assays.

2.2.2.1 Assays that measure DNA synthesis

Studies of cell proliferation *in vivo* as well as on individual cells *in vitro* frequently employ [^{3}H]-TdR to label the DNA of replicating cells and autoradiography to reveal the radioactive label. As a nonradioactive alternative, bromodeoxyuridine (BrdU) can be used to label proliferating cells *in vivo* and *in vitro*. Incorporated BrdU can be detected by immunohistochemistry, immunocytochemistry or flow cytometry[73, 74].

Immunochemical techniques allow both the visualization of dividing cells and the detection of tissue morphology by counterstaining (e.g., with hematoxylin and/or eosin). Thus, it is possible to visualize cells which have incorporated BrdU into DNA in its natural environment and to localize cell position in the tissue.

As only those cells which are actually in the S-phase (DNA-synthesis) of the cell cycle will be labeled, the so-called "labeling index"[75] can be determined if the labeled nucleotide ([^{3}H]-TdR or BrdU) is present for only short periods of time (e.g. 15–60 minutes). The "labeling index" (proportion of S-phase cells in an asynchronously growing population) is calculated by dividing the number of labeled cells by the total number of cells in the entire population.

While short labeling periods (pulse labeling) are suitable to quantify the percentage of S-phase cells within a cellular population, longer labeling periods (e.g. for a whole cell cycle transition) can be used to determine a replicating population.

Roche Molecular Biochemicals offers several kits and reagents for measuring proliferating cells by BrdU incorporation. These products are described on the following pages.

BrdU Labeling and Detection Kit I

Cat. No. 1 296 736 100 tests

BrdU Labeling and Detection Kit II

Cat. No. 1 299 964 100 tests

Type	1st generation immunostaining assays for fluorescence (Kit I) or light (Kit II) microscopy
Useful for	Detection of BrdU-labeled DNA in proliferating individual cells
Samples	Cultured or freshly isolated cells, tissue explants or sections
Method	Incubation of cells with BrdU, or injection into an animal, followed by nuclease digestion of DNA of cells or tissue sections and indirect immunodetection (with anti-BrdU and a secondary antibody) of incorporated BrdU label
Time	approx. 2–3 h (+ BrdU labeling)

Significance of kits: The BrdU Labeling and Detection Kits I and II offer an indirect immunostaining method for visualizing proliferating cells under a fluorescence microscope (Kit I) or under a light microscope (Kit II). The kits detect BrdU-labeled DNA with an anti-BrdU antibody, then make the antibody-labeled DNA visible with either a fluorescein-labeled (Kit I) or an alkaline phosphatase-labeled anti-mouse secondary antibody (Kit II).

Note: *These kits belong to the first generation of kits used to measure DNA synthesis. The same assay procedure has been optimized and improved in the second generation of kits, namely the In Situ Cell Proliferation Kit, FLUOS (for flow cytometry and fluorescence microscopy) and the In Situ Cell Proliferation Kit, AP (for light microscopy). For a detailed description of these second generation kits, see the following pages.*

Other applications: For examples of how the BrdU Labeling and Detection Kits I and II can be used in the laboratory, see Appendix, page 125.

In Situ Cell Proliferation Kit, FLUOS

Cat. No. 1 810 740 100 tests

Type	Direct immuno-fluorescence staining for flow cytometry or fluorescence microscopy
Useful for	Detection of BrdU-labeled DNA in proliferating individual cells
Samples	Cultured or freshly isolated cells, tissue explants or sections
Method	Incubation of cells with BrdU, or injection of BrdU into an animal followed by denaturation of DNA of cells or tissue sections and direct immunodetection of incorporated BrdU label
Time	approx. 2 h (+ 0.5–4 h BrdU labeling)

Significance of kit: Bromodeoxyuridine (BrdU) is only incorporated into the DNA of proliferating cells. Short periods (15–60 min) of incubation *in vitro* with BrdU will tag only cells actually going through the S phase of the cell cycle. Alternatively, BrdU can be injected into an animal to label growing cells *in vivo*. The *In Situ* Cell Proliferation Kit, FLUOS can detect proliferating cells in culture or in tissues which have been tagged by *in vitro* or *in vivo* BrdU labeling. Analysis can be done by flow cytometry or by fluorescence microscopy.

Test principle: The BrdU solution and fluorescein-conjugated anti-BrdU antibody supplied in the kit allow BrdU labeling and detection of proliferating cells. The procedure (Figure 65 and Flow Chart 19) involves:

1. **A:** Incubating growing animal tissue or cells *in vitro* with BrdU
   or
   B: Injecting BrdU into whole animals for *in vivo* labeling, then sacrificing the animal and preparing tissue sections.

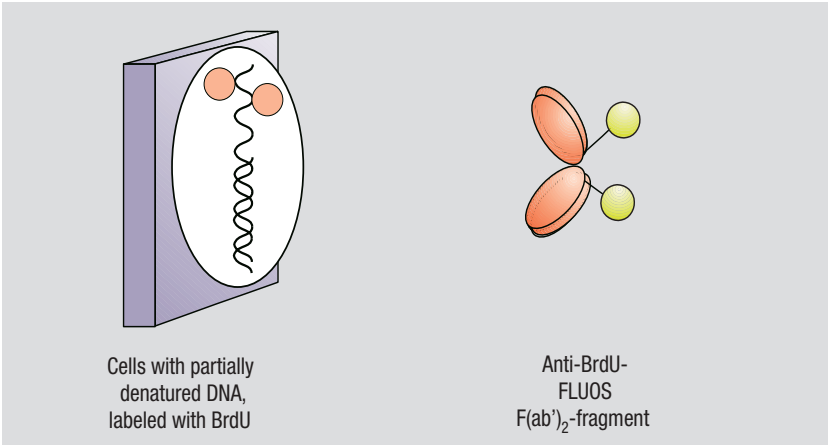
   Note: *Only proliferating cells incorporate BrdU into their DNA.*
2. Fixing BrdU-labeled tissue or cells.
3. Denaturing cellular DNA with acid.
4. Detecting incorporated BrdU with fluorescein-labeled anti-BrdU monoclonal antibody.
5. Analyzing the antibody-labeled samples with a flow cytometer or a fluorescence microscope.

Specificity: The antibody conjugate (anti-BrdU-fluorescein, $F(ab')_2$ fragments) will bind to BrdU-labeled DNA after the DNA is denatured and partially degraded with acid. The antibody specifically recognizes 5-bromo-2'-deoxyuridine; it shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.

Can be used to assay:

- Cell lines (in adherent or suspension cell culture)
- Freshly isolated cells
- Tissue explants labeled with BrdU *in vitro*
- Frozen or paraffin-embedded tissue sections from animals labeled with BrdU *in vivo*.

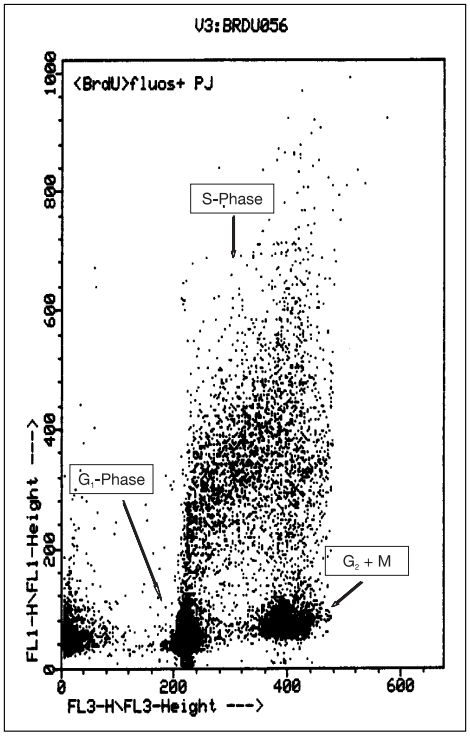
Figure 65: How the *In Situ* Cell Proliferation Kit, FLUOS works. ▼



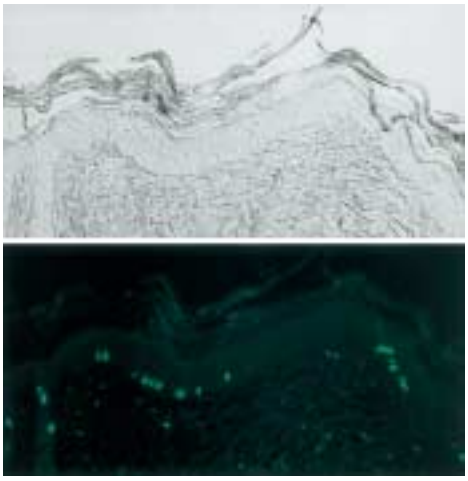
Kit contents

1. BrdU labeling reagent (1000 x), sterile
2. Anti-BrdU-fluorescein, monoclonal, $F(ab')_2$ fragments
3. Antibody incubation buffer

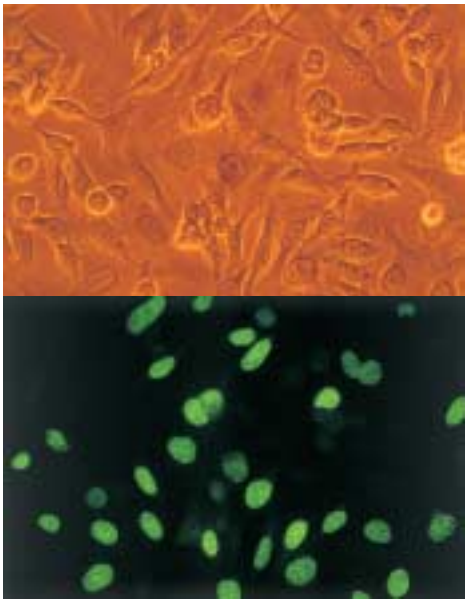
Typical results: see Figures 66–68.



▲ **Figure 66: Flow cytometric measurement of total DNA and incorporated BrdU with the *In Situ* Cell Proliferation Kit, FLUOS.** Exponentially growing U937 cells were incubated with BrdU for 30 min. Incorporated BrdU was measured flow cytometrically with the fluorescein-conjugated anti-BrdU antibody (<BrdU>fluos) from the *In Situ* Cell Proliferation Kit, FLUOS. Total DNA was counterstained with 1 µg/ml propidium iodide (PI). The phase of the cell cycle represented by each population of cells is indicated on the flow cytometric histogram. FL1-H, fluorescein intensity (relative BrdU content); FL3-H, propidium iodide intensity (relative DNA content).
Result: BrdU labeling is confined exclusively to the S-phase (DNA synthesis) of the cell cycle.



▲ **Figure 67: *In vivo* labeling and analysis of dorsal, hyperproliferative epidermis tissue from mouse with the *In Situ* Cell Proliferation Kit, FLUOS.** Undiluted BrdU labeling solution from the kit was injected intraperitoneally into a mouse (1 ml BrdU solution/100 g body weight). After 2 h of *in vivo* BrdU labeling, the mouse was sacrificed and 5 µm thick, paraffin-embedded tissue sections were prepared. Sections were deparaffinized and rehydrated according to standard methods, then digested with trypsin (15 min). DNA was partially denatured with HCl (20 min) and detected with anti-BrdU-fluorescein. Each section was analyzed by differential interference microscopy (upper photo) and epifluorescence microscopy (lower photo). Magnification, 530 x. (Data kindly provided by S. Kaiser and M. Blessing, I. Med. Klinik der Universität Mainz, Germany.)
Result: Proliferating cells (green spots) are clearly visible throughout the tissue under epifluorescence microscopy.



◀ **Figure 68: *In vitro* labeling and analysis of proliferating HeLa cells with the *In Situ* Cell Proliferation Kit, FLUOS.** HeLa cells in culture were labeled with BrdU and the BrdU-labeled DNA detected with anti-BrdU-fluorescein, according to the package insert of the *In Situ* Cell Proliferation Kit, FLUOS. The labeled cell preparation was analyzed under a light microscope (upper photo) and a fluorescence microscope (lower photo).
Result: Proliferating cells (bright green nuclei) within the HeLa preparation are clearly visible under the fluorescence microscope.

