Apoptosis, Cytotoxicity and Cell Proliferation

4th edition

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How cells die: Apoptosis and other cell death pathways

by Klaus Schulze-Osthoff

Introduction

Cell death is an essential part of normal development and continues into adulthood. The human body, for instance, is composed of approximately 10^{14} cells. Every day billions of cells die an altruistic death in order to secure the functionality of the whole organism. Thus, we remain the same size only because cell division exactly balances cell death.

During development, cell death helps sculpt organs or separate fingers and toes. It also eliminates structures that once served a function but are no longer needed, such as the tail of a tadpole during amphibian metamorphosis. Most of the neurons die during development before having any chance to function in the nervous system. Cell death also eliminates most newly formed lymphocytes, especially those that are useless or dangerous, by targeting self-antigens. Neutrophils, for instance, are produced continuously in the bone marrow, but the vast majority die within a few days. This apparently futile cycle of cell proliferation and cell death serves to maintain a supply of cells that can be readily mobilized when needed [1].

As cell death is intimately linked to tissue homeostasis, its disruption has, not surprisingly, been implicated in numerous pathological conditions. A reasonable estimate is that either too little or too much cell death contributes to approximately half of all medical illnesses, for many of which no adequate therapy exists. Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune syndromes, AIDS, ischemia, liver diseases and neurodegenerative disorders including Parkinson's and Alzheimer's disease [2]. Consequently, considerable interest has emerged in devising therapeutic strategies aimed at modulating cellular life-and-death decisions.

Since it was first described more than 40 years ago, tremendous progress has been made in our understanding of programmed cell death [3]. The importance of these discoveries was highlighted by the award of the 2002 Nobel Prize for Physiology or Medicine to S. Brenner, R. Horvitz and J. Sulston for their discoveries of the genetic regulation of organ development and programmed cell death in the nematode worm *Caenorhabditis elegans* [4]. Together with many other investigations, these have underscored the high degree of conservation of cell death pathways from nematodes to humans. Much of the attention in the field has been focused on a major cell death mechanism, apoptosis, which is often considered to be synonymous with cell death

However, the elucidation of additional programmed cell death pathways is gradually changing this notion. Indeed, it is now evident that cells have many options for dying, and that apoptosis is just one particular, albeit certainly the most prominent, form of cell death.

In recent years the molecular mechanisms of apoptosis have been deciphered at an unprecedented rate in biomedical research. As more than 10,000 papers are published annually in cell death research, it is impossible to keep track of all the developments in this exciting research area. In this survey I will try to provide an overview of cell death research that is mainly intended for newcomers to this field. I will focus on the regulation of apoptosis and methods for detecting apoptotic cells, but I will also explain its dynamic crosstalk with other modes of cell death.

Terminology of Cell Death

As our understanding of programmed cell death has evolved, it has become evident that cells can die by various mechanisms. A recent classification delineated eight different types of cell death, while some researchers describe as many as eleven pathways of cell death in mammals [5,6]. However, whether these various cell fates are indeed based on distinct molecular mechanisms remains unclear. Historically, three types of cell death have been distinguished in mammalian cells by morphological criteria.

Apoptosis, also termed type I cell death, is defined by characteristic changes in the nuclear morphology, including chromatin condensation and fragmentation, overall cell shrinkage, blebbing of the plasma membrane and formation of apoptotic bodies that contain nuclear or cytoplasmic material.

Autophagic cell death, also known as type II cell death, is characterized by a massive accumulation of double-membrane containing vacuoles known as autophagosomes, which subsequently fuse with lysosome vacuoles.

Type III cell death, better known as **necrosis**, is often defined in a negative manner as death lacking the characteristics of the type I and type II processes.

To simplify matters I will explain these three major cell death mechanisms (Figure 1). Distinction of different cell death forms is not only relevant for semantic reasons, but can also have important clinical implications when considering the potential therapeutic targeting of cell death processes. Nevertheless, in some conditions these distinct cell modes may only be the extremes, and there are numerous examples in which cell death demonstrates a continuum of intermediate features, for instance of both apoptosis and necrosis. In the literature we occasionally find newly coined expressions such as necrapoptosis or aponecrosis. As we shall discuss later, the inhibition of a particular type of cell death does not prevent cell death in many cases, but simply shifts the phenotype to alternative modes of cell death [7].

Furthermore, in some cases it is the cell type or the nature and duration of cellular injury that determine whether cells die by apoptosis, necrosis or other default mechanisms. At low doses, a variety of harmful stimuli such as radiation, hypoxia and anticancer drugs can induce apoptosis, but the same stimuli can result in necrosis at higher doses. Therefore, in many situations cell death may not occur as a clear-cut and paradigmatic form of cell death.

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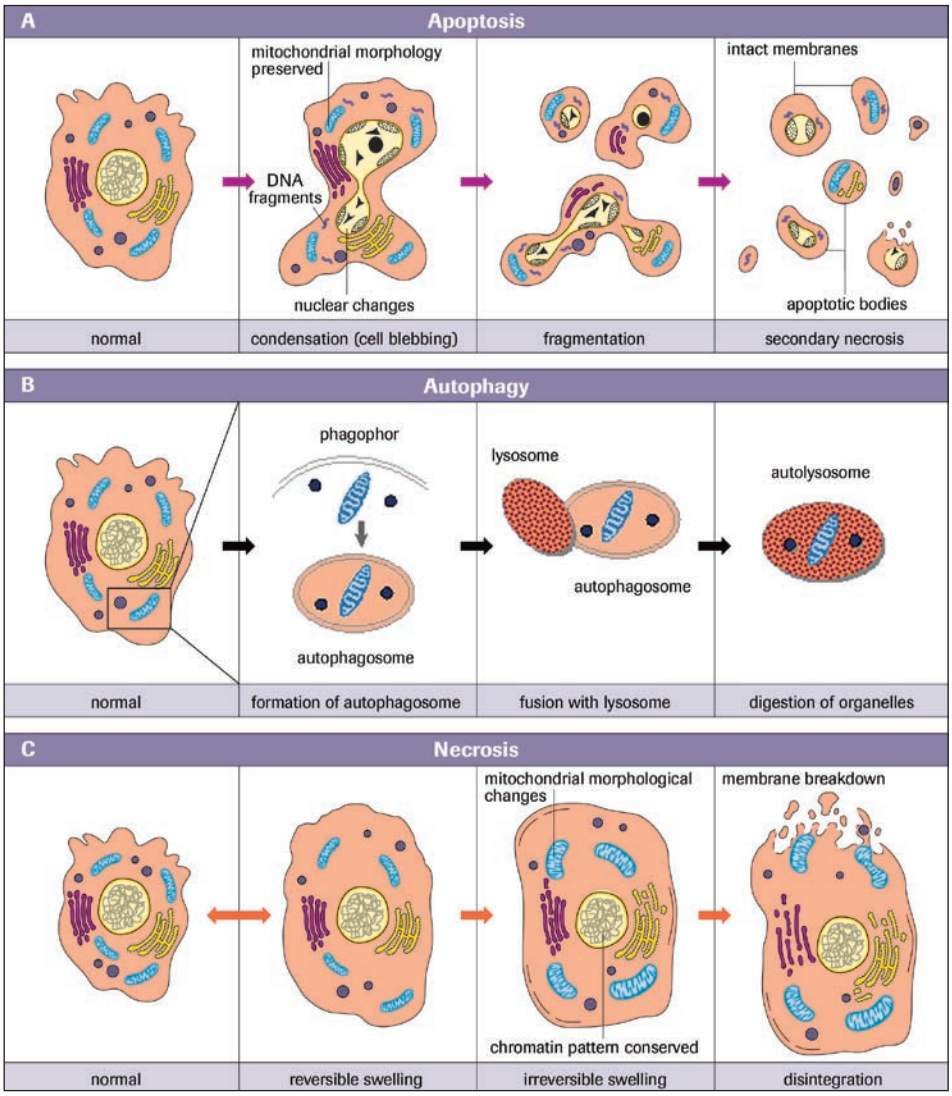

Figure 1: Three forms of cell death: Upon cell death induction cells can undergo different cell fates and morphological alterations including apoptosis, autophagy and necrosis. **A) Apoptotic** cells cells reveal characteristic changes in nuclear morphology, including chromatin condensation and fragmentation, overall cell shrinkage, blebbing of the plasma membrane and formation of apoptotic bodies that contain nuclear or cytoplasmic material. **B) Autophagy** is characterized by a massive accumulation of double-membrane containing vacuoles, called autophagosomes, which originate from phagophores or isolation membranes and encapsulate cytoplasmic material. The autophagosomes subsequently fuse with lysosomes to form autolysosomes, which causes the degradation of the autophagosomal contents. Under physiological conditions, autophagy is a survival mechanism that enables adaptation to starving conditions, whereas excessive autophagy can lead to cell death. **C) Necrosis** is morphologically defined by cytoplasmic swelling, dilation of organelles which causes cellular vacuolation and rupture of the plasma membrane, resulting in the proinflammatory leakage of the intracellular content.

Apoptosis

Apoptosis occurs in a well-choreographed sequence of morphological events. This process usually starts with the blebbing of the plasma membrane, which breaks up into membrane-enclosed particles, termed apoptotic bodies, containing intact organelles as well as portions of the nucleus. In fact the word 'apoptosis' comes from the ancient Greek, meaning 'falling off' (of petals from a flower) and refers to the morphological feature of the formation of apoptotic bodies [8]. These apoptotic bodies are rapidly recognized, ingested and eaten by professional phagocytes or neighboring cells. Under physiological conditions certain modifications occur in the plasma membrane which function as 'eat-me' signals and enable the apoptotic bodies to be recognized by phagocytic cells. Since the apoptotic bodies are surrounded by an intact plasma membrane, apoptosis usually occurs without any leakage of cellular contents and therefore without

provoking an inflammatory response. Moreover, the engulfment of apoptotic cells by macrophages triggers the production of anti-inflammatory cytokines.

Because apoptotic cells are eaten and digested so quickly, there are usually few dead cells to be seen in tissue sections, even when large numbers of cells have died. This probably explains why apoptosis was neglected by pathologists for a long time. Looking inside the cell, one of the most noticeable features of apoptosis is the condensation of the nucleus and its fragmentation into smaller pieces, a highly distinctive event that is not seen in other forms of cell death. Another defining feature is the extensive hydrolysis of nuclear DNA into internucleosomal fragments (Figure 1A).

Apoptosis is the major cell death pathway for removing unwanted and harmful cells in a clean or silent manner during embryonic development, tissue homeostasis and immune regulation. In addition, most anti-cancer therapies rely on the activation of apoptotic pathways. As the alterations of apoptosis are stereotypical and similar in all cell types irrespective of the death stimulus, the biochemical mechanisms underlying these changes also follow a similar built-in program. In nematodes, insects and human cells, most, if not all, morphological alterations of apoptosis are mediated by the activation of an evolutionarily conserved and unique class of intracellular proteases known as caspases [9].

Autophagy

Like apoptosis, autophagy is a highly conserved and genetically controlled process involving a cascade of molecular events [10]. Autophagy (meaning self-eating) is classically activated in response to nutrient starvation, but is also observed during development, differentiation and various forms of environmental stress. In addition, defective autophagy underlies a number of pathological conditions, including myopathies, neurodegenerative diseases, liver diseases, and some forms of cancer [11].

Autophagy is a major catabolic mechanism by which long-lived proteins and organelle components are directed to lysosomes and recycled in order to maintain energy and protein synthesis. It is characterized by the appearance of numerous cytosolic vacuole-like structures known as autophagosomes, which are formed by the assembly of double-layered, membrane-bound structures of still largely undefined origin. The autophagosomes encapsulate cytosolic materials and subsequently fuse with lysosomes, which causes the autophagosomal contents to degrade (Figure 1B).

Although the role of the autophagic process in protein and organelle degradation, and in protection during nutrient starvation is readily accepted, its function in programmed cell death is controversial [12]. This is partly because the term 'autophagic cell death' has been applied to two distinct observations: cell death *associated with* autophagy and cell death *through* autophagy.
Under normal physiological conditions autophagy occurs at low basal levels, contributing to the turnover of cytoplasmic components and promoting cell adaptation and survival during stress, *e.g.* starvation. Excess autophagy, on the other hand, leads to autophagic cell death.

Interestingly, in cells deficient for apoptosis, apoptotic signals can trigger massive autophagy and cell death, which is prevented by the inhibition of autophagosome formation. Thus, a complex relationship also exists between autophagy and apoptosis, in that autophagy can both promote and inhibit apoptotic cell death. Interestingly, some of the regulators of apoptosis also interfere with autophagic processes [13]. At present, it would seem that, while the predominant function of autophagy is to promote cell survival, extended autophagy will result in autophagic cell death. Clearly, more work is needed to elucidate the role of autophagy as a cell death mechanism and the complex crosstalk with other cell death pathways.

Necrosis

Necrotic cell death is often defined negatively as a form of cell death that lacks signs of apoptosis or autophagy. Necrotic cells typically show cytoplasmic swelling and vacuolation, rupture of the plasma membrane, dilation of organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation. When cells swell and burst they spill their contents over their neighbors and elicit a damaging inflammatory response [14] (Figure 1C).

Necrosis is usually considered to be an uncontrolled and accidental cell death which, unlike apoptosis, is not energy-dependent. Biochemically, most prominent features include massive energy depletion, the formation of reactive oxygen species and the activation of non-apoptotic proteases. All these events result in a loss of function of homeostatic ion pumps and damage to membrane lipids with cell membrane swelling and rupture.

Furthermore, during necrosis a substantial rise in intracellular calcium is observed. The elevated calcium levels in the cytosol trigger mitochondrial calcium overload, leading to depolarization of the inner mitochondrial membrane and a shut-down of ATP production [15]. While depletion of ATP impedes the function of membrane channels, increased calcium activates calcium-dependent proteases, *e.g.* calpains. Calcium fluxes, ATP depletion and oxidative stress involve complex and interactive feedback loops that self-amplify and potentiate each other, leading to exaggerated cell death. Such processes are most relevant under conditions of excessive trauma and ischemia-reperfusion. Necrosis, however, can also be observed in response to death receptor activation or chemotherapy, conditions that were originally believed to mediate cell death exclusively via apoptosis.

In addition, the inhibition of specific proteins involved in regulating apoptosis or autophagy can switch the type of cell death to necrosis. Finally, secondary or post-apoptotic necrosis occurs when massive apoptosis overwhelms the scavenging activity of phagocytes, thereby resulting in leakage of the cell contents with induction of inflammatory responses.

There is increasing evidence that necrosis is more tightly regulated than previously thought and underlies a genetic control that might be relevant in multiple physiological and pathological scenarios [14]. Necrosis might therefore serve as a backup cell death mechanism when apoptosis or autophagic cell death fails.

Molecular Basics of Apoptosis

Although any form of regulated cell death is potentially relevant, we will focus below on apoptosis, which is the best-defined and most prevalent form of cell death. In view of the recent explosion of publications, it is often difficult for newcomers in the field to grasp the essentials of apoptosis regulation. As we will see later, the basic molecular machinery of apoptosis is relatively simply, even though its complexity has increased through evolution.

The demolition machinery

Apoptosis results from a collapse of the cellular infrastructure through internal proteolytic digestion, which leads to cytoskeletal disintegration, metabolic derangement and genomic fragmentation [9]. Members of the caspase family of proteases form the core engine of apoptosis and are involved in the initiation, execution and regulatory phases of the pathway. Caspases are cysteine proteases that cleave substrates after certain aspartate

residues within specific tetra- or pentapeptide recognition sequences [16]. The fourteen different caspases identified in mammals are expressed in most cell types. To preclude unwarranted cell death, however, each caspase is maintained as an inactive zymogen consisting of a prodomain followed by two subunits with the catalytic domain. Caspases operate in hierarchical cascades that serve to amplify the apoptotic signal.

Based on their structure and sequence in cell death pathways, caspases can be divided into upstream initiators and downstream effectors of apoptosis. Effector caspases such as caspase-3, -6, and -7 contain only a short prodomain and cleave diverse cellular substrates, whereas initiator caspase-8, -9, and -10 have a long prodomain and exert regulatory roles by activating downstream effector caspases. While it was previously assumed that all caspases are activated by proteolytic cleavage, recent results suggest that the mode of activation differs considerably between initiator and effector caspases [17].

Effector caspases are constitutive homodimers that are always activated by initiator caspases through proteolytic cleavage.