Anti-BrdU, formalin grade

Cat. No. 1 170 376 50 µg

Anti-BrdU-Fluorescein

Cat. No. 1 202 693 50 µg

Anti-BrdU-Peroxidase, Fab fragment

Cat. No. 1 585 860 15 units

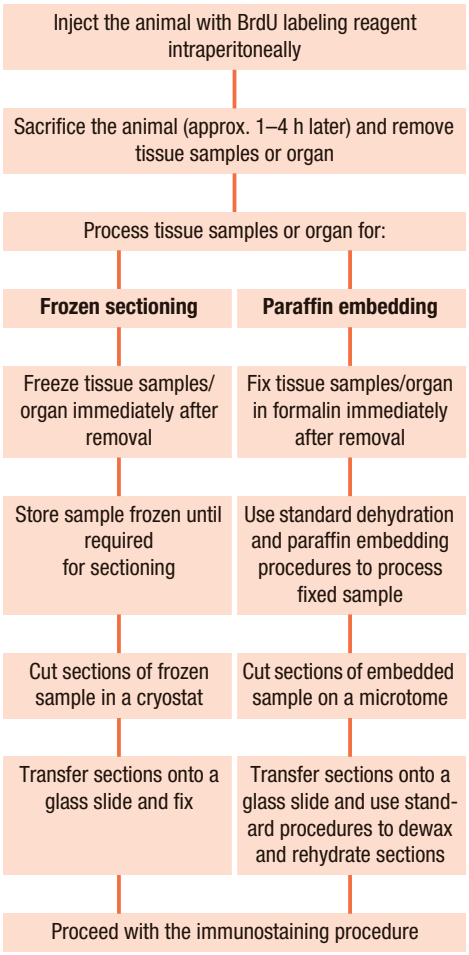
Type	Monoclonal antibodies, from mouse
Useful for	Detection of BrdU-labeled DNA in proliferating individual cells
Samples	Cultured or freshly isolated cells, tissue explants or sections
Method	Incubation of samples with BrdU, followed by denaturation of DNA, detection of BrdU label with anti-BrdU antibody, and (if necessary) visualization of anti-BrdU antibody with secondary antibody
Time	Variable (depending on sample and antibody used)

Significance of antibodies: Bromodeoxyuridine (BrdU) is only incorporated into the DNA of proliferating cells. Short periods (15–60 min) of incubation *in vitro* with BrdU will tag only cells going through the S phase of the cell cycle. Alternatively, BrdU can be injected into an animal to label growing cells *in vivo*. Conjugated or unconjugated anti-BrdU antibody may be used to detect proliferating cells or tissues which have been tagged by *in vitro* or *in vivo* BrdU labeling. Depending on the sample and the antibody used, analysis can be by flow cytometry, fluorescence microscopy, or light microscopy.

Test principle: The anti-BrdU antibodies may be used to detect BrdU-labeled DNA in proliferating cells. The procedure involves (Flow Chart 20):

1. **A:** Incubating growing animal tissue or cells *in vitro* with BrdU
   or
   B: Injecting BrdU into whole animals for *in vivo* labeling, then sacrificing the animal and preparing tissue sections.

   Note: *Only proliferating cells (cells in S-phase) incorporate BrdU into their DNA.*
2. Fixing BrdU-labeled tissue or cells.
3. Denaturing cellular DNA.
4. Detecting incorporated BrdU with conjugated or unconjugated anti-BrdU monoclonal antibody.



▲ **Flow Chart 19:** Assay procedure, *in vivo* labeling of proliferating cells with BrdU.

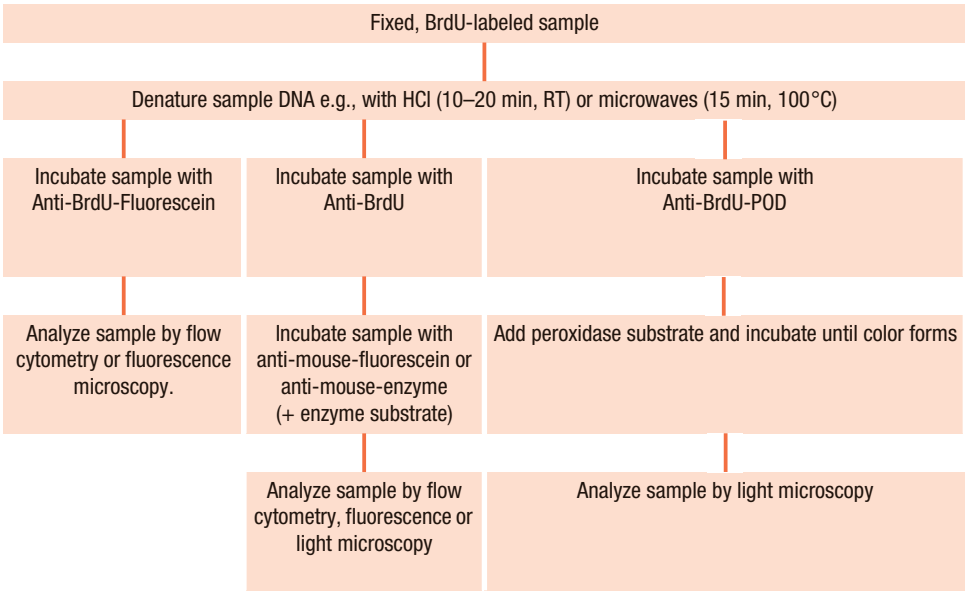
5 (Option) **A:** Localizing unconjugated anti-BrdU antibody with a secondary antibody detection system
or
(Option) **B:** Localizing enzyme-conjugated anti-BrdU antibody with an enzyme substrate.

6 Analyzing the antibody-labeled samples with a flow cytometer, a fluorescence microscope, or a light microscope.

Specificity: Conjugated or unconjugated anti-BrdU antibody will bind to BrdU-labeled DNA after the DNA is denatured and partially degraded (e.g., with DNase, acid or microwaves). The antibody specifically recognizes 5-bromo-2'-deoxyuridine; it shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.

Can be used to assay:

- Cell lines (in adherent or suspension cell culture)
- Freshly isolated cells, or tissue explants labeled with BrdU *in vitro*
- Frozen or paraffin-embedded tissue sections from animals labeled with BrdU *in vivo*.



▲ **Flow Chart 20:** Immunostaining procedure, Anti-BrdU antibody and conjugates.

Typical results: The anti-BrdU antibody has been used to determine the cell cycle position of apoptotic cells[76].

Briefly, the experimental procedure was as follows: Cultured mouse thymocytes were treated with 0.5 µM ionomycin (2 h or 12 h) to induce apoptosis. After treatment, the cells were harvested, fixed in paraformaldehyde and ethanol (two-step fixation), and analyzed for apoptosis and cell cycle position by flow cytometry. As a measure of apoptotic cells, fragmented DNA content was quantitated with the *In Situ* Cell Death Detection Kit, Fluorescein (TUNEL method, according to the kit package insert). Either of two flow cytometric techniques was used to determine the cell cycle position of the cells: 1) Relative DNA content was determined by treating the cells with 5 µg/ml propidium iodide and 200 µg/ml ribonuclease (30 min, room temperature). 2) Cells going through S-phase were identified by labeling with BrdU (10 µM BrdU, 30 min), detection of BrdU-labeled cells with anti-BrdU monoclonal antibody (30 min, 37°C), and visualization of those cells with R-phycoerythrin-conjugated goat anti-mouse antibody (30 min, 37°C). For results, see Figure 69.

Other applications: For examples of how the Anti-BrdU conjugates and the antibodies may be used in the lab, see Appendix, page 126.

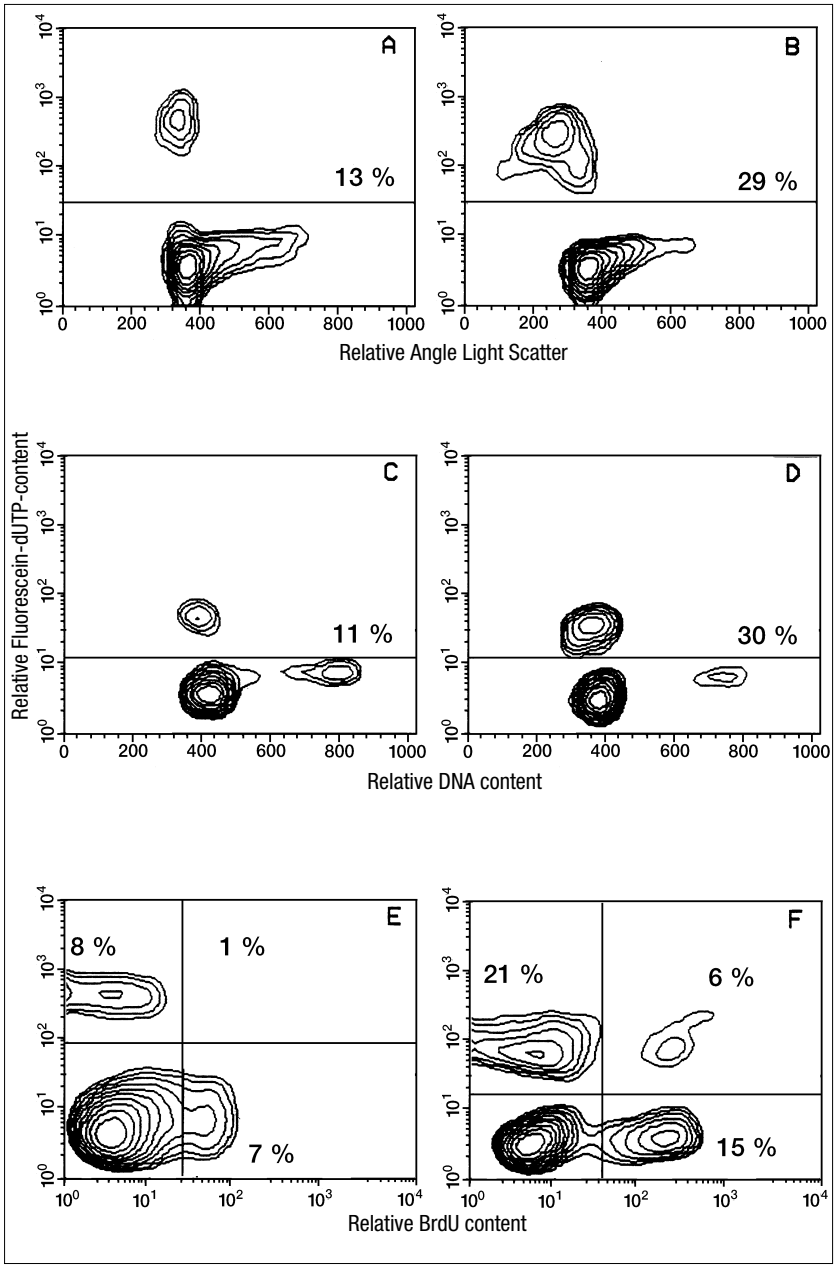


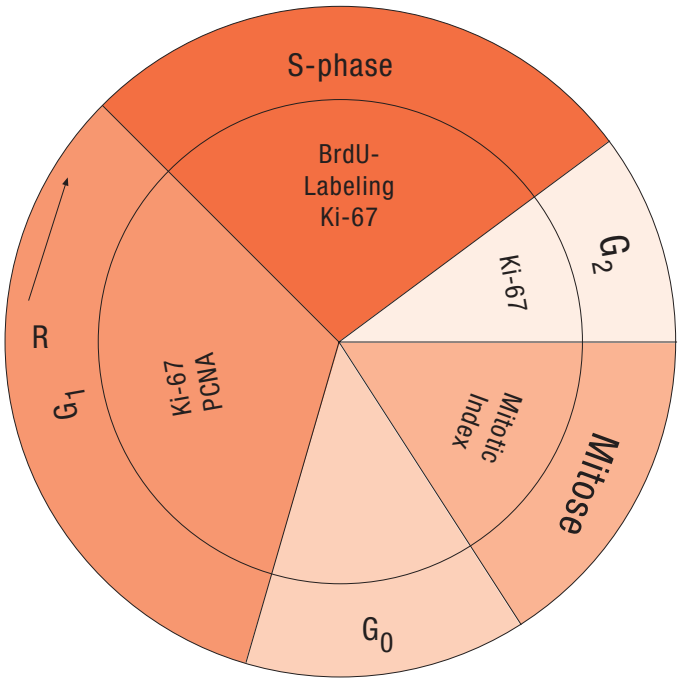
Figure 69: Concomitant flow cytometric analysis of apoptosis and cell cycle position with the anti-BrdU antibody, propidium iodide, and the *In Situ* Cell Death Detection Kit, Fluorescein. Cultured mouse thymocytes were treated with ionomycin (2 h or 12 h) to induce apoptosis. After treatment, the cells were harvested, fixed, and analyzed for apoptosis and cell cycle position by flow cytometry. Histograms A, C, and E show data obtained from cells after 2 h treatment with ionomycin. Histograms B, D, and F show data obtained from cells after 12 h treatment with ionomycin. Histograms A and B show fluorescein intensity (green fluorescence) alone, a measure of DNA fragmentation. Histograms C and D show a two-parameter analysis of fluorescein intensity (green fluorescence, DNA fragmentation) and propidium iodide intensity (red fluorescence, DNA content). Histograms E and F show a two-parameter analysis of fluorescein intensity (green fluorescence, DNA fragmentation) and phycoerythrin intensity (orange fluorescence, BrdU content). The percentage of positive cells is indicated in each panel. [Data from Hanon, E., Vanderplasschen, A. and Pastoret, P.-P. (1996) *Biochemica* **No. 2**, 25–27.]