By contrast, the zymogens of **initiator caspases** are predominantly present as monomers. Their activation is achieved by the binding of their long prodomains to high-molecular weight adaptor complexes that stimulate the homodimerization of the initiator caspases. The dimerization in turn causes a conformational change in the initiator caspases, resulting in their activation. Thus, the most important event for initiator caspase activation is their recruitment into caspase activation platforms.

Once activated, effector caspases cleave an estimated 400 substrates, including proteins involved in scaffolding of the cytoplasm, signal transduction and transcription-regulatory proteins, cell cycle-controlling components and proteins involved in DNA replication and repair [18]. For many of the identified substrates the functional consequences of their cleavage are unknown and have only been inferred from their normal functions. In other cases, proteolysis of certain components can be linked to discrete morphological changes. A classical example is the DNase inhibitor ICAD (inhibitor of caspase-activated DNase). Cleavage of ICAD by caspase-3 liberates the active CAD nuclease which, in turn, mediates apoptotic DNA fragmentation. The cleavage of several other substrates, including gelsolin as well as the kinases ROCK-1 and PAK2, has been implicated in membrane blebbing of apoptotic cells.

Moreover, caspases destroy several proteins required for maintenance of the cytoskeletal architecture, such as the intermediate filament cytokeratin-18, which is involved in filament organization. A large percentage of caspase substrates are further involved in cell adhesion or mediate cell–cell communication in adherens and gap junctions.

However, caspase activation is not necessarily synonymous with cell death: on the one hand, it is apparent that cells can survive limited caspase activation and, conversely, that inhibiting caspases blocks the morphological manifestations of apoptosis, but does often not influence final cell death [7]. Moreover, certain caspases such as caspase-1 are involved in the regulation of inflammation, rather than of apoptosis, by controlling the maturation of cytokine precursors, *e.g.* pro-interleukin-1 . Additionally to their established function in cell death, caspases may also participate in several non-apoptotic processes by controlling events in cell proliferation and differentiation [19,20].

1

Activation pathways

Caspase activation is initiated by two major signaling routes, namely the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (Figure 2). Death receptor-mediated apoptosis plays a fundamental role in the maintenance of tissue homeostasis, especially in the immune system, whereas the mitochondrial pathway is used extensively in response to extracellular cues and internal insults such as DNA damage [21,22].

Death receptors form a subgroup of the tumor necrosis factor (TNF) receptor superfamily that includes TNF-R1, CD95 (Fas/APO-1) and receptors binding to the TNF-related apoptosis-inducing ligand (TRAIL). All death receptors are characterized by an intracellular motif, termed the death domain, which is important for transmission of the apoptotic signal. Upon ligand binding, the death receptors interact via their death domain with a corresponding protein motif in adapter proteins such as FADD. These adapters also contain a second homotypic protein interaction motif, the death effector domain, which facilitates their binding to the prodomain of the initiator caspase-8. Taken together, these components form the death-inducing signaling complex (DISC) which, when assembled, activates caspase-8 through a proximity-induced dimerization mechanism [23,24]. At this point a high local concentration of dimerized caspase-8 leads to its autocatalytic activation and the subsequent activation of downstream effector caspases.

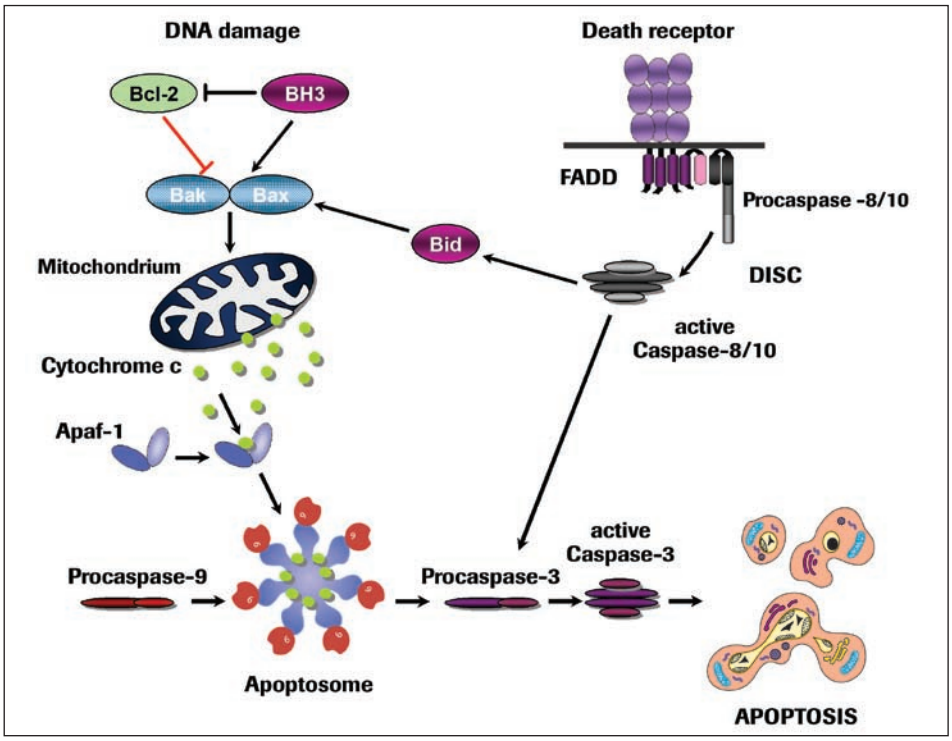

Figure 2: The two major routes to apoptosis. The intrinsic mitochondrial pathway is triggered in response to various forms of cellular stress, such as DNA damage, which provokes the activation of one or more proapoptotic BH3-only proteins. BH3-only proteins act as sensors for various death stimuli and relieve the inhibitory action of antiapoptotic Bcl-2 proteins. The activation of BH3-proteins results in the oligomerization of Bax and Bak, which are thought to form pores in the outer mitochondrial membrane causing the release of cytochrome c. Subsequently, cytosolic cytochrome c binds to the adaptor protein Apaf-1 and initiates formation of the apoptosome and activation of initiator caspase-9. The extrinsic death receptor pathway is initiated by binding of death ligands to their cognate death receptors and subsequent recruitment of the adaptor protein FADD and caspase-8/-10 into the caspase-activating death-inducing signaling complex (DISC). In both pathways effector caspases, such as caspase-3, are activated cleaving selected target proteins. The two pathways are interconnected by the Bcl-2 protein Bid that is cleaved by caspase-8/10 resulting in its mitochondrial translocation and subsequent cytochrome c release.

The death receptor pathway plays an important role in the immune system. For instance, when we become infected by a virus, cytotoxic T lymphocytes are activated and then express CD95 ligand. By binding to its receptor, CD95 ligand induces apoptosis in the infected cells to prevent the virus from replicating and spreading to other cells. Cytotoxic T cells, in addition, utilize a granule-exocytosis pathway for the elimination of virus-infected cells. Cytotoxic granules contain a pore-forming protein, perforin, and serine proteases known as granzymes. Granzyme B can directly cleave proteins after aspartate residues and can therefore also activate caspase-3. In this way the upstream events of death receptor signaling are bypassed, and apoptosis is induced directly (Figure 2).

Most cell death, however, proceeds via the mitochondrial pathway, which integrates signals generated by a variety of stressors including DNA damage, loss of adhesion, growth factor withdrawal and others [25]. As a key event these apoptotic stimuli evoke the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. Cytochrome c normally functions in electron transport processes of the respiratory chain to generate ATP. In the cytosol of apoptotic cells, however, it serves as a cofactor for the adapter protein Apaf-1. Upon binding of cytochrome c, Apaf-1 oligomerizes and recruits the initiator caspase-9 to trigger the formation of the apoptosome [26]. In this case the binding of caspase-9 to Apaf-1 is mediated by a shared homotypic interaction motif known as the caspase recruitment domain (CARD). Thus, like the DISC, the apoptosome is a high-molecular weight complex that serves as a caspase activation platform. Once assembled in the apoptosome, caspase-9 becomes activated and subsequently triggers the caspase cascade (Figure 2).

Although, at first glance, both pathways of caspase activation appear to trigger apoptosis in a straightforward manner, the circuitry of the system is often more complicated. In some cell types, for example, activation of caspase-8 at the DISC is insufficient to stimulate the direct activation of effector caspases, and this reaction requires an amplification step with cytochrome c-dependent apoptosome formation. In this scenario caspase-8 cleaves and activates the pro-apoptotic Bcl-2 protein Bid, which catalyzes cytochrome c release. Bid cleavage thereby links the death receptor to the mitochondrial pathway. Furthermore, in addition to these two pathways, a number of other pathways of caspase activation have been reported, including an initiator role of caspase-2 or of caspase-12 in apoptosis triggered by endoplasmic reticulum stress. However, unlike the well-established role of the death receptor and mitochondrial pathway, the physiological relevance of these death routes remains controversial and is currently not supported by the phenotype of knockout models.

Intracellular controls

The decision to die must be tightly controlled, and so it is not surprising that the death program is regulated not only by proapoptotic mediators, but also by various apoptosis inhibitors. In the death receptor pathway this is accomplished by the caspase-8 inhibitor c-FLIP, which has a structure very similar to caspase-8 but without the catalytic protease domains. Upregulation of c-FLIP expression can therefore compete efficiently with caspase-8 for DISC binding [24].

A crucial point in the intrinsic pathway is an event known as mitochondrial outer membrane permeabilization (MOMP), which often defines the point-of-no return [27,28]. MOMP is thought to involve the formation of mitochondrial membrane-spanning pores that mediate the release of cytochrome c and other cytotoxic proteins. During apoptosis MOMP is essentially controlled by proteins of the Bcl-2 family. The prototype member of this family, Bcl-2 itself, was initially identified in a common form of B-cell lymphoma, where a chromosome translocation causes overproduction of the Bcl-2 protein. The high levels of Bcl-2 promote cancer by inhibiting apoptosis, thereby prolonging cell survival.

Meanwhile, more than 20 members of the Bcl-2 family have been identified, which are all defined by the presence of one to four Bcl-2 homology (BH) domains [29]. Bcl-2 proteins come in two flavors including, firstly, anti-apoptotic proteins that prevent the release of cytochrome c and, secondly, proapoptotic proteins that trigger MOMP.

The proapoptotic Bcl-2 proteins can be further subdivided into two subfamilies based on the sharing of BH domains. BH multidomain proteins, such as Bax and Bak, are the triggers of MOMP, most likely as a result of their ability to form pores in the outer mitochondrial membrane. The other subfamily, the BH3-only proteins, which contain only the BH3 domain, act as upstream regulators by controlling the allosteric activation of the gatekeepers Bax and Bak. It is therefore not surprising that the activity of BH3-only proteins is under tight transcriptional and posttranslational control. While, in healthy cells, BH3-only proteins are either not expressed or are conformationally restrained, they rapidly become activated following exposure to cellular stresses. Interestingly, different types of stresses can evidently activate distinct sets of BH3-only proteins, suggesting that BH3-only proteins act as essential sensors of different death stimuli [29].

In addition to cytochrome c, activation of the proapoptotic Bcl-2 proteins results in the release of toxic mitochondrial mediators, including the protein Smac and the serine protease Omi/HtrA2 [30]. Both proteins can promote apoptosis by counteracting the inhibitor-of-apoptosis proteins (IAPs), which comprise a family of endogenous caspase inhibitors. Interestingly, Omi/HtrA2 is also thought to promote caspase-independent cell death, apparently due to its serine protease activity.

Another group of proteins released from mitochondria comprises apoptosis-inducing factor (AIF) and endonuclease G. Both can promote cell death in a caspase-independent manner by inducing chromatin condensation and DNA degradation. Thus, if for some reason cells do not activate caspases after MOMP, these mediators might still ensure that cell death proceeds.

Assays for Apoptosis

Since apoptosis occurs via a tightly regulated cascade, there are many possibilities to measure the activity of these regulators or the functional consequences of their action (Figure 3). A large number of apoptosis assays for detecting and counting apoptotic cells have been devised. All of these assays have advantages and disadvantages. For instance, certain features of apoptosis might only appear transiently, while others might partially overlap with necrosis. It is therefore crucial to employ two or more distinct assays to confirm that cell death is occurring via apoptosis. In addition, certain assays might be suitable for cultured cells, but inappropriate for investigating apoptosis in tissue sections. Therefore, when choosing methods of apoptosis detection in cells, tissues or organs, we should understand the pros and cons of each assay

Caspase activation

When caspases become activated during apoptosis, they cleave specific substrates, either activating or inactivating them. These cleavages produce the morphologic changes associated with apoptosis [9,18]. Caspase activation also provides us directly or indirectly with markers of apoptosis. An indication that caspase activation has occurred in a cell can be provided by the detection of cleavage of known caspase substrates. Antibodies against a variety of caspase substrates, such as PARP-1 (see Anti-Poly (ADP-Ribose) Polymerase (Anti-PARP), page 34) and many others, are available and can be employed in immunoblot analysis. Moreover, some companies have developed antibodies that solely detect the caspase-cleaved form, but not the native form, of a substrate protein. Such cleavage site-specific antibodies against caspase substrates, *e.g.* those against cleaved

cytokeratin-18 (see M30 CytoDEATH, page 21), are particularly convenient for detecting early apoptosis in cellular assays and even in archived tissue biopsies by means of immunohistochemistry.

An alternative that is often used to obtain evidence for the involvement of a particular caspase is to monitor its proteolytic cleavage as an indicator of caspase activation. While the proteolytic processing of effector caspase-3, -6 and -7 faithfully reflects their activation, this does not hold true for initiator caspases [17]. As mentioned above, in contrast to a widespread misinterpretation seen in many publications, the mere cleavage of an initiator caspase does not activate it, and therefore this cannot be taken as sufficient evidence for its activation. Furthermore, certain caspases are also cleaved by non-caspase proteases at a non-relevant site, which might incorrectly indicate that caspase-mediated proteolytic activation has occurred.

Caspase activity is most frequently detected in synthetic substrates assays. The substrates are generated by fusing a preferred tetrapeptide cleavage sequence at the aspartate residue to a fluorogenic or colorimetric reporter, so that a signal is produced upon substrate cleavage (see Homoegeneous Caspases Assay, fluorimetric, page 30). The knowledge of the inherent substrate specificity of individual caspases has further been employed to construct peptide-based substrate reporters that are often claimed to measure the activity of specific caspases. However, although these substrates are useful for characterizing individual purified caspases, they are unable to distinguish a role of a certain caspase in cell lysates. This is due to a significant overlap between individual consensus sequences, because the caspases are promiscuous on these sequences. This specificity problem, however, can be solved by the use of ELISA-based capture assays for individual caspases that are coupled with a substrate reaction (see Caspase 3 Activity Assay, page 26). Thus, by employing adequate tools and appropriate assays, detection of caspase activity or its cleavage products might still constitute a specific approach for measuring apoptosis.

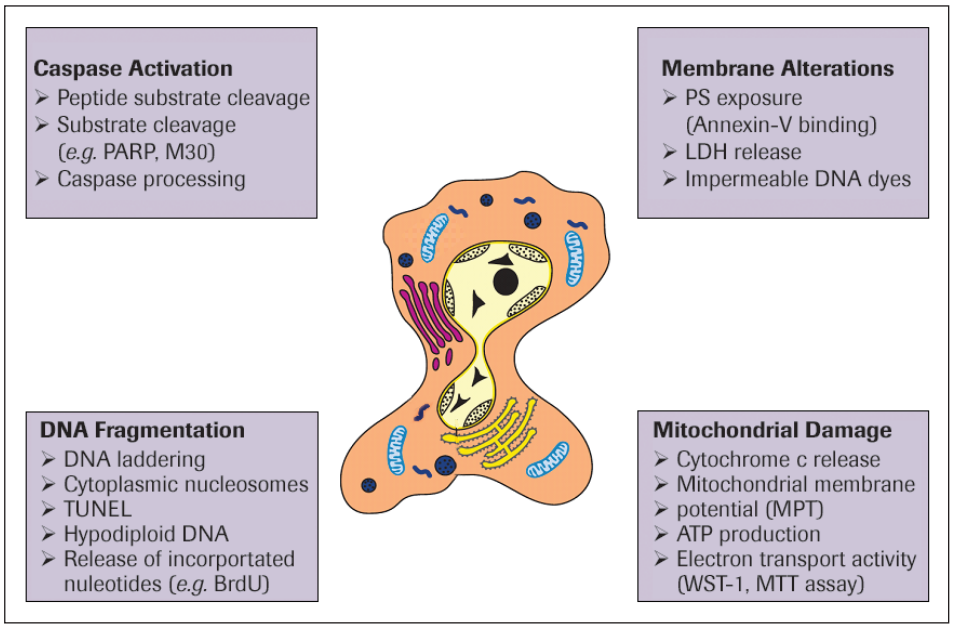

Figure 3: Methods to detect apoptosis. Hallmarks of apoptosis include caspase activation, DNA fragmentation as well as alterations of the plasma membrane and mitochondria, which can be assessed by a variety of methods in cell lysates, cell culture or tissue biopsies.

Membrane alterations

Another characteristic feature of apoptosis is the exposure of the phospholipid phosphatidylserine (PS) to the outer cell membrane. PS is normally confined to the inner plasma membrane in healthy cells, but is translocated to the outer membrane leaflet in response to proapoptotic stimuli. This flip-flop mechanism is caspase-dependent, although how caspases promote PS externalization remains a mystery. PS exposure is important for the elimination of apoptotic cells, because it represents an 'eat-me' signal for the engulfment by professional phagocytes following binding to a putative PS receptor. This process therefore ensures the early uptake of apoptotic cells with no release of cellular contents and without provoking an inflammatory response [31].

PS exposure is experimentally detected most commonly using annexin-V, a PS-binding protein. Various annexin-V derivatives coupled to different fluorochromes are available, providing versatile possibilities for apoptosis measurement, for instance by multicolor flow cytometry or fluorescence microscopy (see Annexin-V products on page 39-45). The advantages are sensitivity and rapidity; the disadvantage is that necrotic cells are labeled upon rupture of their plasma membrane. Therefore, it important to control the membrane integrity of the PS-positive cells by double-staining with membrane-impermeable DNA dyes such as propidium iodide. In these assays, healthy cells are doubly negative to annexin-V and propidium iodide, whereas cells in the early phases of apoptosis are annexin-V-positive but propidium iodide-negative, and secondary necrotic cells are doubly positive to annexin-V and propidium iodide.

DNA fragmentation

The internucleosomal cleavage of DNA is a classical feature of apoptosis and fulfills the physiological function of eliminating DNA that is highly immunogenic. The failure to degrade DNA can give rise to autoantibodies that can cause lupus erythematosus-like autoimmune disease. DNA fragmentation during apoptosis is induced by caspase-mediated cleavage of ICAD (inhibitor of caspase-activated DNase), with the ensuing activation of CAD (caspase-activated DNase). This event then leads to characteristic internucleosomal DNA double-strand breaks with fragments of multiples of 180 base pairs in size. In contrast, necrotic cell death is accompanied by late and random DNA fragmentation through the release of lysosomal DNases. Techniques that detect DNA fragmentation are thus not necessarily specific to apoptosis, but may detect DNA damage in a variety of cell death paradigms.

Detection of DNA fragmentation is currently one of the most frequently used techniques for highlighting apoptotic cells in tissues. A traditional method for demonstrating internucleosomal DNA degradation is gel electrophoresis of genomic DNA (see Apoptotic DNA Ladder Kit, page 57). In these experiments, apoptotic cells show a characteristic DNA ladder, while necrotic cells reveal a smear of randomly degraded DNA. Another assay is the detection of hypodiploid nuclei from apoptotic cells, which is usually performed in a flow cytometer using DNA-binding fluorochromes such as propidium iodide. The technique determines the DNA content, and therefore the number of apoptotic cells, but also allows the simultaneous analysis of cell cycle parameters of surviving cells. It should be stressed, however, that necrotic cells sometimes also display a certain degree of DNA degradation that may result in hypodiploid nuclei. Therefore, the presence of a hypodiploid DNA peak does not provide unequivocal proof of apoptotic cell death but should be adequately controlled.
A very elegant manner for quantifying apoptotic cells is the detection of cytoplasmic mono- and oligonucleosomes that originate from internucleosomal DNA cleavage and disruption of the nuclear envelope during apoptosis (see Cell Death Detection ELISA^{PLUS}, page 62). Cytoplasmic nucleosomes can be measured reliably by sensitive ELISA techniques using a combination of anti-histone and anti-DNA capture and detection antibodies.

In tissue sections, the TUNEL (terminal dUTP nick end-labeling) method is widely used to measure DNA fragmentation (see *In Situ* Cell Death Detection Kits, page 74-79). The principle of the assay is that endonuclease-generated DNA breaks are enzymatically labeled by terminal transferase with UTP derivatives coupled to fluorochromes or biotin that can be detected in an immunoperoxidase reaction. This assay is very sensitive, and allows DNA fragmentation to be assessed quantitatively by light and fluorescence microscopy or by flow cytometry. However, since this method can also be subject to pitfalls, such as false positives from necrotic cells, it should be paired with additional assays.

Mitochondrial changes

Apoptosis-specific alterations of mitochondria are more difficult to detect. The mitochondrial pathway begins with the permeabilization of the mitochondrial outer membrane by proapoptotic members of the Bcl-2 family, resulting in a release of cytochrome c and other toxic proteins from the intermembrane space into the cytosol. The release of these proteins is generally determined by immunocytochemistry or by Western blotting of cytosolic, mitochondrial and nuclear fractions.
In addition to MOMP, another event observed during cell death is the loss of the inner mitochondrial transmembrane potential, the electrochemical proton gradient generated by the respiratory chain. This process is called mitochondrial permeability transition (MPT) and involves the opening of a largely undefined, non-selective pore in the inner mitochondrial membrane [28]. MPT also causes mitochondrial swelling, which can eventually result in rupture of the outer mitochondrial membrane and release of cytochrome c and other intermembrane proteins. It has therefore previously been claimed that MPT and the loss of the mitochondrial transmembrane potential can actively contribute to cytochrome c release.

The mitochondrial transmembrane potential can be measured easily using a variety of potentiometric dyes, together with flow cytometry or fluorescence microscopy. In many publications MPT is considered to be an early marker of mitochondrial alterations, which is thought to precede cytochrome c release and apoptosome formation. However, with rare exceptions, this hypothesis seems to be largely incorrect. Only a few cytotoxic (in fact necrotic rather than apoptotic) stimuli, such as calcium overload or oxidative stress, seem to cause cytochrome c release downstream of permeability transition. In most cases, MOMP caused by proapoptotic Bcl-2 proteins, but not by the transmembrane potential, is the primary mechanism of cytochrome c release. This assumption is supported by the fact that loss of MPT does not precede cytochrome c release and is generally prevented by caspase inhibition. Therefore, the loss of the transmembrane potential should be regarded rather as a good marker of mitochondrial damage that may occur in late apoptosis, but also during necrosis.

Caveats and cautionary notes

The recent interest in cell death pathways other than apoptosis has revealed that cells can die by different modes. For most cells examined, apoptosis is the preferred physiological response to stress, but cells can also undergo autophagy and, failing that, can succumb to necrosis. Importantly, inhibition of one pathway, *e.g.* apoptosis, will mostly not restore clonogenic survival but will just shift a particular mode of cell death to another phenotype. A failure to activate caspase-3 or fragment DNA certainly does not prove that a given cell is rescued from cell death and able to proliferate. Even without caspase activation, MOMP generally results in cell death through the release of multiple caspase-independent death effectors, which will disrupt essential vital functions. Thus, many studies concerned with caspase inhibitors, for example, are flawed when they claim that caspase inhibition is cytoprotective and prevents cell death without a clear demonstration of cell survival. It is therefore crucial that we measure cell death by more than one means and implement complementary methodologies.

Another important issue concerns the timing of the experiments. Apoptosis proceeds very rapidly, often within a few hours, whereas necrosis is typically much slower. Thus, if an injured cell fails to undergo apoptosis and remains viable for a few hours, it might well die through alternative pathways a few hours later. In many instances, the suppression of signs of apoptosis has been misinterpreted as inhibition of cell death, which is often an incorrect statement. Therefore, I strongly recommend the use of additional clonogenicity and viability assays. Traditional assays measuring cell proliferation (see chapter 9), ATP content (see ATP Bioluminescence Assay Kits, page 130) or the release of marker enzymes such as lactate dehydrogenase (see Cytotoxicity Detection Kit^{PLUS} (LDH), page 98) are well-suited for this purpose. Implementation of those methods will provide us not only with better insights into cell death mechanisms, but can also demonstrate whether short-term inhibition of cell death is connected to long-term survival.

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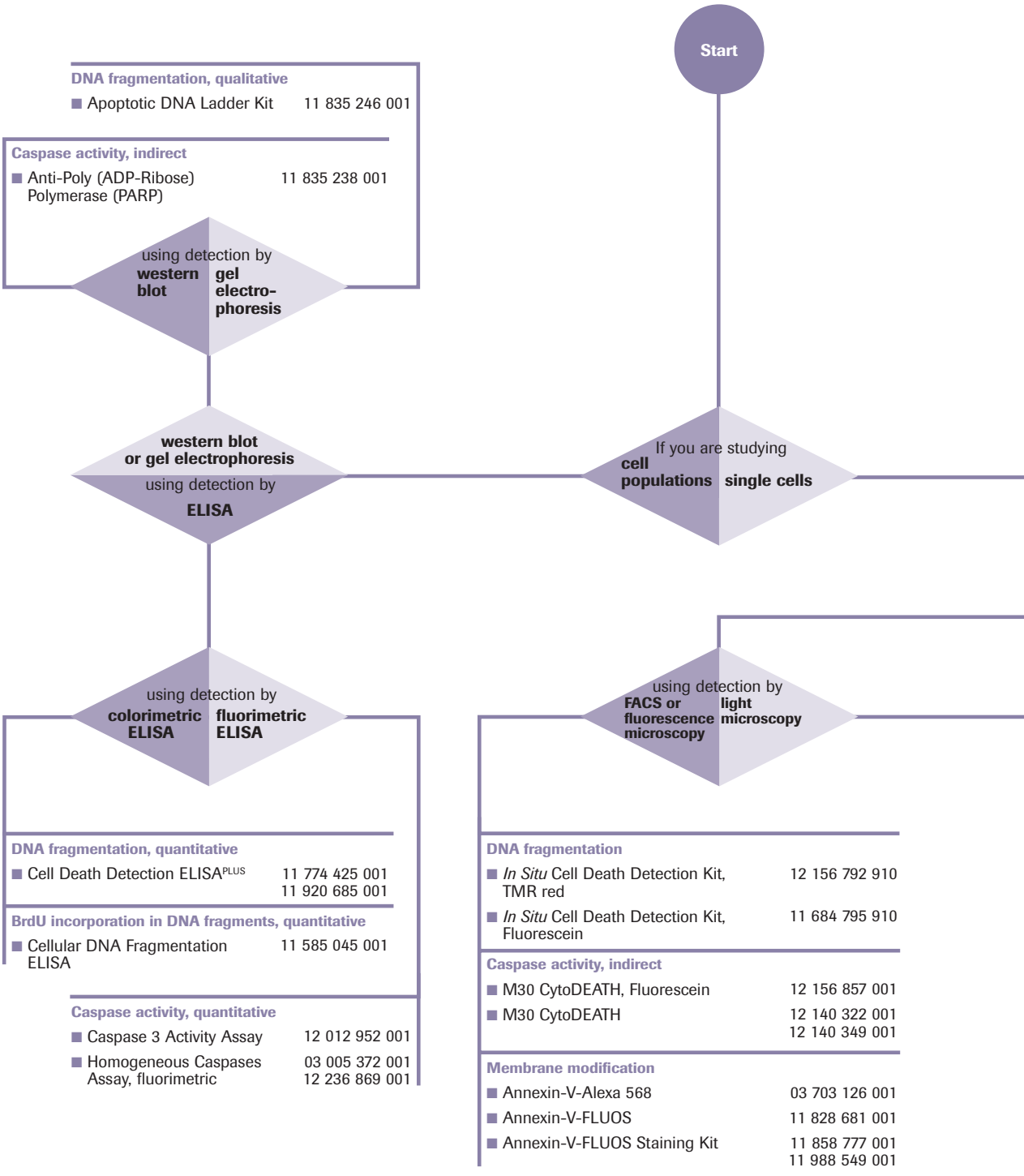
References

1. Raff MC. Social controls on cell survival and cell death. Nature. 1992; 356:397-400.
2. Fischer U, Schulze-Osthoff K. New approaches and therapeutics targeting apoptosis in disease. Pharmacol Rev. 2005; 57:187-215.
3. Lockshin RA, Williams CM. Programmed cell death. I. Cytology of degeneration in the intersegmental muscles of the Pernyi Silkmoth. J Insect Physiol 1965; 11:123–133.
4. Horvitz HR. Nobel lecture. Worms, life and death. Biosci Rep. 2003; 23:239-303.
5. Kroemer G, El Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, Zhivotovsky B, Blagosklonny MV, Malorni W, Knight RA, Piacentini M, Nagata S, Melino G. Classification of cell death: Recommendations of the nomenclature committee on cell death. Cell Death Differ 2005; 12:1463 7.
6. Melino G, Knight RA, Nicotera P. How many ways to die? How many different models of cell death? Cell Death Differ 2005; 12:1457 62.
7. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? Nat Rev Mol Cell Biol. 2005; 6:268-75.
8. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972; 26:239–57.
9. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008; 9:231-41.
10. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol. 2007; 8:931-7.
11. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132:27-42.
12. Levine B, Yuan J. Autophagy in cell death: an innocent convict? J Clin Invest. 2005; 115:2679 –2688.
13. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007; 8:741-52.
14. Festjens N, Vanden Berghe T, Vandenabeele P. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. Biochim Biophys Acta. 2006; 1757:1371-1387.
15. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol. 2003; 4:552–65.

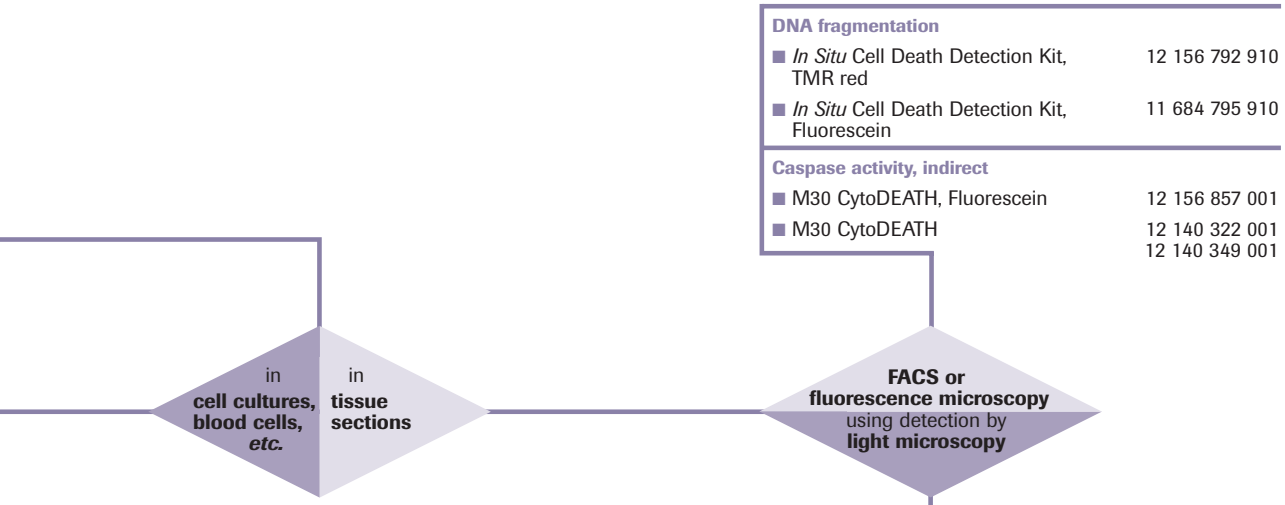
16. Los M, Wesselborg S, Schulze-Osthoff K. The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity. 1999; 10:629-39.
17. Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J. 2004; 384:201-32.
18. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 2003; 10:76-100.
19. Schwerk C, Schulze-Osthoff K. Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol. 2003; 66:1453-8.
20. Kuranaga E, Miura M. Nonapoptotic functions of caspases: caspases as regulatory molecules for immunity and cell-fate determination. Trends Cell Biol. 2007; 17:135-44.
21. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004; 116:205-219
22. Meier P, Vousden KH. Lucifer's labyrinth--ten years of path finding in cell death. Mol Cell. 2007; 28:746-54.
23. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. Oncogene. 2004; 23:2950–2966.
24. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zörnig M, Lynch DH. The CD95 receptor: apoptosis revisited. Cell. 2007; 129:447-50.
25. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004 ;305: 626-9.
26. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem. 2004; 73:87-106.
27. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 2008; 18:157-64.
28. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007; 87:99-163.
29. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008; 9:47-59.
30. Ekert PG, Vaux DL. The mitochondrial death squad: hardened killers or innocent bystanders? Curr Opin Cell Biol. 2005; 17: 626-30.
31. Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. Mol Cell. 2004; 14:277-87.
32. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med. 2005; 11:725-30.