Result: The ionomycin-treated cells contained about 13% apoptotic cells (histogram A) after 2 h and about 29% apoptotic cells (histogram B) after 12 h exposure. Concomitant analysis of apoptosis and total DNA content (histograms C and D) showed that apoptotic cells contained about as much DNA as cells in G_0/G_1 or early S-phase. Concomitant analysis of apoptosis and BrdU content after 12 h ionomycin treatment (histogram F) showed that 6% of the apoptotic cells went through S phase (that is, were positive for BrdU) while 21% of apoptotic cells remained in G_0/G_1 (that is, were negative for BrdU). ▶

2.2.2.2 Assays that monitor expression of cell cycle-associated antigens

Monoclonal antibodies directed against cell cycle antigens can identify cells which are actively cycling[77]. In some cases, these antibodies can distinguish specific phases of the cell cycle. Antibodies against such antigens as Ki-67 are especially useful for the clinical assessment of cell proliferation by immunohistochemical techniques[78–81]. Furthermore, immunocytochemical analysis of cells by flow cytometry allows quantitation of cell proliferation[69].

Important monoclonal antibodies used to study cell proliferation and the cell cycle are summarized in Table 19.



▲ **Figure 70:** Phases of cell cycle.

Monoclonal antibodies to cell cycle-associated antigens

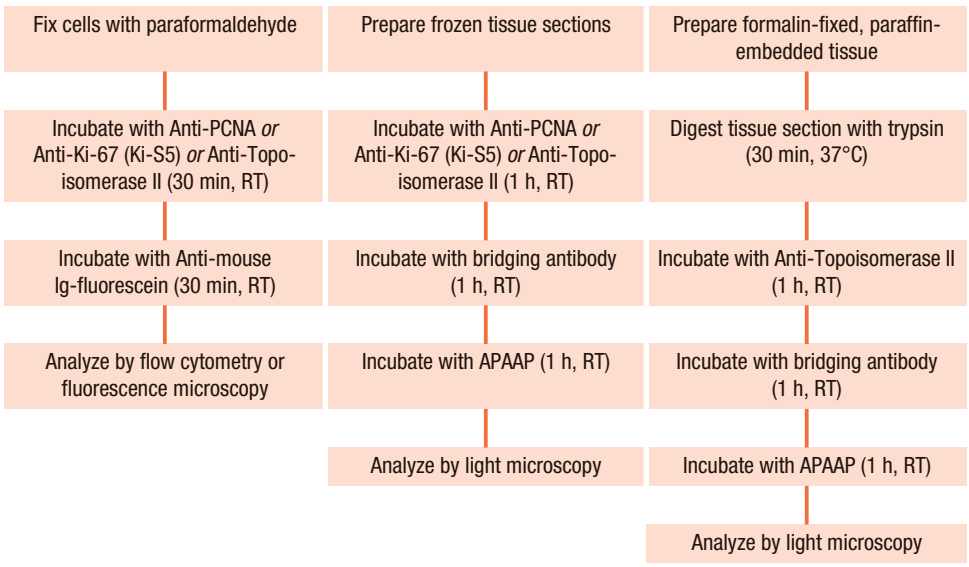
(See Table 19)

Type	Monoclonal antibodies, from mouse
Useful for	Detection of cell cycle-associated antigens which are expressed only in proliferating cells
Samples	Paraformaldehyde-fixed cells in suspension (flow cytometry), cell smears, tissue sections
Method	Incubation of samples with monoclonal antibody, followed by (if necessary) visualization of monoclonal antibody with secondary antibody. Also uselful for immunohistochemistry and cytochemistry , for western blotting in populations of cells.
Time	Variable (depending on sample and antibody used)

Significance of antibodies: Several nuclear antigens [e.g. proliferating cell nuclear antigen (PCNA), Ki-67 and topoisomerase II-alpha (Ki-S1)] are expressed only in proliferating cells. They are absent in resting cells. Thus, antigens that recognize these antigens may be used to selectively mark proliferating cells in cell populations and tissue. Depending on the sample and the antibody used, analysis can be by flow cytometry, fluorescence microscopy, or light microscopy.

Test principle: The antibodies listed in Table 19 may be used to detect nuclear antigens present only in proliferating cells. The procedure involves (Flow Chart 21):

1. Fixing cells or tissue so the target antigen is preserved. (See "Can be used to assay" next page for appropriate samples for each antibody.)
2. Detecting the target antigen with a monoclonal antibody.
3. (Optional) Localizing unconjugated monoclonal antibody with a secondary anti-mouse antibody detection system.
4. Analyzing the antibody-labeled samples with a flow cytometer, a fluorescence microscope, or a light microscope.



◀ **Flow Chart 21:** Immunostaining procedure, unconjugated monoclonal antibodies to cell cycle-associated antigens.

Specificity: All the monoclonal antibodies to cell-cycle associated antigens are mouse monoclonal antibodies which react with nuclear antigens expressed only in proliferating cells. They do not react with cytoplasmic antigens or with resting cells.

Can be used to assay:

- *Anti-PCNA/Cyclin (clone PC10):* Formalin-fixed and paraffin-embedded tissue sections.
- *Anti-Ki-67 (clone Ki-S5):* Paraformaldehyde-fixed cells, cell smears, formalin-fixed or frozen tissue sections.
- *Anti-Topoisomerase II-α (clone Ki-S1):* Paraformaldehyde-fixed cells in suspension, cell smears, formalin-fixed and paraffin-embedded or frozen tissue sections.
- *Anti-Tranferrin-Receptor, human (clone B3/25):* Paraformaldehyde-fixed cells and paraffin-embedded tissue sections.

Supplied as:

Anti-PCNA/Cyclin: 100 µg lyophilizate

Anti-Ki-67 (clone Ki-S5): 100 µg lyophilizate

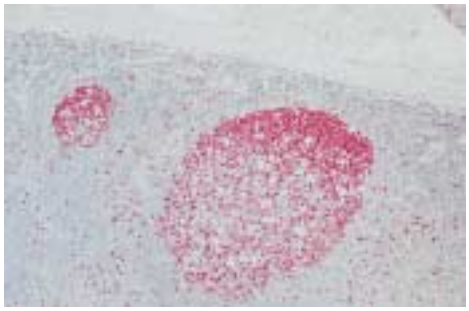
Anti-Topoisomerase II-α (clone Ki-S1): 100 µg lyophilizate

Anti-Tranferrin Receptor, human: 200 µg lyophilizate in 500 µl solution.

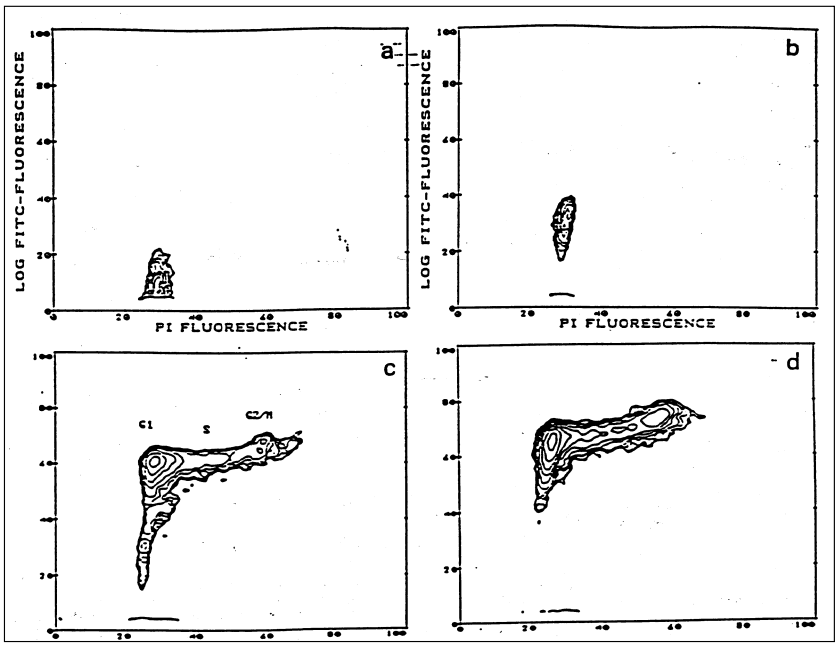
Typical results: see Figures 71–72.

Product	Specificity
Anti-Ki-67 (Ki-S5) (clone Ki-S5) **Formalin grade**	In Western blots the Ki-S5 antibody recognizes a protein of 345 kD and 395 kD identical with the Ki-67 antigen. The immunoreactivity of Ki-S5 is confined to the nuclei proliferating cells and no cross-reactivity with cytoplasmic antigens of epithelial occurs. A comparison of immuno-histochemical labeling of fresh and fixed tissue samples of NHL showed that identical results were obtained with Ki-67 and Ki-S5.
Anti-PCNA/Cyclin (clone PC10) **Formalin grade**	The antibody reacts with proliferating cell nuclear antigen (PCNA = an auxiliary protein of DNA polymerase δ), a polypeptide of 36 kD. Anti-PCNA is used to determine the proliferative cell fraction in various tumors.
Anti-Topoisomerase IIα (clone Ki-S1) **Formalin grade**	The antibody recognizes a major protein of 170 kDA, the α isoform of topoisomerase II. It binds to the carboxyterminal α-isoenzyme specific epitope missing in topoisomerase IIβ. In immunohistochemistry the antibody shows strong nuclear staining also in paraffin-embedded tissue sections. It binds only to proliferating cells, while resting, non-cycling cells are not labeled. This specificity for proliferating cells has allowed the antibody to be used for determination of the proliferative fraction in solid tumors such as mammary carcinomas and gangliomas.
Anti-Transferrin Receptor, human (clone B3/25)	The antibody reacts with the human transferrin receptor glycoprotein. The transferrin receptor participates in the uptake of tranferrin, the major serum iron transport protein. The transferrin receptor is present on all cells (except erythrocytes) but is especially dense on the surface of rapidly proliferating cells. It can be used therefore, as a proliferation marker.

▲ **Table 19:** Specificity of monoclonal antibodies to cell-cycle associated antigens.



▲ **Figure 71: Anti-Ki-67 (clone Ki-S5) staining of formalin-fixed, paraffin-embedded normal lymphatic tissue from tonsil.** The slide shows a secondary follicle with normal cell layering. Slide was counterstained with hematoxylin. (Data kindly provided by Dr. H. Merz). **Result:** Lymphoblasts are observed in the upper right hand corner of the follicle (darkly stained area). The cortex, which is leukocyte-rich and lymphocyte-poor, is much less stained. However, lymphoblasts are seen sporadically in the diffuse cortex.



▲ **Figure 72: Flow cytometric analysis, Anti-Topoisomerase II-alpha (clone Ki-S1) and propidium iodide staining of human peripheral blood lymphocytes.** Human PBL were stimulated with phytohemagglutinin A. At timed intervals, aliquots of the cell preparation were stained for cell cycle position (with Anti-Topoisomerase II and Anti-mouse-Ig-fluorescein, according to the pack insert) and total DNA content (with propidium iodide, according to standard procedures). The histograms show the two-parameter flow cytometric analysis of the cells at the time of stimulation (a = 0 h), and at 24 h intervals after stimulation (b= 24 h, c = 48 h, d = 72 h). The cell cycle phases are indicated on histogram c. FITC-Fluorescein, intensity of Anti-Topoisomerase II staining; PI Fluorescence, intensity of propidium iodide staining.
Result: Topoisomerase IIα can be found during G_1, S, G_2, and M phases in proliferating cells (histograms c-d), but is not expressed in resting (G_0) cells (histogram a).

◀ **Table 20:** Monoclonal antibodies to cell-cycle associated antigens

Antibody to/Conjugated to (Clone)	Antigen expressed during G_0	G_1	S	G_2	M	Cat. No.	No. of-tests*
Anti-PCNA/Cyclin (clone PC10)	–	+	+	+	+	1 486 772	100
Anti-Ki-67 (clone Ki-S5)	–	+	+	+	+	1 742 345	1000
Anti-Topoisomerase IIα (clone Ki-S1)	–	+	+	+	+	1 742 353	1000
Anti-Transferrin-Receptor (clone B3/25)	+	+	+	+	+	1 118 048	200

* Flow cytometric assays

Other applications: For examples of how the Anti-PCNA/Cyclin antibody may be used in the lab, see Appendix, page 126.