Apoptosis Product Selection Guide

1



Follow the selection guide below to determine the appropriate Roche Applied Science product for the study of **apoptosis** to meet your needs.
See page 90 for products to study cytotoxicity and page 114 for products to study cell proliferation. If you need additional help, please visit
www.roche-applied-science.com/apoptosis



DNA fragmentation

■ *In Situ* Cell Death Detection Kit, TMR red	12 156 792 910
■ *In Situ* Cell Death Detection Kit, Fluorescein	11 684 795 910

Caspase activity, indirect

■ M30 CytoDEATH, Fluorescein	12 156 857 001
■ M30 CytoDEATH	12 140 322 001 12 140 349 001

DNA fragmentation

■ *In Situ* Cell Death Detection Kit, POD	11 684 817 910
■ *In Situ* Cell Death Detection Kit, AP	11 684 809 910

Caspase activity, indirect

■ M30 CytoDEATH	12 140 322 001 12 140 349 001

DNA fragmentation

■ *In Situ* Cell Death Detection Kit, POD	11 684 817 910
■ *In Situ* Cell Death Detection Kit, AP	11 684 809 910

Caspase activity, indirect

■ M30 CytoDEATH	12 140 322 001 12 140 349 001

Membrane modification

■ Annexin-V-Biotin	11 828 690 001

Apoptosis	Autophagy	Necrosis
Morphological features		
■ Membrane blebbing, no loss of integrity	■ Cell membrane stays intact, weak membrane blebbing	■ Loss of membrane integrity
■ Aggregation of chromatin at the nuclear membrane	■ Marginal chromatin condensation	■ No chromatin condensation
■ Begins with shrinking of cytoplasm and condensation of nucleus	■ Begins with sequestration of cytoplasmic material in autophagosomes and autolysosomes	■ Begins with swelling of cytoplasm and mitochondria
■ Mitochondria preserve normal ultrastructure, outer membrane permeabilization	■ Damaged mitochondria degraded within autophagosomes	■ Disintegration (swelling) of organelles
■ Ends with fragmentation of cell into small vesicles (apoptotic bodies)	■ No cell lysis, ends with self-digestion	■ Ends with total cell lysis
Biochemical features		
■ Tightly regulated process involving mediators and enzymes	■ Tightly regulated process involving various regulatory steps	■ Loss of ion homeostasis
■ Energy (ATP)-dependent (active process)	■ Generates ATP in the absence of exogenous energy supply	■ No energy requirement (passive process)
■ Non-random internucleosomal DNA fragmentation (ladder pattern after agarose gel electrophoresis)	■ No DNA fragmentation	■ Random digestion of DNA (smear of DNA after agarose gel electrophoresis)
■ Release of several apoptogenic mitochondrial proteins (cytochrome c, AIF etc.)	■ Involvement of Atg (autophagy related gene) products; ubiquitin-like activation cascades	■ Oxidative stress, calcium overload, ATP depletion
■ Activation of caspase cascade	■ Activation of lysosomal proteases and hydrolases	■ Activation of calcium-dependent proteases
■ Loss of plasma membrane asymmetry (*i.e.*, translocation of phosphatidylserine from the inner to the outer side of the membrane)		
Physiological significance		
■ Serves to eliminate damaged, transformed or infected cells; functions in organ development and regulation of immune responses	■ Serves primarily to maintain cellular energy and to recycle damaged organelles	■ Physiological role unclear, potential backup mechanism during apoptosis failure.
■ Induced by physiological stimuli (death ligands), growth factor depletion and different cellular stresses	■ Triggered by nutrient deprivation and other cellular stresses.	■ Evoked by severe injuries (complement attack, lytic pathogens, hypoxia, ischemia, toxins)
■ Phagocytosis by adjacent cells or macrophages	■ Self-digestion, late heterophagy by other cells	■ Phagocytosis by macrophages
■ No inflammatory response	■ No inflammatory response	■ Severe inflammatory response

Table 1: Differential features of apoptosis, necrosis and autophagy

Apoptosis – Caspase Activity

2

Assays that Measure Apoptosis-Induced Proteases (Caspases)

2

Several caspases 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 Applied Science, 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 Applied Science offers a caspase 3 activity assay with highest specificity by the use of an immunosorbent enzyme assay principle.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
Caspase Activity	Caspase cleavage of cytokeratin 18	Western blot, flow cytometry, fluorescence microscopy, or light microscopy	**M30 CytoDEATH**	21
			M30 CytoDEATH, Fluorescein	21
	Caspase 3 activity	Fluorescence ELISA	**Caspase 3 Activity Assay**	26
	Caspases	Fluorescence ELISA	**Homogeneous Caspases Assay, fluorimetric**	30
	Caspase cleavage of PARP	Western blot, immunoprecipitation, immunohistology	**Anti-Poly (ADP-Ribose) Polymerase (PARP)**	34

M30 CytoDEATH*

Cat. No. 12 140 322 001 50 tests
Cat. No. 12 140 349 001 250 tests

M30 CytoDEATH, Fluorescein*

Cat. No. 12 156 857 001 250 tests

Type	Monoclonal antibody, clone M30, IgG2b, mouse
Useful for	Detection of apoptosis in epithelial cells and tissues (formalin grade)
Sample material	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
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.
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.
Time	2 h for immunofluorescence on cells, 3.5 h for staining of tissues (excluding dewaxing)
Antibody supplied as	Mouse monoclonal antibody (clone M30), lyophilized, stabilized. Formalin grade.
Background information	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. 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. 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.
Benefits	■ **Detect early apoptosis** in epithelial cells and tissue sections with high sensitivity. ■ **Determine caspase activity** – even in formalin-fixed, paraffin-embedded tissue. ■ **Choose from two antibody formats** to meet your specific application needs. ■ **Easily perform fluorescence and FACS analysis** with M30 CytoDEATH, Fluorescein. ■ **Use in dual-labeling applications**, for example, in combination with the *In Situ* Cell Death Detection Kit, TMR red (TUNEL technique, see page 71-73). ■ **Save time** with a convenient, easy-to-use protocol.

*The M30 antibody is made under a license agreement from Peviva AB, Sweden. US Patent No. 6,296,850; 6,706,488 and 6,716,968.

2

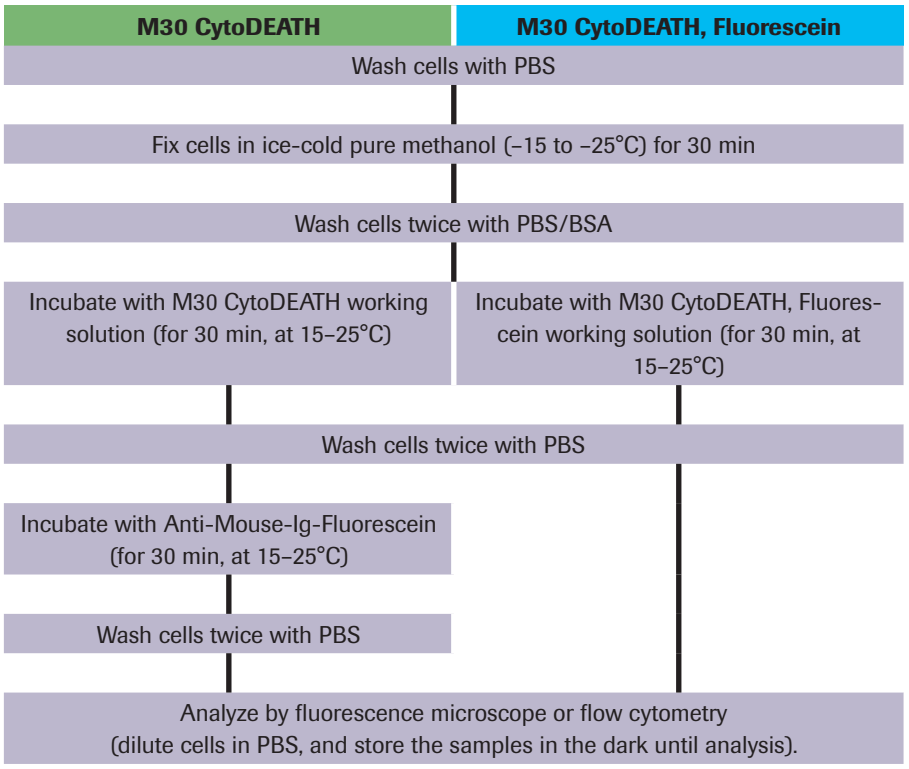
How to use the reagent

I.a Assay procedure overview

for immunofluorescence and flow cytometry on cells for M30 CytoDEATH and M30 CytoDEATH, Fluorescein:

1 Fix cells.	3 Add Anti-Mouse-Ig-Fluorescein (not necessary for M30 CytoDEATH, Fluorescein).
2 Add M30 CytoDEATH antibody or M30 CytoDEATH, Fluorescein	4 Analyze under a fluorescence microscope or in FACS.

Staining procedure for fluorescence microscopy and flow cytometry (FACS)



I.b Assay procedure overview

for formalin-embedded tissue for M30 CytoDEATH

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

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 15–25°C)

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

Block with PBS + 1% BSA (for 10 min, at 15–25°C)

Remove blocking solution. Add M30 CytoDEATH working solution (1 h, 15–25°C)

Wash slides three times in PBS

Cover with Anti-Mouse-Biotin (for 30 min, at 15–25°C)

Wash slides three times in PBS

Cover with Streptavidin-POD (for 30 min, at 15–25°C)

Wash slides three times in PBS

Incubate slides in a freshly prepared substrate solution (DAB or AEC at 15–25°C) until a clearly visible color develops

Counterstain with hematoxilin, and mount the section

Analyze by a light microscope

2

Typical results with the reagent

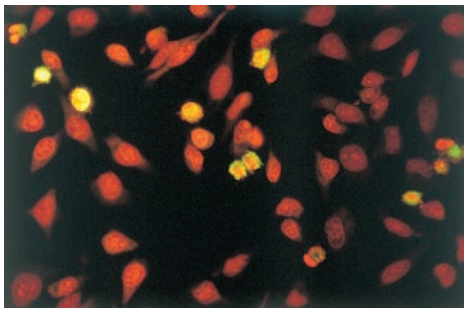


Figure 4: Detection of apoptosis in HeLa cells, treated with TNF and Actinomycin D, using M30 CytoDEATH. Secondary detection with Anti-Mouse-Fluorescein and propidium iodide.

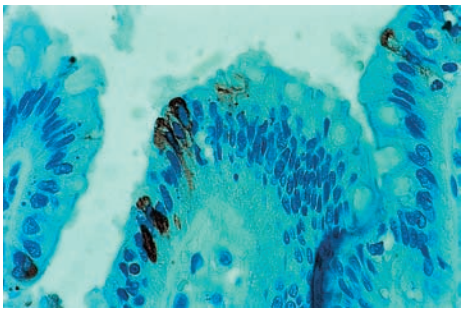


Figure 5: Detection of apoptosis in human colon using M30 CytoDEATH (blue filter). Secondary detection with Anti-Mouse-Biotin, Streptavidin-POD and AEC as substrate, counterstained with hematoxilin.

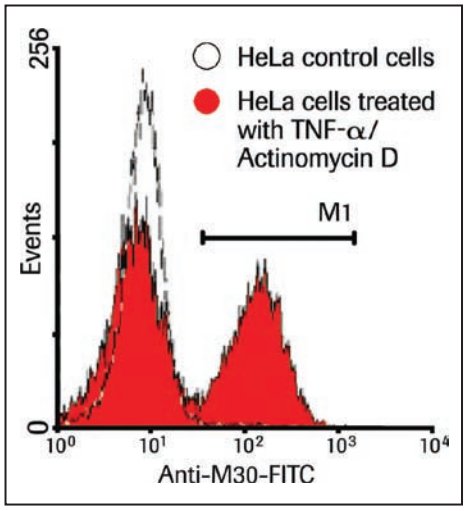


Figure 6: FACS analysis of apoptosis in HeLa cells, using M30 CytoDEATH, Fluorescein. White: untreated control cells. Red: Cells treated with TNF and Actinomycin D.

References

M30 CytoDEATH

1. Epidermal growth factor abrogates hypoxia-induced apoptosis in cultured human trophoblasts through phosphorylation of BAD serine 112
Rachel G. Humphrey, Christina Sonnenberg-Hirche, Steven D. Smith, Chaobin Hu, Aaron Barton, Yoel Sadovsky, and D. Michael Nelson
Endocrinology, Feb 2008; 10.1210/en.2007-1253.
2. Binary PAH mixtures cause additive or antagonistic effects on gene expression but synergistic effects on DNA adduct formation
Yvonne C.M. Staal, Dennie G.A.J. Hebels, Marcel H.M. van Herwijnen, Ralph W.H. Gottschalk, Frederik J. van Schooten, and Joost H.M. van Delft
Carcinogenesis, Dec 2007; 28: 2632 - 2640.
3. Desmoglein-2: A Novel Regulator of Apoptosis in the Intestinal Epithelium
Porfirio Nava, Mike G. Laukoetter, Ann M. Hopkins, Oskar Laur, Kirsten Gerner-Smidt, Kathleen J. Green, Charles A. Parkos, and Asma Nusrat
Mol. Biol. Cell, Nov 2007; 18: 4565 - 4578.

4. NAC-1 Controls Cell Growth and Survival by Repressing Transcription of Gadd45G-IP1, a Candidate Tumor Suppressor
Kentaro Nakayama, Naomi Nakayama, Tian-Li Wang, and Ie-Ming Shih
Cancer Res., Sep 2007; 67: 8058 - 8064.
5. Endothelin-1 Attenuates Apoptosis in Cultured Trophoblasts From Term Human Placentas
M. Cervar-Zivkovic, C. Hu, A. Barton, Y. Sadovsky, G. Desoye, U. Lang, and D.M. Nelson
Reproductive Sciences, Jul 2007; 14: 430 - 439.
6. The ABC transporter BCRP/ABCG2 is a placental survival factor, and its expression is reduced in idiopathic human fetal growth restriction
Denis A. Evseenko, Padma Murthi, James W. Paxton, Glen Reid, B. Starling Emerald, K. M. Mohankumar, Peter E. Lobie, Shaun P. Brennecke, Bill Kalionis, and J. A. Keelan
FASEB J, Nov 2007; 21: 3592 - 3605.
7. Prognostic Value of Apoptosis in Rectal Cancer Patients of the Dutch Total Mesorectal Excision Trial: Radiotherapy Is Redundant in Intrinsically High-Apoptotic Tumors
Elza C. de Bruin, Cornelis J.H. van de Velde, Simone van de Pas, Iris D. Nagtegaal, J. Han J.M. van Krieken, Marleen J.E.M. Gosens, Lucy T.C. Peltenburg, Jan Paul Medema, and Corrie A.M. Marijnen
Clin. Cancer Res., Nov 2006; 12: 6432 - 6436.
8. Effect of Simultaneous Inhibition of Epidermal Growth Factor Receptor and Cyclooxygenase-2 in HER-2/Neu-Positive Breast Cancer
Susan Lanza-Jacoby, Randy Burd, Francis E. Rosato, Jr., Kandace McGuire, James Little, Noel Nougbilly, and Sheldon Miller
Clin. Cancer Res., Oct 2006; 12: 6161 - 6169.
9. The Histidine Triad Protein Hint1 Triggers Apoptosis Independent of Its Enzymatic Activity
Jörg Weiske and Otmar Huber
J. Biol. Chem., Sep 2006; 281: 27356 - 27366.
10. Adenoviral vector saturates Akt pro-survival signaling and blocks insulin-mediated rescue of tumor-necrosis-factor-induced apoptosis
Kathryn Miller-Jensen, Kevin A. Janes, Yun-Ling Wong, Linda G. Griffith, and Douglas A. Lauffenburger
J. Cell Sci., Sep 2006; 119: 3788 - 3798.