Methods for studying cell proliferation and viability in individual cells
Summary of methods for studying cell proliferation and viability in individual cells

2.2.2.3 Summary of methods for studying cell proliferation and viability in individual cells

DNA Synthesis

Method/ Roche Molecular Biochemicals product	Assay principle	Advantages	Limitations	For product information, see
Autoradiography	● The samples are incubated with [^{3}H]-TdR for a certain period of time. If [^{3}H]-TdR is present for 1 h or less, only those cells which are in the S-phase (DNA synthesis) of the cell cycle will be labeled. ● The samples are fixed and immersed in emulsion. ● The radiolabel is visualized as black grains on the film.	● Quantitative detection of S phase cells: Determination of growing fraction in population	● Long exposure time (days) required ● Radioactive isotope, handling and storage problems	
Immunocytochemistry (fluorescence microscopy) ***In Situ* Cell Proliferation Kit, FLUOS** **BrdU Labeling and Detection Kit I**	● The samples are incubated with BrdU for a certain period of time. If BrdU is present for 1 h or less, only those cells which are in the S-phase (DNA synthesis) of the cell cycle will be labeled. ● The samples are fixed and the DNA is denatured. ● Incorporated BrdU is bound by a fluorescein-conjugated monoclonal antibody against BrdU. ● Bound Anti-BrdU-Fluorescein is detected by fluorescence microscopy or flow cytometry.	● Quantitative detection of S-phase cells: Determination of growing fraction in population ● Results within a few hours ● Can counterstain the tissue simultaneously to reveal tissue morphology	● Stained samples cannot be stored for long periods of time ● Histological tissue organization cannot be observed simultaneously	pages 89, 90 of this guide
Immunocyto/histochemistry (light microscopy) **BrdU Labeling and Detection Kit II**	● The samples are incubated with BrdU for a certain period of time. If BrdU is present for 1 h or less, only those cells which are in the S-phase (DNA synthesis) of the cell cycle will be labeled. ● The samples are fixed and the DNA is denatured. ● Incorporated BrdU is bound by an alkaline phosphate (AP)-conjugated monoclonal antibody against BrdU. ● Bound anti-BrdU AP is detected by a substrate reaction and visualized by light microscopy.	● Quantitative detection of S-phase cells: Determination of growing fraction in population ● Results within a few hours ● Can counterstain the tissue simultaneously to reveal tissue morphology		page 89 of this guide

▲ **Table 21:** Summary of methods to study DNA synthesis in individual cells.

Cell cycle-associated antigens

Method/ Roche Molecular Biochemicals product	Assay principle	Advantages	Limitations	For product information, see
Immunocytochemistry monoclonal antibodies	● The fixed and permeabilized cells are incubated with an antibody directed against a cell cycle/proliferation-associated antigen (e.g., Ki-67, PCNA, Topoisomerase IIα). ● The antibody bound to the intracellular antigen is detected by a fluorescein-conjugated anti-mouse Ig antibody. ● Bound fluorescein-conjugated antibody is visualized by fluorescence microscopy or measured by flow cytometry.	● No prelabeling of the cells required: each cell type/tissue may be analyzed ● Quantitative detection of the proliferative cell fractions (e.g., in solid tumors) ● Results within a few hours ● Can counterstain the tissue simultaneously to reveal tissue morphology	● Detection of the antigen strongly depends on the fixation procedure: some antibodies may not work on some tissue sections when the antigen is altered by the fixation step (e.g., formalin fixed paraffin-embedded tissue sections)	page 97 of this guide
Immunohistochemistry monoclonal antibodies	● The fixed tissue sections are incubated with an antibody directed against a cell cycle/proliferation-associated antigen (e.g., Ki-67, Topoisomerase IIα). ● The antibody bound to the intracellular antigen is detected by an alkaline phosphatase (AP)- or peroxidase (POD)-conjugated anti-mouse Ig antibody. ● Bound anti-mouse Ig-AP or anti-mouse Ig-POD is detected by a substrate reaction and visualized by light microscopy.	See above	See above	See above

▲ **Table 22:** Summary of methods to study cell cycle-associated antigens in individual cells.

3

Appendix

3.1 Technical tips

3.1.1 Selected frequently asked questions (FAQs) about cell death assays

The questions below were chosen from those received by our Technical Services representatives. Wherever possible, the answers will direct you to pages and sections of this guide which can provide more information.

① Can I determine the number of apoptotic cells using the Cell Death Detection ELISA^{PLUS}?

A: No. The ELISA data is interpreted as a change in the level of death in an apoptotic population compared to an uninduced control population. It does not provide data on individual cells.

② What is the best way to get rid of nonspecific (false-positive) background in the TUNEL (*In Situ* Cell Death Detection) kits?

A: The best approach to reducing background depends on the results you obtain with the controls:

- If cells incubated with fluorescein-dUTP but without terminal transferase are false positive, try washing the cells more thoroughly, reducing the concentration of fluorescein-dUTP, or using an alternative permeabilization procedure.
- If false positives are produced only in reactions which include both fluorescein-dUTP and terminal transferase, the best means of reducing false positives is a reduction in enzyme concentration or a change in permeabilization procedure.

Note: For further tips on obtaining the best results with the TUNEL method, see page 113 of this Appendix.

③ What types of sample can be assayed with the TUNEL method?

A: Tissue sections, adherent cell cultures, cytospins and cell smears have all been used with this assay (page 24, Section 1.2.2.1). Note, however, that the sample material must be preserved with a cross-linking fixative (such as paraformaldehyde).

④ Why isn't substrate included in the TUNEL kits (*In Situ* Cell Death Detection Kits, AP or POD)?

A: These kits will work with a variety of common alkaline phosphatase or peroxidase substrates. Since many laboratories already have these substrates, and know how these substrates work in "their" system we decided to leave them out. In addition this gives the researcher the flexibility for secondary staining.

⑤ How long and at what temperature can I store my samples before analyzing them with the various kits that you offer?

A: Table 24 gives some general guidelines for sample storage. Note however that some samples may be more or less stable than others.

⑥ Is a special wash/stop buffer required for the TUNEL kits?

A: Our procedure does not require an equilibration buffer. Our wash buffer is PBS, a commonly used solution.

Kit	Storage of samples before assay
Cell Death Detection ELISA^{PLUS}	Purified cytoplasmic samples can be stored at –20°C for 2 weeks (with some reduction of signal)
Apoptotic DNA Ladder Kit	Purified DNA can be stored at –20°C for 1 year
Annexin-V-FLUOS Staining Kit; Annexin V-Biotin	Cells must be used live, directly after induction of apoptosis
Cellular DNA Fragmentation ELISA	Purified cytoplasmic samples can be stored at –20°C for at least 2 weeks

▲ **Table 23:** Storage of samples for apoptosis assay

3.1.2 Technical tips on the TUNEL method

3.1.2.1 TUNEL: Improvement and evaluation of the method for *in situ* apoptotic cell identification

[from Adrien Negoescu, Philippe Lorimier, Francoise Labat-Moleur, Laurent Azoti, Catherine Robert, Christiane Guillermat, Christian Brambilla, and Elisabeth Brambilla; of Groupe de recherche sur le cancer du poumon, Institut Albert Bonniot, Faculte de Medecine, Domaine de la Merci, 38706 Grenoble cedex, France, and Laboratoire de Pathologie cellulaire, CHRU, BP 217X, 38043 Grenoble cedex 09, France]

Note: *This is a summary of an article that appeared in the Biochemica No. 2 (1997). For further experimental detail and background, see the full Biochemica article.*

Summary: TUNEL or **t**erminal deoxynucleotidyl transferase-mediated d**U**TP **n**ick **e**nd-**l**abeling, is a preferred method for rapid identification and quantification of the apoptotic cell fraction in cultured cell preparations. However, the accessibility of DNA breaks to enzymatic reactions is reduced by the nuclear protein environment (Kerrigan et al., 1987) and impaired by cell fixation (Gold et al., 1994) and postfixation (Gorczyca et al., 1994). Thus, several sample pretreatments have been devised to improve TUNEL sensitivity (Desjardins and MacManus, 1995; Kerrigan et al., 1987; Lucassen et al., 1995). An optimized TUNEL protocol for cultured cells has been developed.

3.1.2.2 TUNEL protocol for tissues which tend to give false positives

[from Dr. Georg Fertig, Roche Molecular Biochemicals]

The protocol given below has been found to eliminate the TUNEL labeling "false positives" seen with certain paraffin-embedded tissue sections (for example, of rabbit endometrium). The key step is pretreatment of the slide with microwave irradiation rather than proteinase K.

Sample: Paraffin-embedded tissue sections (e.g., of rabbit endometrium)

Reagents: *In Situ* Cell Death Detection Kit, POD, Cat. No. 1 684 817
DAB Substrate,
Cat. No. 1 718 096

1. Dewax paraformaldehyde- or formalin-fixed tissue sections according to standard procedures.

2. Place the slide(s) in a plastic jar containing 200 ml 0.1 M citrate buffer, pH 6.0, put the jar in a microwave oven, and apply 750 W (high) microwave irradiation for 1 min. For rapid cooling, immediately add 80 ml redist. water (20°–25°C) to the jar, then transfer the slide(s) into PBS (20°–25°C).
Caution: *DO NOT perform a proteinase K treatment!*

3. Immerse the slide(s) for 30 min at room temperature (RT) in a blocking solution containing 0.1 M Tris-HCl, 3% BSA, and 20% normal bovine serum, pH 7.5.

TUNEL protocol for tissues which tend to give false positives

4. Rinse the slide(s) twice with PBS at RT. Let excess fluid drain off.

5. Apply 50 µl of TUNEL reaction mixture to the section and incubate for 60 min at 37°C in a humidified atmosphere.

6. Rinse slide(s) three times in PBS (5 min for each wash).
Note: *At this stage, you can evaluate the section under a fluorescence microscope.*

7. Block endogenous peroxidase activity by incubating slides for 10 min at RT with 0.3% H_2O_2 in methanol.

8. Repeat steps 3 and 4 to block nonspecific binding of the anti-fluorescein-antibody to the tissue.

9. Add 50 µl Converter-POD, pre-diluted 1:2 in blocking solution (from Step 3), and incubate for 30 min at 37°C in a humidified atmosphere.

10. Rinse slide(s) three times in PBS at RT for 5 min each.

11. Add 50 µl DAB substrate solution and incubate for 1–3 min at RT.

12. Wash slide(s) extensively in tap water and counterstain if needed.

3.1.2.3 Tips for avoiding or eliminating potential TUNEL labeling artifacts

To avoid this artifact	Which may be caused by	Try the following
Nonspecific TUNEL labeling	● DNA strand breaks induced by UV irradiation during tissue embedding (UV used to polymerize tissue embedding material such as methacrylate)	● Use a different embedding material, which does not require UV irradiation ● Use an alternate polymerization method
	● Acid tissue fixatives (e.g., mathacarn, Carnoy's fixative) cause DNA strand breaks	● Use buffered 4% paraformaldehyde as fixative
	● Endogenous nuclease activity which occurs soon after tissue preparation (e.g., in smooth muscle tissue slices)	● Fix tissue immediately after organ harvest ● Perfuse fixative through liver vein in intact animal
	● TdT concentration too high during TUNEL labeling	● Reduce concentration of TdT by diluting it 1:2 or 1:3 with TUNEL Dilution Buffer (Cat.No. 1966 006) containing 30 mM Tris (pH 7.2) containing 140 mM sodium cacodylate and 1 mM $CoCl_2$
	● Endogenous alkaline phosphatase activity during converter reaction	● Block endogenous AP activity by adding 1 mM levamisole to the AP substrate solution
	● Endogenous peroxidase activity during converter reaction	● Before permeabilizing cells, block endogenous POD activity by immersing the slides in a solution of 0.3% H_20_2 in methanol
	● Nonspecific binding of anti-fluorescein antibody conjugate during converter reaction	● Block nonspecific sites with normal anti-sheep serum ● Block nonspecific sites with PBS containing 3% BSA (20 min) ● Use 1:2 dilution of converter solution in PBS
High background	● Formalin fixation, which causes yellow staining of cells containing melanin precursors	● Use methanol fixation ***Note:*** *This fixation may lead to a reduction in TUNEL labeling sensitivity*
	● TUNEL labeling mix too concentrated (e.g., for carcinomas)	● Reduce concentration of labeling mix by diluting it 1:2 with TUNEL Dilution Buffer (Cat. No. 1966 006) containing 30 mM Tris (pH 7.2) containing 140 mM sodium cacodylate and 1 mM $CoCl_2$
	● Endogenous alkaline phosphatase activity during converter reaction	● Block endogenous AP activity by adding 1 mM levamisole to the AP substrate solution
	● Endogenous peroxidase activity during converter reaction	● Before permeabilizing cells, block endogenous POD activity by immersing the slides in a solution of 0.3% H_20_2 in methanol
	● Nonspecific binding of anti-fluorescein antibody conjugate during converter reaction	● Block nonspecific sites with normal anti-sheep serum ● Block nonspecific sites with PBS containing 3% BSA (20 min) ● Use 1:2 dilution of converter solution in PBS

Tips for avoiding or eliminating potential TUNEL labeling artifacts

To avoid this artifact	Which may be caused by	Try the following
Low TUNEL labeling (low sensitivity)	● Ethanol and methanol fixation	● Use buffered 4% paraformaldehyde as fixative
	● Extensive crosslinking during prolonged fixation reactions	● Reduce fixation time ● Use buffered 2% paraformaldehyde as fixative
	● Insufficient permeabilization of cells, so TUNEL reagents cannot reach nuclei	● Pretreat with proteinase K (concentration and time must be optimized empirically) ***Note:*** *To avoid possible nuclease contamination, use only Proteinase K from Roche Molecular Biochemicals, Cat. No. 161 519* ● Pretreat with 0.01 M sodium citrate for 30 min at 70°C ● Increase TUNEL incubation time
	● Restricted access of TUNEL reagents to nuclei, caused by paraffin-embedding	● After dewaxing tissue sections, treat with proteinase K (concentration, time, and temperature must be optimized empirically) ***Note:*** *To avoid possible nuclease contamination, use only Proteinase K from Roche Molecular Biochemicals, Cat. No 161 519* ● Immerse dewaxed tissue sections in 200 ml 0.01 M citrate buffer (pH 6.0) and treat with microwave irradiation (370 W, 5 min) ***Note:*** *Conditions must be experimentally optimized for each tissue*
No signal on positive control	● Inadequate DNase treatment (DNase concentration too low)	● For cryosections, apply 1 µg/ml DNase ● For paraffin-embedded tissue sections, apply 0.5 mg/ml DNase ● For many other samples, apply 1 U/ml DNase in a solution of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM $MnCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl; incubate 30 min at 37°C ● As an alternative DNase buffer, use a solution of 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mg/ml BSA
Diminished TUNEL staining during DNA counterstaining	● Quenching of fluorescein signal by propidium iodide (PI)	● Use 0.5 µg/ml PI as DNA stain ● Substitute TO-PRO-3 (from Molecular Probes) in place of PI

3.1.3 Technical tips on the use of Annexin-V-Biotin for light microscope detection

The following protocol provides a method for the detection of Annexin-V-Biotin-binding to cell culture cells with light microscopy. The percentage of necrotic cells is determined by trypan blue staining.

Preparation of solutions

- Annexin-V-Biotin working solution: Dilute 20 µl. Annexin-V-Biotin labeling reagent in 1000 µl incubation buffer (sufficient for 10 samples).
- HEPES buffer: Prepare according to the instructions in the Annexin-V-Biotin pack insert.

All steps can be performed at room temperature

1. Incubate 1 x 10^6 cells in 100 µl Annexin-V-Biotin working solution for 10–15 min.
2. Wash 2 times with HEPES buffer.
   For suspension cells: Continue with step 3.
   For adherent cells: Continue with step 4.
3. Resuspend suspension cells in 1 ml HEPES buffer. Transfer 2 x 10^5 cells to a slide using cytospin device.
4. Air dry cells. Fix with methanol/ethanol 1:1 for 90 sec.
5. Air dry cells. Add 100 µl Streptavidin-POD (Cat. No. 1 089 153) working solution, incubate for 1 h.
6. Rinse with HEPES buffer
7. Add DAB substrate solution (Cat. No. 1 718 096) working solution, incubate for 10–15 min.
8. Rinse with HEPES buffer
9. Analyze samples under a light microscope.

3.1.4 Technical tips on the use of the Apoptotic DNA Ladder Kit on tissue samples

The package insert for our Apoptotic DNA-Ladder Kit, Cat. No. 1 835 246, describes the purification of nucleic acids from whole blood and cultured cells. By following the modified procedure decribed here it is also possible to use tissue samples.

Preliminary Information

- **Weight of sample:** The tissue sample should weigh between 25 and 50 mg.
- **Additional required solutions:**
  - Lysis buffer: Prior to extraction of DNA, prepare a lysis buffer. 200 µl of this buffer are sufficient for one tissue sample. The lysis buffer consists of 4 M urea, 100 mM Tris, 20 mM NaCl and 200 mM EDTA, pH 7.4 (25°C).
  - Proteinase K solution: 20 mg/ml in 50 mM Tris-HCl (pH 8.0) and 1 mM $CaCl_2$.

Protocol for isolation of DNA from tissue samples

1. Add 200 µl lysis buffer and 40 µl proteinase K solution to 25–50 mg tissue, mix.
2. Incubate for 1 h at 55°C.
3. Add 200 µl binding buffer, mix.
4. Incubate for 10 min at 72°C.
5. Proceed with the addition of 100 µl isopropanol as described in the pack insert (3rd. step of section 5).

Note: *Be aware, that apoptosis is a single cell event, and therefore in most tissues you will not find a sufficient number of apoptotic cells to produce a DNA ladder.*

3.1.5 Technical tips on the Cell Proliferation ELISA kits

How to interrupt the proliferation assay
The detection of BrdU-labeled DNA with the Cell Proliferation ELISAs does not take more than 1.5–3 hours. Nevertheless, the labeling period which may vary between 2 and 24 hours can get the scientist in time trouble. Our assay can be interrupted after the labeling process: After the removal of the culture medium, the protocol proceeds with the drying of the labeled cells using e.g. a hair-dryer. The dry cells stay safe and sound up to one week when stored at 4°C in the microtiter plate before they are fixed and denatured according to the provided protocol.

A tip for measuring lymphocyte proliferation
To study the proliferation of lymphocytes, the cells are stimulated e.g. with growth factors, cytokines or mitogens. The increase in cell numbers can (in special cases) lead to cluster formation of the lymphocytes: Cells from the same progenitor stick together and form aggregates in the culture plate.This effect may disturb the antibody recognition of the ELISA system and thereby result in an underestimation of response. To avoid signal variation: Carefully resuspend the cells after the BrdU-labeling period and before centrifugation for removing the culture medium. This will enable the equal accessibility of each cell for the antibody recognizing the BrdU-label.

3.2 Special applications of cell death and cell proliferation methods

This section of the Appendix contains condensed versions of articles that appeared in the Roche Diagnostics Biochemica newsletter. For further experimental detail and background, see the full *Biochemica* articles.

3.2.1 TUNEL assays

3.2.1.1 Discrimination between dead and viable apoptotic cells using two-color TdT assay and surface labeling as detected by flow cytometry

[from Earl A. Timm, Jr. and Carleton C. Stewart, Laboratory of Flow Cytometry, Roswell Park Cancer Institute, Buffalo, N.Y., USA]

Note: *This article appeared in Biochemica No. 1 (1996), 44–47.*

Summary: The TUNEL method uses terminal dideoxynucleotidyl transferase (TdT) to incorporate hapten-tagged nucleotides into the 3'-strand breaks that occur in DNA during apoptosis (Gorczyca et al., 1993; Chapman et al., 1995). If these nucleotides are coupled to a fluorescent molecule, or if the hapten can be detected by a fluorescent secondary reagent, the apoptotic cells can be analyzed by flow cytometry.

Flow cytometry permits not only the detection of apoptotic populations, but also the simultaneous detection and immunophenotyping of necrotic populations. The drawback to using the current TdT method, however, is that the ethanol permeabilization of the cells is incompatible with immunophenotyping because it denatures cellular epitopes (Darzynkiewicz et al., 1992; Li et al., 1995).

A protocol has been developed that both preserves the surface markers and detects apoptotic cells. In addition, it is possible to discriminate between dead apoptotic cells and viable apoptotic cells with a second hapten-tagged nucleotide that labels dead cells. The method also can distinguish dead apoptotic cells from cells that have died by other mechanisms (e.g., necrosis).

3.2.1.2 The use of flow cytometry for concomitant detection of apoptosis and cell cycle analysis

[from E. Hanon, A. Vanderplasschen and P.-P. Pastoret, Department of Immunology/Vaccinology, Faculty of Veterinary Medecine, University of Liège, Liège, Belgium]

Note: *This article appeared in Biochemica No. 2 (1996), 25–27.*

Summary: Two distinct modes of cell death, apoptosis and necrosis, can be distinguished on the basis of differences in morphological, biochemical, and molecular changes occuring in the dying cells (Duvall and Wyllie, 1986).

Cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of the plasma membrane and nuclear disintegration (Duvall and Wyllie, 1986). Extensive damage to chromatin and cleavage of DNA into oligonucleosomal-length fragments both occur during apoptosis (Duvall and Wyllie, 1986).

Several flow cytometric methods for identifying cells undergoing DNA fragmentation have been described recently. These include DNA content analysis and *in situ* labeling of DNA fragments with tracer-dUTP. The former is based on the accumulation of ethanol-fixed apoptotic cells in the sub-G_0/G_1 peak of DNA content histogram as a result of loss of DNA fragments out of the cells and because of a reduced DNA "stainability" (Telford et al.,1991, 1992). The latter uses exogenous terminal deoxynucleotidyl transferase (TdT) to label *in situ* the DNA strand breaks with a tracer-dUTP (Gorczyca et al., 1993; Sgonc et al., 1994).

Recent observations have revealed a profound regulatory interrelationship between apoptosis and the cell cycle (Gorczyca et al.). The investigation of this relationship ideally requires techniques that permit concomitant apoptosis detection and cell cycle analysis at a single-cell level.

Two flow cytometric techniques are usually used to investigate the cell cycle: DNA quantification to identify the cell cycle position (Vindelov et al., 1990) and detection of bromodeoxyuridine (BrdU) incorporation to reveal cells going through the S phase (Gratzner, 1982). In this investigation, the development of flow cytometric techniques that permit concomitant detection of apoptosis and cellular DNA content or BrdU content analysis by adapting the apoptosis detection protocol of the Roche Diagnoctics *In Situ* Cell Death Detection Kit, Fluorescein is reported.

3.2.1.3 Comparison of two cell death detection methods: *In situ* nick translation and TUNEL

[from Maria Pihlgren, Joelle Thomas, and Jaqueline Marvel, Immunologie cellulaire, Lyon Cedex, France]

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Summary: Apoptosis is a form of regulated cell death characterized by specific morphological changes. These include cell shrinkage, membrane blebbing, chromatin condensation, and cell fragmentation into small apoptotic bodies. At the molecular level, the activation of an endogenous endonuclease results in the fragmentation of cellular DNA into oligosomal length fragments (Martin et al. 1994). These can be readily detected by DNA gel electrophoresis. However, gel electrophoresis does not allow the detection of apoptosis in individual cells.

In contrast, techniques that use enzymatic labeling of DNA strand breaks can provide information regarding apoptosis at a single-cell level. The TdT-mediated dUTP Nick End Labeling (TUNEL) technique uses terminal deoxnucleotidyl transferase (TdT) and allows the labeling of double-stranded DNA breaks (free 3'-OH DNA ends), while the *In Situ* Nick Translation (ISNT) method employs DNA Polymerase I and detects single-stranded DNA breaks. Another advantage of these techniques is that they can be used in combination with cell surface staining or cell cycle analysis. The abilities of the TUNEL and ISNT techniques to detect apoptosis in two types of cells: the IL-3-dependent cell line BAF-3 and freshly isolated $CD8^+$ lymphocytes from mouse spleen are compared.

3.2.1.4 Fixation of tissue sections for TUNEL combined with staining for thymic epithelial cell marker

[from Olav Schreurs, Trond S. Halstensen, Zlatko Dembic, Bjarne Bogen, Karl Schenck, Department of Oral Biology, University of Oslo, Oslo, Norway]

Note: *This article appeared in Biochemica No. 4 (1997), 19–21.*

Summary: In the thymus, positive and negative selection of thymocytes are important forces that shape the repertoire of mature T lymphocytes in the immune system. In studies on negative selection, it is of great interest to determine whether apoptotic cells reside in thymic cortex or medulla (Surh et al. 1994; Kisielow et al. 1995). Terminal dUTP nick end labeling (TUNEL) is a technique, that is well suited to demonstrate apoptosis *in situ*, and the method may be combined with labeling of other markers. In order to distinguish between thymic cortex and medulla, differential expression of cytokeratin, MHC class II molecules and epithelial cell markers have been used (Surh et al. 1994; Wack et al. 1996; Douek et al. 1996). In the course of an investigation on deletion of tumor-specific TCR-transgenic T-cells in the thymus (Lauritzsen et al.), TUNEL has been combined with commercially available monoclonal antibodies, that are monospecific for thymic epithelial cells, to unambiguously localize T-cell deletion. During the course of these studies, we established fixating conditions, that gave us superior results.