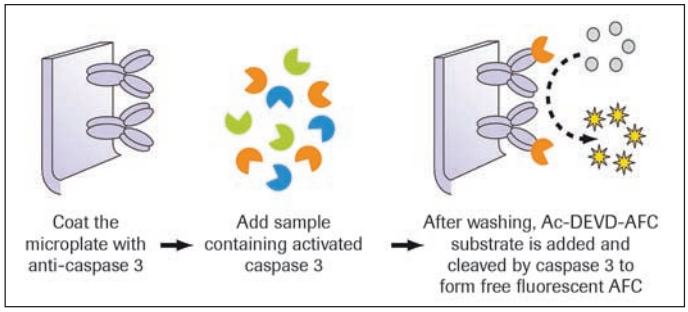
M30 CytoDEATH, Fluorescein

1. Epithelial Cells Remove Apoptotic Epithelial Cells During Post-Lactation Involution of the Mouse Mammary Gland
Jenifer Monks, Christine Smith-Steinhart, Ellen R. Kruk, Valerie A. Fadok, and Peter M. Henson
Biol Reprod, Dec 2007; 10.1095/biolreprod.107.065045.
2. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts
Faiy H. Psahoulia, Konstantinos G. Drosopoulos, Lenka Doubravska, Ladislav Andera, and Alexander Pintzas
Mol. Cancer Ther., Sep 2007; 6: 2591 - 2599
3. The Induction and Suppression of the Apoptotic Response of HSV-1 in Human Corneal Epithelial Cells
David H. Miles, Archana Thakur, Nerida Cole, and Mark D. P. Willcox
Invest. Ophthalmol. Vis. Sci., Feb 2007; 48: 789 - 796.
4. Zinc modulates cytokine-induced lung epithelial cell barrier permeability
Shenying Bao and Daren L. Knoell
Am J Physiol Lung Cell Mol Physiol, Dec 2006; 291: L1132 - L1141.

2

Caspase 3 Activity Assay

Cat. No. 12 012 952 001 96 tests

Type	Immunosorbent enzyme assay, fluorometric
Useful for	Specific, quantitative *in vitro* determination of caspase 3 activity
Sample material	Cell lysates, recombinant caspase 3
Method	Cell lysis, followed by capturing of caspase 3 by a specific antibody and fluorometric determination of proteolytic cleavage of the substrate
Test principle	
Significance of kit	This kit allows specific, quantitative detection of caspase 3 activity in cellular lysates after induction of apoptosis. Caspase 3 activation play a key role in initiation of cellular events during the early 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.
Sensitivity	In a model system, caspase 3 activity was clearly detectable in lysates of 10^6 cells with 5 % apoptotic cells (Figure 7). 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.
Time	Approx. 5 h (after induction of apoptosis)
Benefits	■ **Specifically detect caspase 3 activity.** Detect only natural and recombinant human caspase 3 activity in research samples. ■ **Detect low levels of caspase 3 activity** – even in populations where as little as 5% of cells are apoptotic. ■ **Detect and obtain semiquantitative data** from samples undergoing the early stages of apoptosis. ■ **Perform kinetic assays.** Lysates are stable for up to six months, allowing studies at multiple time points. ■ **Screen multiple samples simultaneously.** Take advantage of a convenient 96-well microplate format when screening multiple samples.

How to use the kit

I. Assay procedure overview

The assay uses a fluorometric immunosorbent enzyme assay (FIENA) principle. The procedure 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.

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, 15–25°C)

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

Centrifuge at maximum speed in a tabletop centrifuge (1 min, 15–25°C)

Remove 100 µl sample for direct analysis or storage for 1 week at –15 to –25°C

Coating microplate

Incubate microplate with anti-caspase 3 coating solution (either at 37°C for 1h or at 2-8°C over night)

Block unspecific binding by incubation with blocking buffer (30 min, 15–25°C)

Remove blocking solution and wash 3 times with incubation buffer (3 x 1 min, 15–25°C)

Assaying protease activity

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

Remove sample and wash 3 times with incubation buffer (3 x 1 min, 15–25°C)

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

II. Kit content

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

2

Typical results with the kit

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 (CAM) (Figure 7, 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.

Figures 7 and 8, demonstrate that Caspase 3 activity and Annexin-V binding correlate very closely in both dose-response and kinetic studies

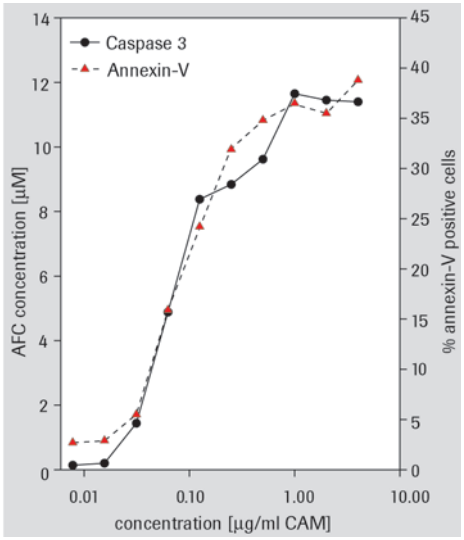


Figure 7: Dose-response experiment analyzed by the caspase 3 Activity Assay. U937 cells were exposed to different concentrations of camptothecin (CAM) for 4 h at 37°C. Lysates were analyzed for caspase 3 activity and standardized values are plotted versus concentration. Additionally, an aliquot of the same cells was analyzed for Annexin-V binding.

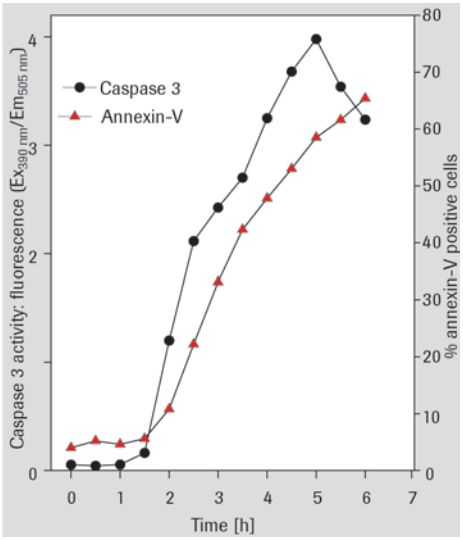


Figure 8: Kinetic study of caspase 3 activation by camptothecin exposure in U937 cells. U937 cells were exposed to 4 µg/ml camptothecin for different time intervals at 37°C. Lysates were analyzed for caspase 3 activity and fluorescence (minus fluorescence of blank) is plotted versus time. Additionally, an aliquot of the same cells was analyzed for Annexin-V binding in parallel.

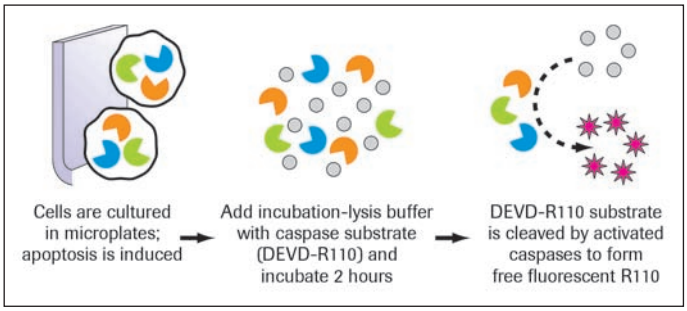
References

1. Proliferative and Protective effects of Growth Hormone Secretagogues on Adult Rat Hippocampal Progenitor cells
   Inger Johansson, Silvia Destefanis, N. David Åberg, Maria A.I. Åberg, Klas Blomgren, Changlian Zhu, Corrado Ghè, Riccarda Granata, Ezio Ghigo, Giampiero Muccioli, Peter S. Eriksson, and Jörgen Isgaard
   Endocrinology, Jan 2008; 10.1210/en.2007-0733.
2. ALL1 fusion proteins induce deregulation of EphA7 and ERK phosphorylation in human acute leukemias
   Hiroshi Nakanishi, Tatsuya Nakamura, Eli Canaani, and Carlo M. Croce
   PNAS, Sep 2007; 104: 14442 - 14447.
3. Ginkgolide B induces apoptosis and developmental injury in mouse embryonic stem cells and blastocysts
   Wen-Hsiung Chan
   Hum. Reprod., Nov 2006; 21: 2985 - 2995.
4. The mechanism of methylselenocysteine and docetaxel synergistic activity in prostate cancer cells
   Rami G. Azrak, Cheryl L. Frank, Xiang Ling, Harry K. Slocum, Fengzhi Li, Barbara A. Foster, and Youcef M. Rustum
   Mol. Cancer Ther., Oct 2006; 5: 2540 - 2548.
5. Plasminogen inhibits TNF-induced apoptosis in monocytes
   Jennifer W. Mitchell, Nagyung Baik, Francis J. Castellino, and Lindsey A. Miles
   Blood, Jun 2006; 107: 4383 - 4390.
6. Tumour necrosis factor signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice
   K Tomita, G Tamiya, S Ando, K Ohsumi, T Chiyo, A Mizutani, N Kitamura, K Toda, T Kaneko, Y Horie, J-Y Han, S Kato, M Shimoda, Y Oike, M Tomizawa, S Makino, T Ohkura, H Saito, N Kumagai, H Nagata, H Ishii, and T Hibi
   Gut, Mar 2006; 55: 415 - 424.
7. Inhibition of lung cancer cell growth by quercetin glucuronides via G2/M arrest and induction of apoptosis
   Jen-Hung Yang, Te-Chun Hsia, Hsiu-Maan Kuo, Pei-Dawn Lee Chao, Chi-Chung Chou, Yau-Huei Wei, and Jing-Gung Chung
   Drug Metab. Dispos., Feb 2006; 34: 296 - 304.
8. BRCA1 Phosphorylation Regulates Caspase-3 Activation in UV-Induced Apoptosis
   Sarah A. Martin and Toru Ouchi
   Cancer Res., Dec 2005; 65: 10657 - 10662.
9. Hyperglycemia Reduces Survival and Impairs Function of Circulating Blood-Derived Progenitor Cells
   Nicolle Kränkel, Volker Adams, Axel Linke, Stephan Gielen, Sandra Erbs, Karsten Lenk, Gerhard Schuler, and Rainer Hambrecht
   Arterioscler. Thromb. Vasc. Biol., Apr 2005; 25: 698 - 703.
10. Endogenous IGF-I protects human intestinal smooth muscle cells from apoptosis by regulation of GSK-3 activity
    John F. Kuemmerle
    Am J Physiol Gastrointest Liver Physiol, Jan 2005; 288: G101 - G110.

Homogeneous Caspases Assay, fluorimetric

Cat. No. 03 005 372 001	100 assays (96-well plates) 400 assays (384-well plates)
Cat. No. 12 236 869 001	1,000 assays (96-well plates) 4,000 assays (384-well plates)

2

Type	One step assay, fluorimetric
Useful for	Specific, quantitative *in vitro* determination of caspases in microplates
Sample material	Cell cultures, recombinant caspases
Method	Cell lysis, followed by detection of caspases activity (fluorimeric determiantion of proteolytic cleavage of the substrate)
Test principle	
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.
Specificity	Specifically detects caspases 2, 3 and 7, caspases 6, 8, 9 and 10 to a lesser extent
Time	Approx. 2 h (after induction of apoptosis)
Benefits	■ **Detect several types of activated caspases in a single assay.** ■ **Easily obtain results from this one-plate assay** by performing experiments directly in a culture plate or a microplate (MP). ■ **Use a convenient, one-step fluorimetric assay** – no washing step is needed. ■ **Save time** with an assay that takes just 2.5 hours. ■ **Simultaneously analyze large numbers of samples** in this high-throughput format.

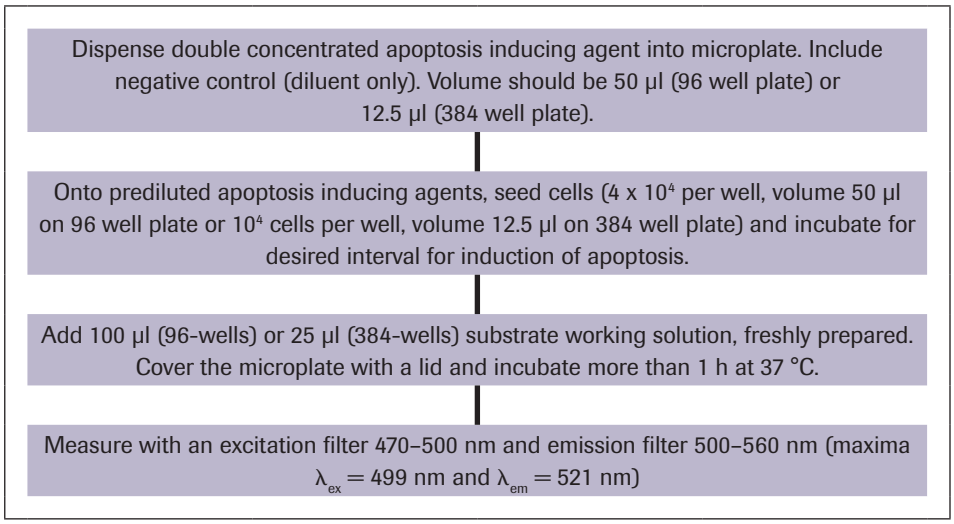
HTS = Suitable for high-throughput screening.

How to use the assay

I. Assay procedure overview

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

1	Cells are cultured in microplates and apoptosis is induced, causing an activation of caspases.
2	Caspase substrate, prediluted in Incubation buffer, is added and incubated for 2 h at 37°C. The incubation buffer is lysing the cells during this incubation.
3	Free R110 is determined fluorimetrically at $\lambda_{max} = 521$ nm. The developed fluorochrome is proportional to the concentration of activated caspases and could be quantified by a calibration curve.



II. Assay content

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

2

Typical results with the assay

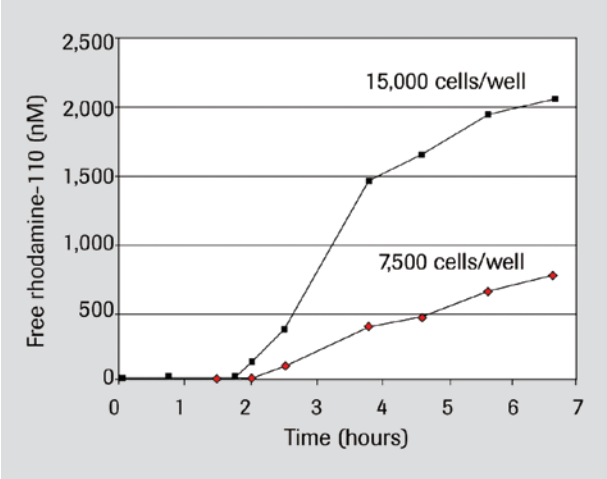


Figure 9: Kinetics of caspase activation in U937 cells by camptothecin (384-well plate). U937 cells were exposed to 4 µg/ml camptothecin for different time intervals at 37 °C, analyzed for caspase activity with the Homogeneous Caspases Assay and fluorescence plotted versus time.

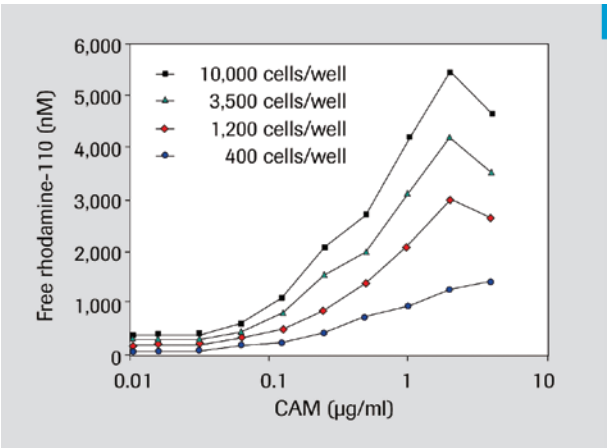


Figure 10: Dose-response curve of U937 cells exposed to different concentrations of camptothecin (CAM) (384-well plate). U937 cells were exposed to camtothecin for 4 h at 37 °C, analyzed for caspase activity with the Homogeneous Caspases Assay and standardized values plotted versus concentration.