3.2.2 Metabolic assays

3.2.2.1 Biochemical and cellular basis of cell proliferation assays that use tetrazolium salts

[from Michael V. Berridge, An S. Tan, Kathy D. McCoy, and Rui Wang, Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington South, New Zealand]

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Summary: Tetrazolium salts (such as MTT, XTT, and WST-1) are used extensively in cell proliferation and cytotoxicity assays, enzyme assays, histochemical procedures, and bacteriological screening. In each, these tetrazolium salts are metabolically reduced to highly colored end products called formazans. Yet, the nature of their cellular bioreduction is poorly understood despite their long-time use (Stoward and Pearse, 1991).

In our laboratory, we demonstrated that most cellular reduction of MTT was dependent on the reduced pyridine nucleotides NADH and NADPH, not on succinate as had been previously believed (Berridge et al., 1993, 1994; Berridge and Tan, 1993). Cellular reduction of MTT was associated with enzymes of the endoplasmic reticulum and was more related to NADH production through glycolysis than to respiration.

Recently, assays have been introduced based on tetrazolium salts (such as XTT and WST-1) that are reduced to soluble formazans. These assays depend on intermediate electron acceptors such as phenazine methosulfate (PMS).

The question arises: Is the cellular reduction of these new salts similar to that of MTT? In this article, the answer to that question is attempted.

In summary, it could be shown that, unlike MTT, XTT and WST-1 are efficiently reduced by NADH and NADPH in the absence of cells or enzymes, and their reduction involves superoxide. Cellular reduction of WST-1 occurs at the cell surface and also involves superoxide.

3.2.3 Annexin assays

3.2.3.1 The use of annexin for concomitant detection of apoptosis and cellular phenotype

[from S. Hoornaert, E. Hanon, J. Lyaku, and P.-P. Pastoret, Department of Immunology/Vaccinology, Faculty of Veternary Medicine, University of Liège, Liège, Belgium]

Note: *This article appeared in Biochemica No. 3 (1997), 19–20.*

Summary: Two distinct modes of cell death, apoptosis and necrosis, can be distinguished on the basis of differences in morphological and biochemical characteristics. Under the elctron microscope, cells undergoing apoptosis display cell shrinkage, apoptotic body formation, and chromatin condensation. Biochemically, the apoptotic process is charaterized by fragmentation of DNA into oligonucleosomal fragments. Furthermore, during the early stages of apoptosis, changes also occur at the cell surface membrane (Andree et al. 1990; Creutz, 1992; Fadok et al. 1992). One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part to the outer layer of the plasma (Vermes et al., 1995), thus exposing PS at the external surface of apoptotic cells, where it can be specifically recognized by macrophage (Fadok et al. 1992).

Annexin V, a Ca^{2+}-dependent phospholipid-binding protein, possesses high affinity for PS (Vermes et al., 1995) and can thus be used for detecting early apoptotic cells (Koopman et al. 1992, Verhoven et al. 1995, Vermes et al. 1995, Homburg et al. 1995). Since annexin V can also detect necrotic cells as a result of the loss of membrane integrity, apoptotic cells have to be differentiated from these necrotic cells by the use of propidium iodide (PI). Indeed, PI selectively labels necrotic, but not apoptotic cells.

Several studies have revealed a correlation between apoptosis and cell phenotype (Carbonari et al. 1995, Lewis, et al. 1994). The investigation of this relationship ideally requires techniques that permit the concomitant detection of apoptosis and cell phenotype analysis at a single cell level. In this report, the development of a procedure which permits concomitant detection of apoptosis and cell phenotype characterization by flow cytometry is described.

3.2.4 BrdU assays

3.2.4.1 Detection of bromodeoxyuridine in paraffin-embedded tissue sections using microwave antigen retrieval is dependent on the mode of tissue fixation

[from Wesley M. Garrett and H.D. Guthrie, Germplasm and Gamete Physiology, Agricultural Research Service, Beltsville, United States]

Note: *This article appeared in Biochemica No. 1 (1998), 17–20.*

Summary: A simple routine microwave antigen retrieval procedure allows the sensitive detection of incorporated BrdU in pulse labeled cells. Of the two fixatives tested, Carnoy's offers superior nuclear morphology, but with a sacrifice of immunostaining intensity. For investigations where animals are sacrificed within several hours after pulse labeling, Carnoy's fixative may prove adequate for a general fixative, but it is not known what effect it has on cellular antigens of interest. For our purposes, 10% neutral buffered formalin was found to be a superior fixative, because of its ability to cross-link nuclear proteins and associated chromatin, resulting in more intense immunostaining for BrdU. In addition, we have found that formalin fixation coupled with microwave antigen retrieval is completely compatible with immunostaining of other antigens of interest.

3.3 References

3.3.1 Apoptosis-related parameters – Abbreviations and References

Parameter	Full length name	Reference	Roche Molecular Biochemicals product
AIF	Apoptosis inducing factor	Susin S. A. et al. (1996) *J. Exp. Med.* **184**, 1331.	
Apaf	Apoptotic protease activating factor	Zou H. et al. (1997) *Cell* **90**, 405. Li P. et al. (1997) *Cell* **91**, 479.	
APO-2 (L)	Apoptosis receptor/ligand	Masters S. A. et al. (1996) *Curr. Biol.* **6**, 750. Pit R. M. et al. (1996) *J. Biol. Chem.* **271**, 12687.	
APO-3 (L)	Apoptosis receptor/ligand	Masters S. A. et al. (1996) *Curr. Biology* **6**, 1669. Chinnaiyan A. M. et al. (1996) *Science* **274**, 990.	
Apopain		Schlegel J. et al. (1996) *J. Biol. Chem.* **271**, 1841.	
Bad		Yang E. et al. (1995) *Cell* **80**, 285.	
Bak		Sattler M. et al. (1997) *Science* **275**, 983. Orth R. & Dixit V. M. (1997) *J. Biol. Chem.* **272**, 8841.	
Bax		Bargou R. C. et al. (1995) *Eur. J. Immunol.* **25**, 770. Zhan Q. M. et al. (1994) *Oncogene* **9**, 3743. Yang E. et al. (1995) *Cell* **80**, 285.	
Bcl-2		Craig W. C. (1995) *Cancer Biology* **6**, 35. Yang E. et al. (1995) *Cell* **80**, 285.	Anti-bcl-2, Cat. No. 1 624 989
Bcl-x_L		Yang E. et al. (1995) *Cell* **80**, 285.	
Bcl-x_S		Williams G. T. & Smith C. A. (1993) *Cell* **74**, 777. Yang E. et al. (1995) *Cell* **80**, 285.	
bik		Orth R. & Dixit V. M. (1997) *J. Biol. Chem.* **272**, 8841.	
Ca^{2+}		McConkey D. J. et al. (1995) *J. Immunology* **155**, 5133. Kataoka A. et al. (1995) *FEBS Letters* **364**, 264. Sokolova I. A. et al. (1995) Biochimica et Biophysica Acta – Mol. *Cell Res.* **1266**, 135.	
CAD	Caspase activated DNase	Enari, M. et al. (1998) *Nature* **391**, 43.	
Calpain		Kikuchi H. & Imajohohmi S. (1995) *Cell Death and Differentiation* **2**, 195. Slukvin I. I. & Jerrelis T. R. (1995) *Immunopharmacology* **31**, 43.	Calpain inhibitor I, Cat. No. 1 086 090 Calpain inhibitor II, Cat. No. 1 086 103
Caspase	Cysteine protease cleaving an aspartic acid residue	Cohen G. M. (1997) *Biochem. J.* **326**, 1. Alnemri E. S. et al. (1996) *Cell* **87**, 171. Nicholson D. W. & Thornberry N. A. (1997) *TIBS* **22**, 299.	
ced-3	Caenorhabditis elegans cell death gene	Yuan J. et al. (1993) *Cell* **75**, 641. Miura M. et al. (1993) *Cell* **75**, 653.	
ced-9	Caenorhabditis elegans cell death gene	Henegartner M. O. & Horovitz H. R. (1994) *Cell* **76**, 665.	
Ceramide		Wiegmann K. et al. (1994) *Cell* **78**, 1005.	
c-Jun		Grand R. J. A. et al. (1995) *Exp. Cell Res.* **218**, 439.	
c-Myc		Wang Y. et al. (1993) *Cell Growth Differ.* **4**, 467. Schwartz L. M. & Osborne B. A. (1993) *Immunol. Today* **14**, 582.	
CPP32		Darmon A. J. et al. (1995) *Nature* **377**, 446.	Anti-PARP, Cat. No. 1 835 238
crm A	Cytokine response modifier A	Zhou Q. et al. (1997) *J. Biol. Chem.* **272**, 7797. Ogasawara J. et al. (1993) *Nature* **364,** 806.	

Apoptosis-related parameters

Parameter	Full length name	Reference	Roche Molecular Biochemicals product
Cytochrome C		● Liu X. et al. (1996) *Cell* **86**, 147. ● Krippner A. et al. (1996) *J. Biol. Chem.* **271**, 21629. ● Yang J. et al. (1997) *Science* **275**, 1129. ● Li P. et al. (1997) *Cell* **91**, 479.	
D4-GDP-DI	DI = dissociation inhibitor	● Danley D. E. et al. (1996) *J. Immunology* **157**, 500.	
Daxx	Death-domain-associated protein xx	● Yang X. L. et al. (1997) *Cell* **89**.	
DcR1	Decoy receptor 1	● Pan G. et al. (1997) *Science* **277**, 815. ● Sheridan J. P. et al. (1997) *Science* **277**.	
DD	Death Domain	● Muzio M. et al. (1996) *Cell*, **85**, 817.	
DED	Death Effector Domain	● Chinnaiyan A. M. et al. (1996) *J. Biol. Chem.* **271**, 4961.	
DISC	Death Inducing Signal Complex	● Muzio M. et al. (1996) *Cell*, **85**, 817.	
DNA-Fragmentation		● Wyllie A. H. et al. (1980) *Int. Rev. of Cytol.* **68**, 251. ● Burgoyne L. A. et al. (1974) *Biochem. J.* **143**, 67. ● Stach R. W. et al. (1979) *J. Neurochem.* **33**, 257.	● Apoptotic DNA Ladder Kit, Cat. No. 1 835 246 ● Cell Death Detection ELISA^{PLUS}, Cat. No. 1 744 425 ● Cell Death Detection ELISA, Cat. No. 1 544 675 ● Cellular DNA Fragmentation ELISA, Cat. No. 1 585 045 ● *In Situ* Cell Death Detection Kit, Fluorescein, Cat. No. 1 684 795 ● *In Situ* Cell Death Detection Kit, TMR, Cat. No. 2 156 792 ● *In Situ* Cell Death Detection Kit, AP, Cat. No. 1 684 809 ● *In Situ* Cell Death Detection Kit, POD, Cat. No. 1 684 817
DNA-PK$_{CS}$	DNA-dependent protein kinase catalytic subunit	● Casiolarosen L. et al. (1996) *J. Exp. Med.* **183**, 1957.	
DNA-repair		● De Murcia G. & De Murcia J. (1994) *TIBS* **19**, 172.	● Anti-PARP, Cat. No. 1 835 238
DR3	Death Receptor	● Chinnaiyan A. M. et al. (1996) *Science* **274**, 990.	
DR4	Death Receptor	● Pan G. H. et al. (1997) *Science* **276**, 111.	
DR5	Death Receptor	● Walczak H. et al. (1997) *EMBO J.* **16,** 5386. ● Sheridan J. P. et al. (1997) *Science* **277**.	
Endonuclease		● Walker P. R. & Sikorska (1994) *Biochem. and Cell Biology* **72**, 615. ● Dini L. et al. (1996) *Exp. Cell Res.* **223**, 340.	● Nuclease S7, Cat. No. 107 921 ● Nuclease P1, Cat. No. 236 225 ● Nuclease S1, Cat. No. 818 348 ● DNase I, RNase free, Cat. No. 776 785 ● DNase I, grade I, Cat. No. 104 132 ● DNase I, grade II, Cat. No. 104 159
FADD/ MORT-1	FADD = Fas-associated death domain	● Chinnaiyan A. M. et al. (1995) *Cell* **81**, 505. ● Chinnaiyan A. M. et al. (1996) *J. Biol. Chem.* **271**, 4961. ● Vincenz C. & Dixit V. M. (1997) *J. Biol. Chem.* **272**, 6578.	
FAK	Focal adhesion kinase	● Crouch D. H. et al. (1996) *Oncogene* **12**, 2689. ● Hungerford J. E. et al. (1996) *J. Cell Biol.* **135**, 1383.	
Fas	Synonyms: Fas = CD 95 = Apo1	● Trauth et al. (1989) *Science* **245**, 301.	● Anti-Fas, Cat. No. 1 922 432
Fas-ligand CD 95/fas (receptor)	Synonyms: Fas = CD 95 = Apo1	● Nagata S. & Goldstein P. (1995) *Science* **267**, 1449. ● Lynch D. H. et al. (1995) *Immunol. Today* **16**, 569. ● Tanaka M. et al. (1998) *Nature Medicine* **4**, 1, 31.	

Parameter	Full length name	Reference	Roche Molecular Biochemicals product
FLICE/MACH	FADD like ICE	● Muzio M. et al. (1996) *Cell* **85**, 817. ● Boldin M. P. et al. (1996) *Cell* **85**, 803. ● Fernandes-Alnemri T. et al. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 7464. ● Scaffidi C. et al. (1997) *J. Biol. Chem.* **272**, 43, 26953.	
FLIP	FLICE-inhibitory proteins	● Thome M. et al. (1997) *Nature* **386**, 517. ● Irmler M. et al (1997) *Nature* **388**, 190.	
Fodrin		● Martin S. J. et al. (1995) *J. Biol. Chemistry* **270**, 6425.	
fos		● Smeyne R. J. et al. (1995) *Nature* **363**, 166 and Erratum *Nature* **365**, 279. ● Colotta F. et al. (1992) *J. Biol. Chem.* **267**, 18278.	
G-Actin		● Boone D. L. & Tsang B. K. (1997) *Biology and Reproduction* **57**, 813.	
Gas-2		● Brancolini C. et al. (1997) *Cell Death and Diff.* **4**, 247.	
Gelsolin		● Kothakota S. et al. (1997) *Science* **278**, 294.	
Glucocorticoid/ Glucocorticoid-Receptor		● Schwartzman R. A. & Cidlowski J. A. (1994) *Int. Arch. of Allergy and Immunology* **105**, 347. ● Perrinwolff M. et al. (1995) *Biochem. Pharmacology* **50**, 103. ● Kiefer J. et al. (1995) *J. Immunology* **155**, 4525.	
Granzyme A, B		● Irmler M. et al. (1995) *J. Exp. Med.* **181**, 1917. ● Peitsch M. C. & Tschopp J. (1994) *Proteolytic Enzymes* **244**, 80. ● Nakajima H. et al. (1995) *J. Exp. Med.* **181**, 1037. ● Smyth M. J. & Trapani J. A. (1995) *Immunology Today* **16**, 202. ● Darmon A. J. et al. (1995) *Nature* **377**, 446. ● Quan L. T. et al. (1996) *Proc. Nat. Acad. Sci.* **93**, 1972.	
hnRNPs C1/C2	Heteronuclear Ribonucleoproteins	● Waterhaus N. et al. (1996) *J. Biol. Chem.* **271**, 29335.	
ICAD	Inhibitor of CAD	● Enari M. et al. (1998) *Nature* **391**, 43.	
ICE	Interleukin-1β/ converting enzyme	● Whyte M. & Evan G. (1995) *Nature* **376**, 17. ● Atkinson E. A. & Bleackley R. C. (1995) *Critical Reviews in Immunology* **15**, 359. ● Kumar S. & Harvey N. L. (1995) *FEBS Letters* **375**, 169.	● Interleukin-1β, human, Cat. No. 1 457 756 ● Interleukin-1β, mouse, Cat. No. 1 444 590 ● Interleukin-1β, ELISA, Cat. No. 1 600 729
JNK	Jun N-terminal kinase	● Hibi M. et al. (1993) *Genes Dev.* **7 (11)**, 2135.	
Lamin A, B		● Weaver V. M. et al. (1996) *J. of Cell Science* **109**, 45.	
MAP	Mitogen activated protein kinase	● Meyer C. F. et al. (1996) *J. Biol. Chem.* **271**, 8971.	
MCL-1		● Williams G. T. & Smith C. A. (1993) *Cell* **74**, 777.	
Mdm-2		● Chen J. D. et al. (1996) *Mol. and Cellular Biol.* **16**, 2445. ● Yu K. et al. (1997) *Cell Growth & Diff.* **8**.	
MEKK-1	MAP Kinase Kinase 1	● Cardone M. H. et al. (1997) *Cell* **90**. ● Meyer C. F. et al. (1996) *J. Biol. Chem.* **271**, 8971.	
MORT-1 (see FADD)		● Boldin M. P. (1995) *J. Biol. Chem.* **270**, 7795. ● Chinnaiyan A. M. et al. (1995) *Cell* **81**, 505. ● Chinnaiyan A. M. et al. (1996) *J. Biol. Chem.* **271**, 4961.	
NEDD		● Gu Y. et al. (1995) *J. Biol. Chemistry* **270**, 18715.	
NF-κB	Nuclear factor kappaB	● Wiegmann K. et al. (1994) *Cell* **78**, 1005.	
NuMa	Nuclear matrix protein	● Guethhallonet C. et al. (1997) *Exp. Cell Res.* **233**. ● Weaver V. M. et al. (1996) *J. Cell science* **109**, 45. ● Hsu H. L. & Yeh N. H. (1996) *J. Cell science* **109**, 277.	

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Parameter	Full length name	Reference	Roche Molecular Biochemicals product
p53		● Yonish-Rouach E. et al. (1993) *Mol. Cell Biol.* **13**, 1415. ● Zambetti G. P. (1993) *FASEB J.* **7**, 855. ● Lowe S. W. et al. (1993) *Cell* **74**, 957.	● Anti-p53, pan, Cat. No. 1 810 928 ● Anti-p53-Biotin, Cat. No. 1 810 936 ● Anti-p53-POD, Cat. No. 1 810 944 ● p53 ELISA, Cat. No. 1 828 789
PAK-2	p21 activated kinase	● Rudel T. & Bokoch G. M. (1997) *Science* **276**.	
PARP	Poly-ADP-ribose-polymerase	● Lippke J. A. et al. (1996) *J. Biol. Chem.* **271**, 1825. ● De Murcia G. & De Murcia J. (1994) *TIBS* **19**, 172.	
Perforin		● Nakajima H. et al. (1995) *J. Exp. Med.* **181**, 1037. ● Schroter M. et al. (1995) *Europ. J. Immunol.* **25**, 3509. ● Lowin B. et al. (1996) *Int. Immunology* **8**, 57.	
Phosphatidyl-serine		● Vermes I. et al. (1995) *J. Immunol. Methods* **184**, 39.	● Annexin-V-Alexa 568, Cat. No. 1 985 485 ● Annexin-V-Fluos, Cat. No. 1 828 681 ● Annexin-V-Biotin, Cat. No. 1 828 690
PITSLRE		● Beyaert R. et al. (1997) *J. Biol. Cem.* **272**, 11694.	
PKC δ	Protein kinase C	● Emoto Y. et al. (1995) *EMBO J.* **14**, 6148. ● Ghayur T. et al. (1996) *J. Exp. Med.* **184**, 2399.	
pRb	Retinoblastoma protein	● Hansen R. et al. (1995) *Oncogene* **11**, 2535. ● Haaskogan D. A. et al. (1995) *EMBO J.* **14**, 461. ● Picksley S. M. (1994) *Curr. Opinion in Cell Biology* **6**, 853.	
Presenilin		● Loetscher H. et al. (1997) *J. Biol. Chem.* **272**.	
prICE		● Smyth M. J. et al. (1996) *Biochem. Journal* **316**, 25.	
RAIDD	RIP associated ICH-1/CED-3 homologous protein with a death domain	● Duan & Dixit (1997) *Nature* **385**, 86.	
Ras		● Krueger G. R. F. et al. (1995) *Pathologe* **16**, 120. ● Wang H. G. et al. (1995) *J. Cell Biol.* **129**, 1103. ● Fernandez A. et al. (1995) *Oncogene* **10**, 769.	
RIP	Receptor interacting protein	● Stanger B. Z. et al. (1995) *Cell* **81**, 513. ● Hsu H. et al. (1996) *Immunity* **4,** 387. ● Grimm S. et al. (1996) *Proc. Natl. Acad. Sci.* **93**, 10923.	
Sphingo-myelinase		● Heller R. A. & Kronke M. (1994) *J. Cell Biol.* **126**, 5. ● Kolesnik R. & Golde D. W. (1994) *Cell* **77**, 325.	
SREBPs	Sterol-regulatory element binding proteins	● Wang X. D. et al. (1996) *EMBO J.* **15**, 1012.	
TNF-α	Tumor necrosis factor	● Leist M. et al. (1994) *J. Immunol.* **153**, 1778.	● TNF-α, human, Cat. Nos. 1 371 843, 1 088 939
TNF-α receptor		● Nagata S. (1997) *Cell*, **88**, 355. ● Tartaglia L. A. et al. (1993) *Cell* **74**, 845.	● TNF-α, mouse, Cat. No. 1 271 156 ● TNF-α ELISA, human, Cat. No. 1 425 943
TRADD	TNFR1-associated death domain	● Hsu H. et al. (1995) *Cell* **81**, 495.	
TRAF2	TNF receptor associated factor	● Liu Z.-G. et al. (1996) *Cell* **87**, 565.	

Parameter	Full length name	Reference	Roche Molecular Biochemicals product
TRAIL -R1, -R2, -R3	TNF-related apoptosis inducing ligand	Wiley S. R. et al. (1995) *Immunity* **3**, 673. Walczak H. et al. (1997) *EMBO Journal* **16**, 5386. Deglli-Esposti M. A. et al. (1997) *J. Exp. Med.* **186**, 1165. Sheridan J. P. et al. (1997) *Science* **277**, 818.	
Trans-glutaminase		Zhang L.-X. et al. (1995) *J. Biol. Chemistry* **270**, 6022. Melino G. et al. (1994) *Mol. and Cell Biology* **14**, 6584.	
U1-70 kDa snRNP	U1 small nuclear ribonucleoprotein protein	Rosena & Casciolarosen L. (1997) *J. Biol. Chem.* **64**, 50.	
YAMA	Synonyms: CPP32, Apopain	Tewari M. et al (1995) *Cell* **81**, 801.	

▲ **Table 24:** Published sources that contain more information about the components of the apoptosis pathways (Figure 2, page 4).

Synonyms

Proteases	Synonyms
Caspase-1	ICE
Caspase-2	ICH-1
Caspase-3	CPP32, Yama, Apopain
Caspase-4	ICErel-II, TX, ICH-2
Caspase-5	ICErel-III, TY
Caspase-6	Mch2
Caspase-7	Mch3, ICE-LAP3, CMH-1
Caspase-8	FLICE, MACH, Mch5
Caspase-9	ICE-LAP6, Mch6
Caspase-10	Mch4 / FLICE 2
Caspase-11	ICH-3
Caspase-12	
Caspase-13	ERICE
Caspase-14	MICE
Granzyme B	CTL proteinase-1, Fragmentin-2, RNKP-1

Receptor	Synonyms
CD95	APO-1, Fas
DcR1	TRID, LIT and TRAIL-R3
DcR2	TRAIL-R4
DcR3	
DR-3	APO-3, TRAMP, WSL-1, LARD
DR-4	TRAIL-R1
DR-5	TRAIL-R2, TRICK2, KILLER
DR-6	
DR-1	TNF-R1
DR-2	CD95
RANK	

Ligands	
CD95L	**Fas ligand, APO-1L**
TRAIL	**APO-2L**
TWEAK	**APO-3L**
RANK L	**TRANCE**

Apaf	Synonyms
Apaf-1	(no alternative, homologue to ced-4)
Apaf-2	Cytochrome C
Apaf-3	Caspase 9 (homologue to ced-3)

3.3.2 Examples for applications of Roche Molecular Biochemicals products

Cell Death

Annexin-V-Alexa 568, Cat. No. 1 985 485
Annexin-V-Biotin, Cat. No. 1 828 690
Annexin-V-FLUOS, Cat. No. 1 828 681
Annexin-V-FLUOS Staining Kit, Cat. No. 1 858 777

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3.4 General abbreviations

ABTS 2,2'-azino-di-[3-ethylbenzthiazoline-sulfonate (6)]