References

1. p75 Neurotrophin Receptor Mediates Neuronal Cell Death by Activating GIRK Channels through Phosphatidylinositol 4,5-Bisphosphate
   Elizabeth J. Coulson, Linda M. May, Shona L. Osborne, Kate Reid, Clare K. Underwood, Frederic A. Meunier, Perry F. Bartlett, and Pankaj Sah
   J. Neurosci., Jan 2008; 28: 315 - 324.
2. Competing Interactions between Micro-RNAs Determine Neural Progenitor Survival and Proliferation after Ethanol Exposure: Evidence from an Ex Vivo Model of the Fetal Cerebral Cortical Neuroepithelium
   Pratheesh Sathyan, Honey B. Golden, and Rajesh C. Miranda
   J. Neurosci., Aug 2007; 27: 8546 - 8557.
3. Amphipathic Peptide-Based Fusion Peptides and Immunoconjugates for the Targeted Ablation of Prostate Cancer Cells
   Kaushal Rege, Suraj J. Patel, Zaki Megeed, and Martin L. Yarmush
   Cancer Res., Jul 2007; 67: 6368 - 6375.
4. Inhibition of GSK3 Promotes Replication and Survival of Pancreatic Beta Cells
   Rainer Mussmann, Marcus Geese, Friedrich Harder, Simone Kegel, Uwe Andag, Alexander Lomow, Ulrike Burk, Daria Onichtchouk, Cord Dohrmann, and Matthias Austen
   J. Biol. Chem., Apr 2007; 282: 12030 - 12037.
5. Nucleosomal DNA Fragments in Autoimmune Diseases
   Stefan Holdenrieder, Peter Eichhorn, Ulrich Beuers, Walter Samtleben, Ulf Schoenermarck, Reinhart Zachoval , Dorothea Nagel, and Petra Stieber
   Ann. N.Y. Acad. Sci., Sep 2006; 1075: 318 - 327.
6. Bovine CD14 receptor produced in plants reduces severity of intramammary bacterial infection
   Lev G. Nemchinov, Max J. Paape, Eun J. Sohn, Douglas D. Bannerman, Dante S. Zarlenga, and Rosemarie W. Hammond
   FASEB J, Jul 2006; 20: 1345 - 1351.
7. Constitutive NF-B activation, normal Fas-induced apoptosis, and increased incidence of lymphoma in human herpes virus 8 K13 transgenic mice
   Priti Chugh, Hittu Matta, Sandra Schamus, Sunny Zachariah, Arvind Kumar, James A. Richardson, Alice L. Smith, and Preet M. Chaudhary
   PNAS, Sep 2005; 102: 12885 - 12890.
8. T-cadherin protects endothelial cells from oxidative stress-induced apoptosis
   Manjunath B. Joshi, Maria Philippova, Danila Ivanov, Roy Allenspach, Paul Erne, and Thérèse J. Resink
   FASEB J, Aug 2005; 10.1096/fj.05-3834fje.
9. Functional Proteomic Screen Identifies a Modulating Role for CD44 in Death Receptor–Mediated Apoptosis
   Robert S. Hauptschein, Kevin E. Sloan, Claudia Torella, Roya Moezzifard, Maryann Giel-Moloney, Carol Zehetmeier, Christine Unger, Leodevico L. Ilag, and Daniel G. Jay
   Cancer Res., Mar 2005; 65: 1887 - 1896.
10. Immunohistochemical profiling of caspase signaling pathways predicts clinical response to chemotherapy in primary nodal diffuse large B-cell lymphomas
    Jettie J. F. Muris, Saskia A. G. M. Cillessen, Wim Vos, Inge S. van Houdt, J. Alain Kummer, Johan H. J. M. van Krieken, N. Mehdi Jiwa, Patty M. Jansen, Hanneke C. Kluin-Nelemans, Gert J. Ossenkoppele, Chad Gundy, Chris J. L. M. Meijer, and Joost J. Oudejans
    Blood, Apr 2005; 105: 2916 - 2923.

Anti-Poly (ADP-Ribose) Polymerase (Anti-PARP)

Cat. No. 11 835 238 001 100 µl (50 blots)

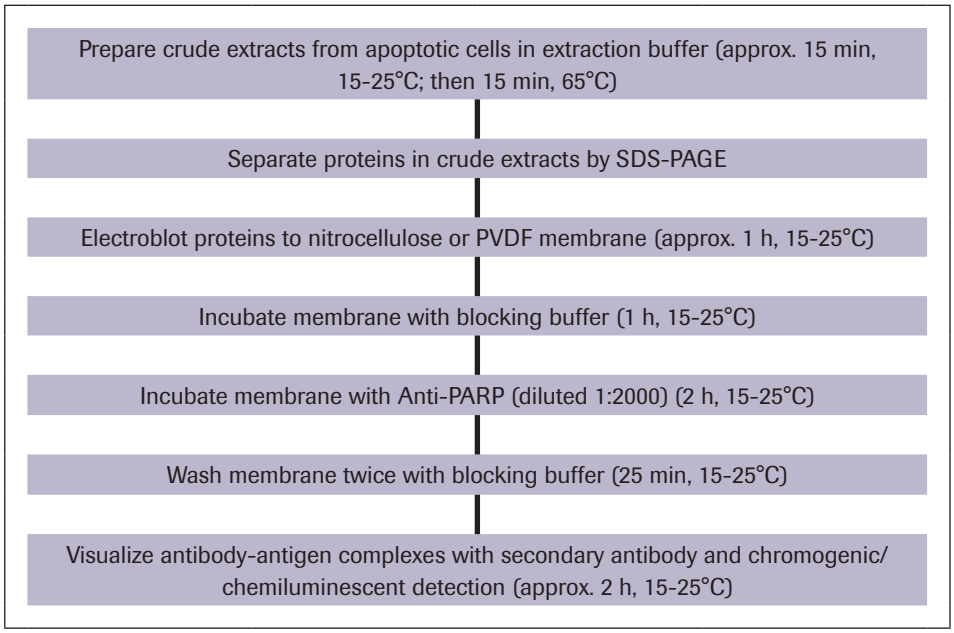
Type	Polyclonal antiserum, from rabbit
Useful for	Detection on Western blots of PARP cleaved by caspases during early stages of apoptosis
Sample material	Crude cell extracts
Method	Western blot of apoptotic cell extracts, followed by indirect immunodetection of PARP cleavage fragment
Significance of 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.
Sensitivity	PARP cleavage fragments from 3 x 10^5 apoptotic cells could be detected on a Western blot (Figure 11).
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.
Time	Approx. 5.5 h (immunodetection only)
Antibody supplied as	Polyclonal antiserum from rabbit, stabilized.
Benefits	■ **Detect early apoptosis** by determining caspase cleavage of PARP. ■ **Use in dual-labeling applications**, for example, in combination with the *In Situ* Cell Death Detection Kit, TMR red (TUNEL technique, page 74). ■ **Detect full-length PARP**, as well as large PARP fragments generated by caspases.

How to use the reagent

I. Assay procedure overview

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 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 page 36).



2

Typical result with the reagent

The appearance of a large (89 kD) cleavage fragment is indicative of caspase proteolytic activity.

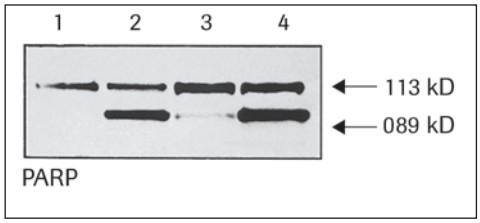


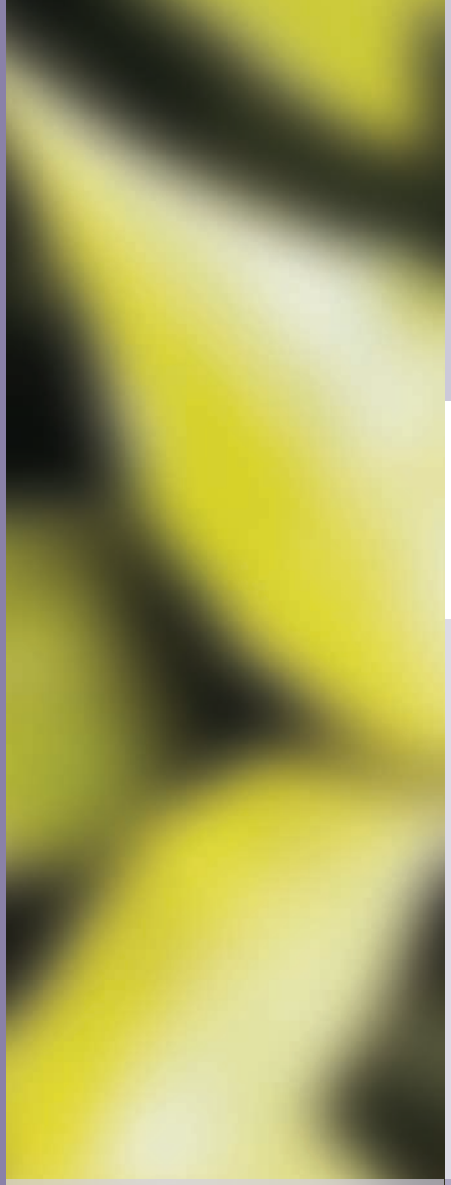
Figure 11: Detection of cleaved PARP in cell extracts of apoptotic CEM T cells. CEM T cells were incubated with one of three apoptosis-inducing drugs. Cell extracts from 3 x 10^5 treated or untreated cells were fractionated on an 10% polyacrylamide gel in the presence of SDS. After electrophoresis, proteins 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)

Related products for visualization of Anti-PARP

Product	Cat. No.	Pack Size
BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)	11 520 709 001	for 2,000 cm^2 membrane
BM Chemiluminescence Blotting Substrate (POD)	11 500 708 001 11 500 694 001	for 1,000 cm^2 membrane for 4,000 cm^2 membrane
CSPD (chemiluminescent AP substrate), ready-to-use	11 755 633 001	2 x 50 ml
CDP-*Star* (chemiluminescent AP substrate) ready-to-use	11 685 627 001 11 759 051 001	1 ml 2 x 1 ml
BM Blue POD Substrate, precipitating	11 442 066 001	100 ml
BM Purple AP Substrate, precipitating	11 442 074 001	100 ml

References

1. A Functional Nuclear Localization Signal in Insulin-Like Growth Factor Binding Protein-6 Mediates Its Nuclear Import
Cristiana Iosef, Theofanis Gkourasas, Christina Y. H. Jia, Shawn S.-C. Li, and Victor K. M. Han
Endocrinology, Mar 2008; 149: 1214 - 1226.
2. Acetyl-Keto-β-Boswellic Acid Induces Apoptosis through a Death Receptor 5–Mediated Pathway in Prostate Cancer Cells
Min Lu, Lijuan Xia, Huiming Hua, and Yongkui Jing
Cancer Res., Feb 2008; 68: 1180 - 1186.



Apoptosis – Membrane Alterations

3

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 *in vivo*. Thus, apoptotic cells are removed from the population without spilling their contents and eliciting an inflammatory response.

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.
- Emergence of surface glycoproteins that may serve as receptors for macrophage-secreted adhesive molecules such as thrombospondin.
- Loss of asymmetry in cell membrane phospholipids, altering both the hydrophobicity and charge of the membrane surface.
- 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 12, 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 12, right diagram) however, the amount of phosphatidylserine (PS) on the outer surface of the membrane increases, exposing PS to the surrounding liquid.

Annexin-V, a calcium-dependent phospholipid-binding protein, has a high affinity for PS. Although it will not bind to normal living cells, Annexin-V will bind to the PS exposed on the surface of apoptotic cells (Figure 13, 14). Thus, Annexin-V has proved suitable for detecting apoptotic cells. Roche Applied Science supplies a number of products for the detection of PS translocation by Annexin-V.

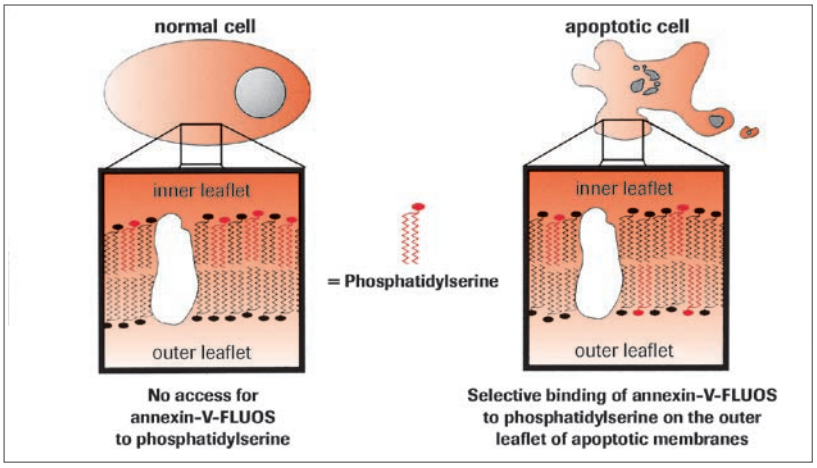


Figure 12: Detection of surface morphology changes during apoptosis. During apoptosis, the distribution of neutral phospholipids (black symbols) and anionic phospholipids such as phosphatidylserine (red symbols) in the cell membrane changes. Phosphatidylserine is present in the outer membrane of apoptotic 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.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
Membrane Alterations	Phosphatidylserine in the outer layer of the plasma membrane	Flow cytometry, fluorescence microscopy, or light microscopy	**Annexin-V-FLUOS**	39
			Annexin-V-FLUOS Staining Kit	39
			Annexin-V-Alexa 568	39
			Annexin-V-Biotin	45

Annexin-V-FLUOS

Cat. No. 11 828 681 001 250 tests

Annexin-V-FLUOS Staining Kit

Cat. No. 11 858 777 001 50 tests
11 988 549 001 250 tests

Annexin-V-Alexa 568

Cat. No. 03 703 126 001 250 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
Sample material	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)
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 (green) and the DNA stain propidium iodide (red) or Annexin-V-Alexa 568 (red) and BOBO-1 (green). Exclusion of propidium iodide or BOBO-1, coupled with binding of Annexin-V-FLUOS or Annexin-V-Alexa 568, indicates an apoptotic cell (Table 2).
Significance of reagent	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.
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.
Time	Approx. 15 min (after induction of apoptosis)
Benefits	**Detect apoptosis using flow cytometry or microscopy with your choice of Annexin-V conjugates** ■ **Rapidly quantify apoptotic cells in cell suspensions** by flow cytometry with Annexin-V-FLUOS. ■ **Distinguish necrotic cells from apoptotic cells** by using the Annexin-V-FLUOS Staining Kit, which includes propidium iodide. ■ **Analyze cell cultures or tissues** by fluorescence microscopy using the FLUOS or Alexa 568 conjugates.

3

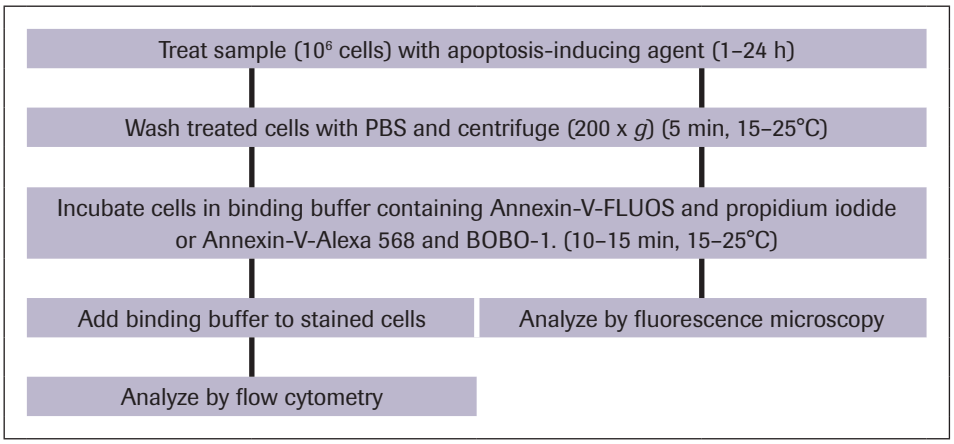
How to use the reagents and the kit

I. Assay procedure overview

The procedure 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.

   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	–	–	+
BOBO-1	–	–	+

Table 2: Distinguishing apoptosis from necrosis using Annexin-V, propidium iodide, or BOBO-1.

IIa. Reagent content

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

IIb. Kit content

Annexin-V-FLUOS Staining Kit

1. Annexin-V-FLUOS, 50 x concentrated
2. Propidium iodide solution, 50 x concentrated
3. Incubation buffer (Hepes Buffer), ready-to-use

Typical results with the reagents and the kit

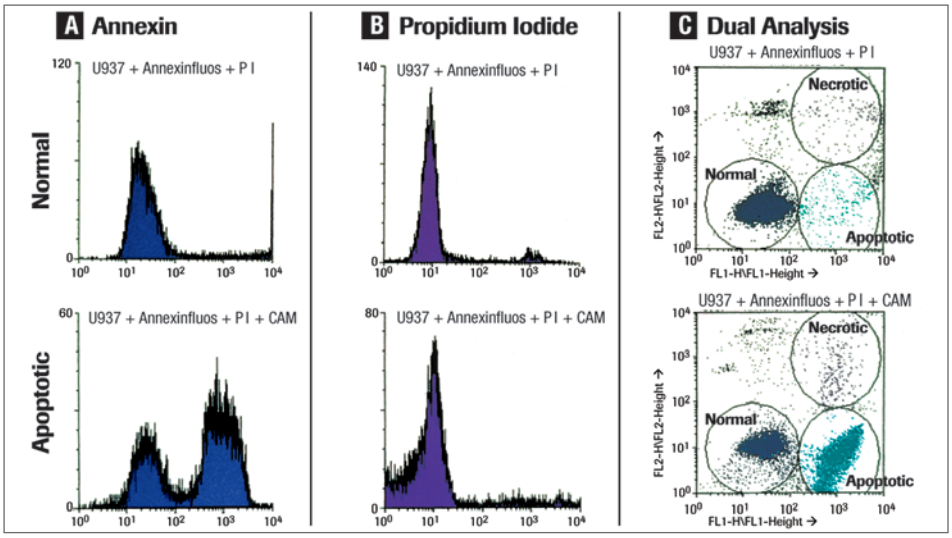


Figure 13: Apoptotic and necrotic U937 cells identified in FACS analysis after staining with Annexin-V-FLUOS and propidium iodide (PI). Cells were then stained with the components of the Annexin-V-FLUOS Staining Kit and analyzed.
Panels A (upper and lower), single parameter analysis, Annexin-V-FLUOS 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 with low Annexin and low PI staining, apoptotic cells with high Annexin and low PI staining, and necrotic cells with high Annexin and high PI staining.

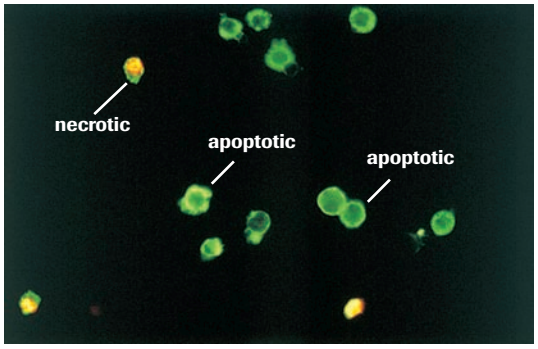


Figure 14: Discrimination between apoptotic and necrotic U937 cells treated with camptothecin. Early-stage apoptosis detected with Annexin-V-FLUOS (green), and counterstained with propidium iodide (red cells).
Results: The apoptotic cells are visible in green and can be differentiated from necrotic cells by the propidium staining. Necrotic cells take up propidium iodide and stain orange/green, while apoptotic cells stain green only.

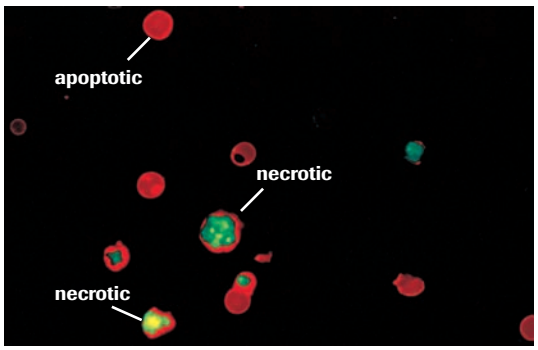


Figure 15: Discrimination between apoptotic and necrotic U937 cells treated with camptothecin (CAM) and stained with Annexin-V-Alexa 568 (red) and BOBO-1 (green).
Results: The apoptotic cells are visible in red and can be differentiated from necrotic cells by the BOBO-1 staining. Necrotic cells take up BOBO-1 and stain green/red, while apoptotic cells stain red only.

References

Annexin-V-FLUOS

1. B-crystallin promotes tumor angiogenesis by increasing vascular survival during tube morphogenesis
   Anna Dimberg, Svetlana Rylova, Lothar C. Dieterich, Anna-Karin Olsson, Petter Schiller, Charlotte Wikner, Svante Bohman, Johan Botling, Agneta Lukinius, Eric F. Wawrousek, and Lena Claesson-Welsh
   Blood, Feb 2008; 111: 2015 - 2023.
2. Mutant FLT3: A Direct Target of Sorafenib in Acute Myelogenous Leukemia
   Weiguo Zhang, Marina Konopleva, Yue-xi Shi, Teresa McQueen, David Harris, Xiaoyang Ling, Zeev Estrov, Alfonso Quintás-Cardama, Donald Small, Jorge Cortes, and Michael Andreeff
   J Natl Cancer Inst, Feb 2008; 100: 184 - 198
3. LETM1, deleted in Wolf–Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability
   Kai Stefan Dimmer, Francesca Navoni, Alberto Casarin, Eva Trevisson, Sabine Endele, Andreas Winterpacht, Leonardo Salviati, and Luca Scorrano
   Hum. Mol. Genet., Jan 2008; 17: 201 - 214.
4. Interferon Induces Nucleus-independent Apoptosis by Activating Extracellular Signal-regulated Kinase 1/2 and c-Jun NH2-Terminal Kinase Downstream of Phosphatidylinositol 3-Kinase and Mammalian Target of Rapamycin
   Theocharis Panaretakis, Linn Hjortsberg, Katja Pokrovskaja Tamm, Ann-Charlotte Björklund, Bertrand Joseph, and Dan Grandér
   Mol. Biol. Cell, Jan 2008; 19: 41 - 50.
5. Serum amyloid A inhibits apoptosis of human neutrophils via a P2X7-sensitive pathway independent of formyl peptide receptor-like 1
   Karin Christenson, Lena Björkman, Carolina Tängemo, and Johan Bylund
   J. Leukoc. Biol., Jan 2008; 83: 139 - 148
6. Erythropoietin Protects against Doxorubicin-Induced Cardiomyopathy via a Phosphatidylinositol 3-Kinase-Dependent Pathway
   Kyoung-Han Kim, Gavin Y. Oudit, and Peter H. Backx
   J. Pharmacol. Exp. Ther., Jan 2008; 324: 160 - 169
7. The Shc-binding site of the ßc subunit of the GM-CSF/IL-3/IL-5 receptors is a negative regulator of hematopoiesis
   Hayley S. Ramshaw, Mark A. Guthridge, Frank C. Stomski, Emma F. Barry, Lisa Ooms, Christina A. Mitchell, C. Glenn Begley, and Angel F. Lopez
   Blood, Nov 2007; 110: 3582 - 3590.
8. Amphipathic Peptide-Based Fusion Peptides and Immunoconjugates for the Targeted Ablation of Prostate Cancer Cells
   Kaushal Rege, Suraj J. Patel, Zaki Megeed, and Martin L. Yarmush
   Cancer Res., Jul 2007; 67: 6368 - 6375.
9. Kringle 5 of human plasminogen, an angiogenesis inhibitor, induces both autophagy and apoptotic death in endothelial cells
   Tri Minh Bui Nguyen, Indira V. Subramanian, Ameeta Kelekar, and Sundaram Ramakrishnan
   Blood, Jun 2007; 109: 4793 - 4802.
10. A Cell-permeable Fusion Toxin as a Tool to Study the Consequences of Actin-ADP-ribosylation Caused by the Salmonella enterica Virulence Factor SpvB in Intact Cells
    Sascha Pust, Henrike Hochmann, Eva Kaiser, Guido von Figura, Karin Heine, Klaus Aktories, and Holger Barth
    J. Biol. Chem., Apr 2007; 282: 10272 - 10282.

Annexin-V-FLUOS Staining Kit

1. Imatinib mesylate-resistant human chronic myelogenous leukemia cell lines exhibit high sensitivity to the phytoalexin resveratrol
   Alexandre Puissant, Sebastien Grosso, Arnaud Jacquel, Nathalie Belhacene, Pascal Colosetti, Jill-Patrice Cassuto, and Patrick Auberger
   FASEB J, Feb 2008; 10.1096/fj.07-101394.
2. Murine -defensin 2 promotes TLR-4/MyD88-mediated and NF-B-dependent atypical death of APCs via activation of TNFR2
   Arya Biragyn, Marta Coscia, Kunio Nagashima, Michael Sanford, Howard A. Young, and Purevdorj Olkhanud
   J. Leukoc. Biol., Jan 2008; 10.1189/jlb.1007700.
3. Triptolide sensitizes AML cells to TRAIL-induced apoptosis via decrease of XIAP and p53-mediated increase of DR5
   Bing Z Carter, Duncan H Mak, Wendy D Schober, Martin F Dietrich, Clemencia Pinilla, Lyubomir T Vassilev, John C Reed, and Michael Andreeff
   Blood, Jan 2008; 10.1182/blood-2007-05-091504.
4. Comparison of conditions for cryopreservation of testicular tissue from immature mice
   J.P. Milazzo, L. Vaudreuil, B. Cauliez, E. Gruel, L. Massé, N. Mousset-Siméon, B. Macé, and N. Rives
   Hum. Reprod., Jan 2008; 23: 17 - 28.
5. The mitochondrial respiratory chain is a modulator of apoptosis
   Jennifer Q. Kwong, Matthew S. Henning, Anatoly A. Starkov, and Giovanni Manfredi
   J. Cell Biol., Dec 2007; 179: 1163 - 1177.
6. Signal sequence mutation in autosomal dominant form of hypoparathyroidism induces apoptosis that is corrected by a chemical chaperone
   Rupak Datta, Abdul Waheed, Gul N. Shah, and William S. Sly
   PNAS, Dec 2007; 104: 19989 - 19994
7. Resistance to an Irreversible Epidermal Growth Factor Receptor (EGFR) Inhibitor in EGFR-Mutant Lung Cancer Reveals Novel Treatment Strategies
   Zhiwei Yu, Titus J. Boggon, Susumu Kobayashi, Cheng Jin, Patrick C. Ma, Afshin Dowlati, Jeffrey A. Kern, Daniel G. Tenen, and Balázs Halmos
   Cancer Res., Nov 2007; 67: 10417 - 10427
8. Hepatocyte Growth Factor Receptor c-MET Is Associated with FAS and When Activated Enhances Drug-induced Apoptosis in Pediatric B Acute Lymphoblastic Leukemia with TEL-AML1 Translocation
   Benedetta Accordi, Serena Pillozzi, Marta Campo Dell'Orto, Giovanni Cazzaniga, Annarosa Arcangeli, Geertruy te Kronnie, and Giuseppe Basso
   J. Biol. Chem., Oct 2007; 282: 29384 - 29393.
9. Genetic Analysis of the Short Splice Variant of the Inhibitor of Caspase-activated DNase (ICAD-S) in Chicken DT40 Cells
   Alexander V. Ageichik, Kumiko Samejima, Scott H. Kaufmann, and William C. Earnshaw
   J. Biol. Chem., Sep 2007; 282: 27374 - 27382.
10. Regulation of Antibody-Dependent Cellular Cytotoxicity by IgG Intrinsic and Apparent Affinity for Target Antigen
    Yong Tang, Jianlong Lou, R. Katherine Alpaugh, Matthew K. Robinson, James D. Marks, and Louis M. Weiner
    J. Immunol., Sep 2007; 179: 2815 - 2823

Annexin-V-Alexa 568

1. Calpain counteracts mechanosensitive apoptosis of vascular smooth muscle cells in vitro and in vivo
   Daniel G. Sedding, Matthias Homann, Ulrike Seay, Harald Tillmanns, Klaus T. Preissner, and Ruediger C. Braun-Dullaeus
   FASEB J, Feb 2008; 22: 579 - 589.
2. Involvement of a guanine nucleotide-exchange protein, ARF-GEP100/BRAG2a, in the apoptotic cell death of monocytic phagocytes
   Akimasa Someya, Joel Moss, and Isao Nagaoka
   J. Leukoc. Biol., Oct 2006; 80: 915 - 921.
3. Human Macrophages Constitute Targets for Immunotoxic Inorganic Arsenic
   Anthony Lemarie, Claudie Morzadec, Emilie Bourdonnay, Olivier Fardel, and Laurent Vernhet
   J. Immunol., Sep 2006; 177: 3019 - 3027.
4. Mirk/Dyrk1b Mediates Cell Survival in Rhabdomyosarcomas
   Stephen E. Mercer, Daina Z. Ewton, Sejal Shah, Asghar Naqvi, and Eileen Friedman
   Cancer Res., May 2006; 66: 5143 - 5150.
5. Nuclear localization of glyceraldehyde-3-phosphate dehydrogenase is not involved in the initiation of apoptosis induced by 1-Methyl-4-phenyl-pyridium iodide (MPP+)
   Rieko Kodama, Tomoyoshi Kondo, Hideyuki Yokote, Xuefeng Jing, Takahiro Sawada, Masaya Hironishi, and Kazushige Sakaguchi
   Genes Cells, Dec 2005; 10: 1211 - 1219
6. Activation of liver X receptors and retinoid X receptors prevents bacterial-induced macrophage apoptosis
   Annabel F. Valledor, Li-Chung Hsu, Sumito Ogawa, Dominique Sawka-Verhelle, Michael Karin, and Christopher K. Glass
   PNAS, Dec 2004; 101: 17813 - 17818
7. Anthrolysin O and Other Gram-positive Cytolysins Are Toll-like Receptor 4 Agonists
   Jin Mo Park, Vincent H. Ng, Shin Maeda, Richard F. Rest, and Michael Karin
   J. Exp. Med., Dec 2004; 200: 1647 - 1655.
8. Anthrolysin O and Other Gram-positive Cytolysins Are Toll-like Receptor 4 Agonists
   Jin Mo Park, Vincent H. Ng, Shin Maeda, Richard F. Rest, and Michael Karin
   J. Exp. Med., Dec 2004; 200: 1647 - 1655
9. Involvement of Estrogen Receptor ß in Ovarian Carcinogenesis
   Aurélie Bardin, Pascale Hoffmann, Nathalie Boulle, Dionyssios Katsaros, Françoise Vignon, Pascal Pujol, and Gwendal Lazennec
   Cancer Res., Aug 2004; 64: 5861 - 5869
10. Loss of Tumorigenicity and Metastatic Potential in Carcinoma Cells Expressing the Extracellular Domain of the Type 1 Insulin-Like Growth Factor Receptor
    Amir Abbas Samani, Eric Chevet, Lucia Fallavollita, Jacques Galipeau, and Pnina Brodt
    Cancer Res., May 2004; 64: 3380 - 3385.