Ac N-acetyl

ActD actinomycin D

ALT alanine aminotransferase

AP alkaline phosphatase

APAAP alkaline phosphatase anti-alkaline phosphatase

APES aminopropyl-triethoxysilane

BCIP 5-bromo-4-chloro-3-indolyl phosphate

B-CLL chronic lymphocytic leukemia (B-type)

Bio biotin

BrdU 5-bromo-2'-deoxyuridine

BSA bovine serum albumin

CAM campothecin

Con A concanavalin A

cpm counts per minute

CTL cytotoxic T lymphocytes

DAB 3,3'-diaminobenzidine

DES diethylstilbestrol

DX dexamethasone

ELISA enzyme-linked immunosorbent assay

Fab protease-generated antibody fragments

$F(ab')_2$ protease-generated antibody fragment

FACS fluorescence activated cell sorter

FAQs frequently asked questions

FITC fluorescein isothiocyanate

FLUOS 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester

FSC forward light scatter

G_0 resting phase

G_1 gap between mitosis and DNA synthesis

G_2 gap between DNA synthesis and mitosis

h hour

HMW DNA high molecular weight DNA

HSV herpes simplex virus type I antigen

[^{3}H]-TdR tritiated thymidine (2'-deoxy)

ICE interleukin-1β-converting enzyme

INT 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride

INV-A influenza A virus antigen

INV-B influenza B virus antigen

INV-KA influenza control antigen

ISNT *in situ* nick translation

kD kilodalton

LAK cells lymphokine-activated killer cells

LDH lactate dehydrogenase

LMW DNA low molecular weight DNA

LSC liquid scintillation counting

M-phase mitosis

MTP microtiter plate

MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NBT 4-nitro-blue tetrazolium chloride

NK cells natural killer cells

OKT3 anti-CD3 monoclonal antibody

PARP poly(ADP-ribose) polymerase

PBL peripheral blood lymphocytes

PBS phosphate buffered saline

PFA paraformaldehyde

PHA phytohemagglutinin

PI propidium iodide

PMS phenazine methosulfate

pNA 4-nitranilide

POD peroxidase

PS phosphatidylserine

PVDF polyvinylidene difluoride

PWM pokeweed mitogen

ref. reference

rlu/s relative light units/second

RT room temperature

RUV rubella virus antigen

SA streptavidin

SAC Staphylococcus aureus Cowan I

SN supernatant

SOD superoxide dismutase

S-phase DNA synthesis (replication)

SSC side light scatter

TdR thymidine

TdT terminal deoxynucleotidyltransferase

TMB tetramethylbenzidine

TNF tumor necrosis factor

TRITC tetramethylrhodamine isothiocyanate

TUNEL terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

WST-1 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

X-dUTP hapten-labeled deoxyuracil triphosphate

X-dNTP hapten-labeled deoxynucleoside triphosphate

XTT 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

Z carbobenzoxy

Amino acids

Name	3-letter	1-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Homoserine	Hse	–
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Methionine sulfoxide	Met (O)	–
Methionine methylsulfonium	Met (S-Me)	–
Norleucine	Nle	–
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
α-aminoisobutyric acid	Aib	–

3.5 Ordering Guide

Products for Measuring Apoptosis in Cell Populations	Cat. No.	Pack Size
Apoptotic DNA Ladder Kit	1 835 246	1 kit (20 tests)
Cell Death Detection ELISA^{PLUS}	1 774 425 1 920 685	1 kit (96 tests) 10 x 96 tests
Anti-PARP	1 835 238	100 µl
Caspase 3 Activity Assay	2 012 952	1 kit (96 tests)

	Products for Measuring Apoptosis in Individual Cells	Cat. No.	Pack Size
TUNEL related reagents	***In Situ* Cell Death Detection Kit, Fluorescein**	1 684 795	1 kit (50 tests)
	***In Situ* Cell Death Detection Kit, AP**	1 684 809	1 kit (50 tests)
	***In Situ* Cell Death Detection Kit, POD**	1 684 817	1 kit (50 tests)
	TUNEL Label	1 767 291	3 x 550 µl (30 tests)
	TUNEL Enzyme	1 767 305	2 x 50 µl (20 tests)
	TUNEL POD	1 772 465	3.5 ml (70 tests)
	TUNEL AP	1 772 457	3.5 ml (70 tests)
	TUNEL Dilution Buffer	1 966 006	2 x 10 ml
	DAB substrate, precipitating (POD substrate)	1 718 096	1 pack
	NBT/BCIP Stock Solution (AP substrate)	1 681 451	8 ml
	Fast Red Tablets (AP substrate)	1 496 549	20 Tablets
Fluorescence labels	**Propidium iodide solution***	1 348 639	20 ml
	DAPI	236 276	10 mg
Annexin-related reagents	**Annexin-V-FLUOS**	1 828 681	250 tests
	Annexin-V-FLUOS Staining Kit	1 858 777	1 kit (50 tests)
	Annexin-V-Biotin	1 828 690	250 tests
	Annexin-V-Alexa 568	1 985 485	250 tests
	Streptavidin-Fluorescein	1 055 097	1 mg
	Streptavidin-POD	1 089 153	500 U (1 ml)
	Streptavidin-AP	1 089 161	1000 U (1 ml)
Antibodies	**M30 CytoDEATH**	2 140 322 2 140 349	50 tests 250 tests
	M30 CytoDEATH, Fluorescein	2 156 857	250 tests
	Anti-Fas (CD95/Apo-1)	1 922 432	100 µg
	Anti-p53 protein, pan	1 413 147	100 µg
	p53 pan ELISA	1 828 789	1 kit (96 tests)
	Anti-p53 protein pan, polyclonal		
	unlabeled	1 810 928	200 µg
	biotin-labeled	1 810 936	150 µg
	POD-labeled	1 810 944	50 U
	Anti-p53 protein, mutant	1 699 823	100 µg
	Anti-bcl-2 oncoprotein, human (clone 124)	1 624 989	1 ml

* only sold in the US.

Products for Measuring Cytotoxicity	Cat. No.	Pack Size
Cytotoxicity Detection Kit (LDH)	1 644 793	1 kit (2000 tests)
Lactate dehydrogenase	107 077	25 mg (2.5 ml)
Cellular DNA Fragmentation ELISA	1 585 045	1 kit (500 tests)
Cell Proliferation Kit I (MTT)	1 465 007	1 kit (2500 tests)
Cell Proliferation Kit II (XTT)	1 465 015	1 kit (2500 tests)
Cell Proliferation Reagent WST-1	1 644 807	2500 tests

Products for Measuring Cell Proliferation in Cell Populations	Cat. No.	Pack Size
Cell Proliferation Kit I (MTT)	1 465 007	1 kit (2500 tests)
Cell Proliferation Kit II (XTT)	1 465 015	1 kit (2500 tests)
Cell Proliferation Reagent WST-1	1 644 807	2500 tests
Cell Proliferation ELISA, BrdU (colorimetric)	1 647 229	1 kit (1000 tests)
Cell Proliferation ELISA, BrdU (chemiluminescent)	1 669 915	1 kit (1000 tests)
FixDenat	1 758 764	4 x 100 ml (2000 tests)
BrdU Labeling and Detection Kit III	1 444 611	1 kit (1000 tests)

Products for Measuring Cell Proliferation in Individual Cells	Cat. No.	Pack Size
***In Situ* Cell Proliferation Kit, FLUOS**	1 810 740	1 kit (100 tests)
BrdU Labeling and Detection Kit I	1 296 736	1 kit (100 tests)
BrdU Labeling and Detection Kit II	1 299 964	1 kit (100 tests)
Anti-BrdU (clone BMC 9318)		
unlabeled, formalin grade	1 170 376	50 µg (500 µl)
fluorescein-labeled, formalin grade	1 202 693	50 µg (500 µl)
Anti-BrdU, POD-labeled (clone BMC 6H8), Fab fragments, formalin grade	1 585 860	15 U
Anti-Ki-67 (Ki-S5), formalin grade	1 742 345	100 µg
Anti-PCNA/Cyclin, formalin grade	1 486 772	100 µg
Anti-Topoisomerase II alpha, human (clone Ki-S1), formalin grade	1 742 353	100 µg
Anti-Transferrin Receptor, human	1 118 048	200 µg

Additional Products for Cell Death/Cell Proliferation Studies	Cat. No.	Pack Size
Actinomycin C_1	102 008	10 mg
Calpain inhibitor I	1 086 090	25 mg
Calpain inhibitor II	1 086 103	25 mg
DNase I, RNase free	776 785	10 000 units
DNase I, grade I	104 132	20 000 units
DNase II, grade II	104 159	100 mg
Interleukin-1β, human, recombinant *(E. coli)*	1 457 756	10 0000 units
Interleukin-1β, mouse recombinant *(E. coli)*	1 444 590	10 0000 units
Interleukin-1β, human, ELISA	1 600 729	1 kit (96 tests)
Nuclease S7	107 921	15 000 units
Nuclease P1	236 225	1 mg
Proteinase K, lyophilizate	161 519	25 mg
Staurosporine	1 055 682	500 µg
TNF-α, human, recombinant *(E. coli)*	1 371 843	10 µg (1 000 000 units)
TNF-α, human, recombinant *(yeast)*	1 088 939	10 µg (1 000 000 units)
TNF-α, mouse, recombinant *(E. coli)*	1 271 156	5 µg (2 000 000 units)
TNF-α ELISA, human	1 425 943	1 kit (96 tests)

3.6 Index

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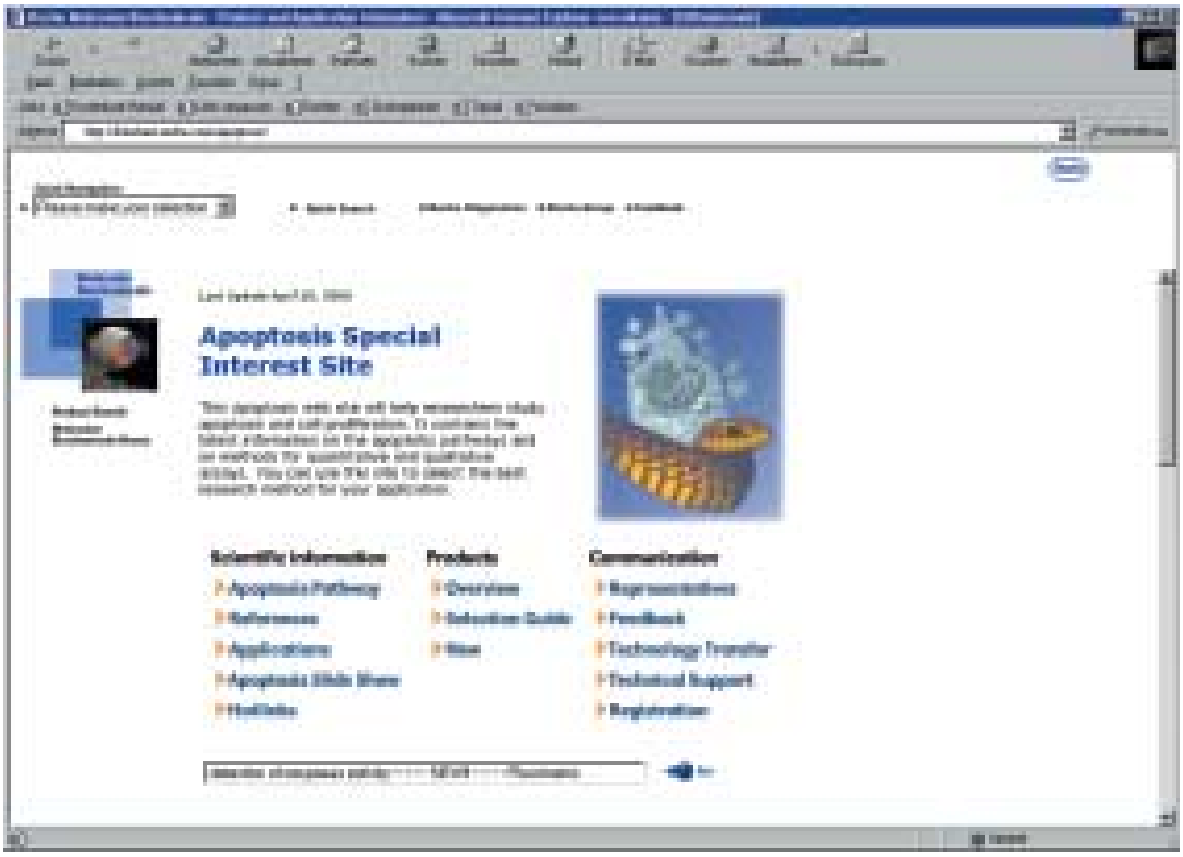
Apoptosis on the Internet

http://biochem.roche.com/apoptosis



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