Annexin-V-Biotin

Cat. No. 11 828 690 001 250 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
Sample material	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)
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, Avidin-Fluorescein 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/avidin conjugate. Analysis may be by flow cytometry, by fluorescence microscopy, or by light microscopy.
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. For distinguishing apoptosis using Annexin-V, see Table 2, page 40.
Specificity	Annexin-V-Biotin binds 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.
Time	Approx. 75 min (after induction of apoptosis)
Benefits	■ **Identify individual apoptotic cells** by light microscopy

3

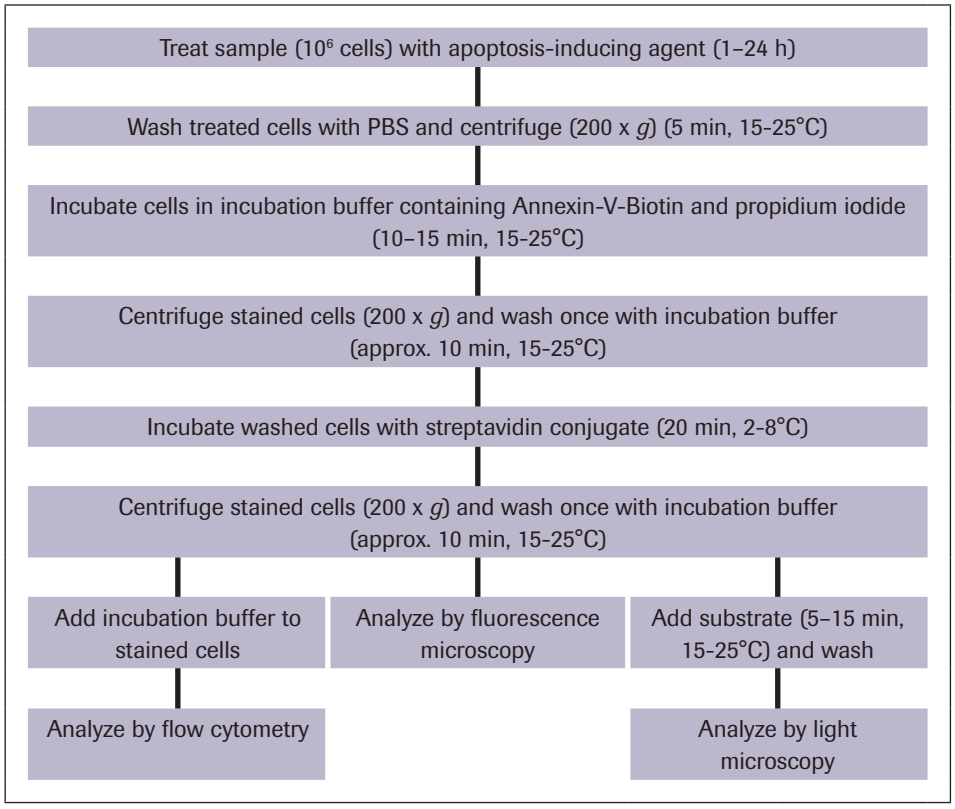
How to use the kit

I. Assay procedure overview

The procedure involves:

1. Washing suspended cells, then pelleting the cells.
2. Resuspending cells in a staining solution containing Annexin-V-Biotin and propidium iodide.

   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) conjugate (Table 3).
5. Analyzing samples in a flow cytometer, under a fluorescence microscope, or under a light microscope (depending on the SA conjugate).



II. Reagent content

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

Related products for visualisation of Annexin-V-Biotin

Product	Application	Cat. No.	Pack Size
SA-Peroxidase	light microscopy	11 089 153 001	500 U (1 ml)
SA-Alkaline Phosphatase	light microscopy	11 089 161 001	1000 U (1 ml)
SA-β-Galactosidase	light microscopy	11 112 481 001	500 U

Table 3: Streptavidin (SA) conjugates available for the indirect assay of apoptotic cells with Annexin-V-Biotin.

III. Protocol for staining of cell suspensions

Preparation of solutions

- Predilute 20 µl Annexin-V-Biotin labeling reagent in 1 ml incubation buffer and add 20 µl Propidium iodide solution.

1 ml is enough for 10 samples.

1	Wash 10^6 cells with PBS and centrifuge cells at 200 × *g* for 5 min.
2	Resuspend the cell pellet in 100 µl of Annexin-V-Biotin labeling solution. Incubate 10 – 15 min at +15 to +25°C.
3	Centrifuge cells at 200 × *g* and wash once in incubation buffer.
4	Incubate in Avidin-Fluorescein staining solution for 20 min at +2 to +8°C with occasionally gentle shaking.
5	Centrifuge cells at 200 × *g* and wash once in incubation buffer.
6	Analyze by fluorescence microscopy or on a flow cytometer.

Typical results with the reagent

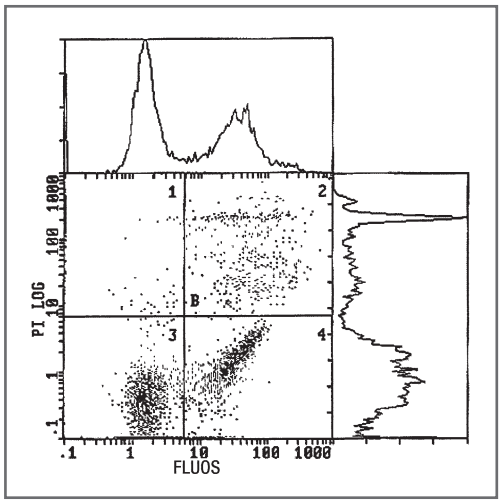


Figure 16: Flow cytometric analysis of apoptotic U937 cells stained with Annexin-V-Biotin, Avidin-FLUOS and propidium iodide. U937 cells (a leukemic cell line) were cultivated for 4 h with 4 µg/ml campothecin. Cells were stained with Annexin-V-Biotin and propidium iodide (PI), then incubated with Avidin-fluorescein and analyzed. Single parameter histograms are shown at the top (Annexin-V-Biotin/Avidin-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.

References

1. Independent and Cooperative Antiviral Actions of Beta Interferon and Gamma Interferon against Herpes Simplex Virus Replication in Primary Human Fibroblasts
   Tao Peng, Jia Zhu, Yon Hwangbo, Lawrence Corey, and Roger E. Bumgarner
   J. Virol., Feb 2008; 82: 1934 - 1945
2. Death Receptor Ligation or Exposure to Perforin Trigger Rapid Egress of the Intracellular Parasite Toxoplasma gondii
   Emma K. Persson, Abela Mpobela Agnarson, Henrik Lambert, Niclas Hitziger, Hideo Yagita, Benedict J. Chambers, Antonio Barragan, and Alf Grandien
   J. Immunol., Dec 2007; 179: 8357 - 8365.
3. Dendritic Cells Loaded with Stressed Tumor Cells Elicit Long-Lasting Protective Tumor Immunity in Mice Depleted of CD4+CD25+ Regulatory T Cells
   Simon J. Prasad, Kathryn J. Farrand, Stephanie A. Matthews, Joe H. Chang, Rebecca S. McHugh, and Franca Ronchese
   J. Immunol., Jan 2005; 174: 90 - 98.
4. Peroxisome proliferator-activated receptor and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias
   Marina Konopleva, Elena Elstner, Teresa J. McQueen, Twee Tsao, Andrey Sudarikov, Wei Hu, Wendy D. Schober, Rui-Yu Wang, David Chism, Steven M. Kornblau, Anas Younes, Steven J. Collins, H. Phillip Koeffler, and Michael Andreeff
   Mol. Cancer Ther., Oct 2004; 3: 1249 - 1262.
5. Dynamic interaction between airway epithelial cells and Staphylococcus aureus
   Mauricio C. A. da Silva, Jean-Marie Zahm, Delphine Gras, Odile Bajolet, Michel Abely, Jocelyne Hinnrasky, Magali Milliot, Maria Cristina de Assis, Coralie Hologne, Noël Bonnet, Marc Merten, Maria Cristina Plotkowski, and Edith Puchelle
   Am J Physiol Lung Cell Mol Physiol, Sep 2004; 287: L543 - L551
6. Targeted Bioactivity of Membrane-Anchored TNF by an Antibody-Derived TNF Fusion Protein
   Stefan Bauer, Nicole Adrian, Barbara Williamson, Con Panousis, Natalie Fadle, Joanna Smerd, Ilknur Fettah, Andrew M. Scott, Michael Pfreundschuh, and Christoph Renner
   J. Immunol., Mar 2004; 172: 3930 - 3939.

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 4).

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 G1 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 17). 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 4: Common fluorochromes used to stain the genomic DNA of viable and/or non-viable cells.

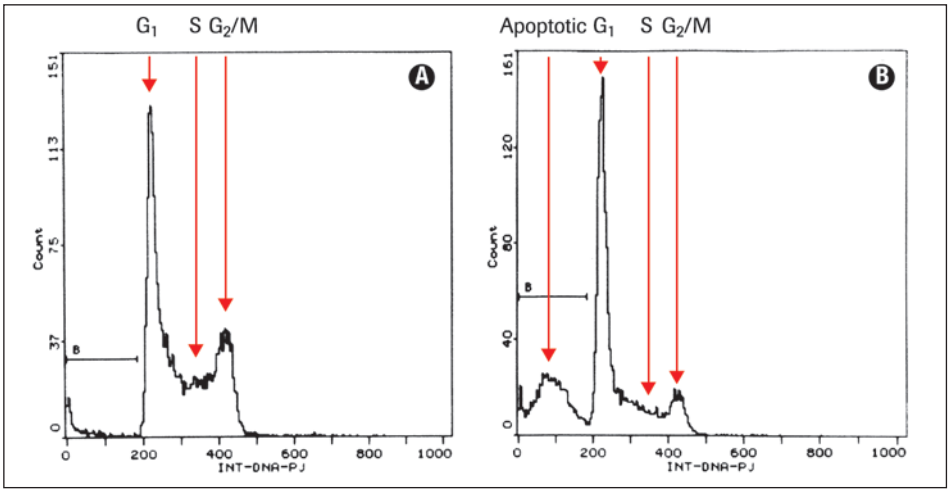


Figure 17: Typical flow cytometric profile of the DNA content in normal (A) and apoptotic cells (B), stained with PI.
Result: A prominent "sub-G1" peak (earliest peak) appears in apoptotic cells, but not in normal cells.

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 18).

During a short exposure to Hoechst 33342, apoptotic cells have stronger blue fluorescence compared to non-apoptotic cells. Co-staining of the cells with propidium iodide (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.

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.

Reagent	Cat. No.	Pack size	Fluorescence	Typical results
Propidium iodide*	11 348 639 001	20 ml	red orange	See Table 4, Figure 13 and 14
DAPI 4',6-Diamidine-2'-phenylindole dihydrochloride	10 236 276 001	10 mg	blue	See Table 4 and Figure 18

Table 5: Fluorescent dyes that stain double-stranded DNA
*Only sold in the US

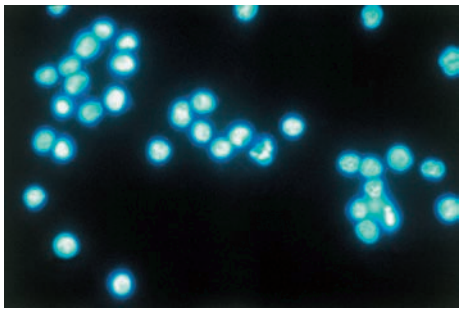


Figure 18: Fluorescent microscopic analysis of apoptotic cells stained with DAPI. DAPI stains the nucleic of all cells (blue).
Result: The characteristic condensed nuclei of apoptotic cells are clearly visible here.

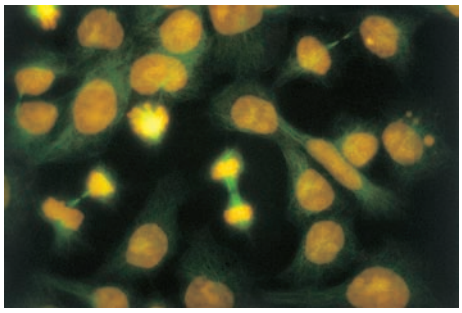


Figure 19: Fluorescent microscopic analysis of mitotic cells stained with ethidium bromide. DNA was stained with ethidium bromide (orange). Mitotic spindles were stained with anti-tubulin antibody (green).
Result: Mitotic cells (with condensed DNA) are brightly stained. Without the double stain, mitotic cells could be mistaken for apoptotic cells, since both have condensed DNA.

3

Apoptosis – DNA Fragmentation in Cell Populations

4

Assays that Measure DNA Fragmentation in Cell Population

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 20). These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit.

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 21). 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.

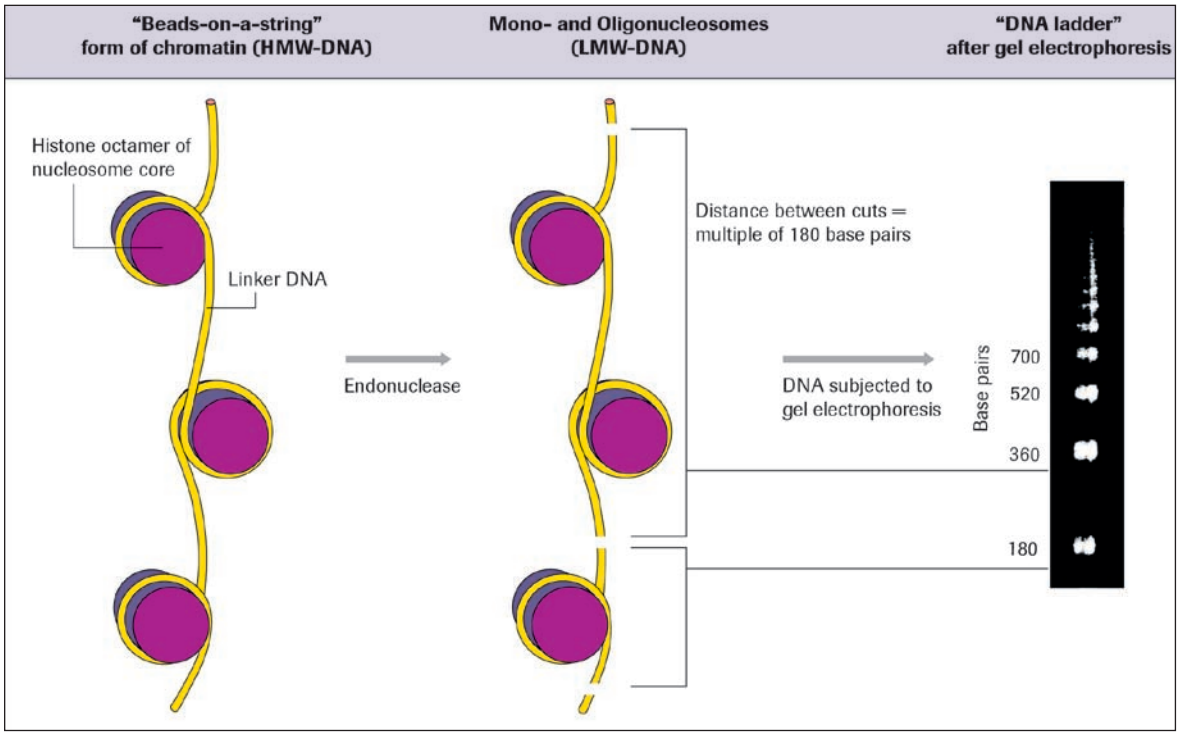


Figure 20: The biochemistry of DNA fragmentation and the appearance of the "DNA ladder".

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".

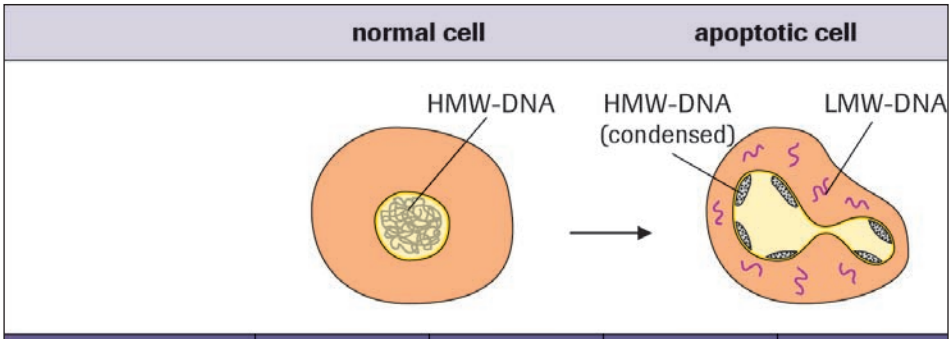
The prelabeling of one cell population (*e.g.*, the target cells) allows the behavior of the labeled cells to be traced specifically when different cell populations are mixed.

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 6). 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 "Cellular DNA Fragmentation ELISA" page 103).

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 20, see also Figure 22). Roche Applied Science offers a kit, the Apoptotic DNA Ladder Kit, that simplifies this assay.



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

Figure 21: 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 6: Distribution of HMW and LMW DNA in cells undergoing apoptosis and target cells during cell mediated cytotoxicity.

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 62).

This nonradioactive immunoassay, offered by Roche Applied Science quantifies that hallmark of apoptosis. The Cell Death Detection ELISA^{PLUS} has been designed for relative quantification of 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.

4

If you are studying…	and you wish to detect…	using detection by…	then use …	page
DNA Fragmentation	DNA fragments	Gel electrophoresis	**Apoptotic DNA Ladder Kit**	57
		ELISA	**Cell Death Detection ELISA^{PLUS}**	62
			Cell Death Detection ELISA	67

Apoptotic DNA Ladder Kit

Cat. No. 11 835 246 001 20 tests

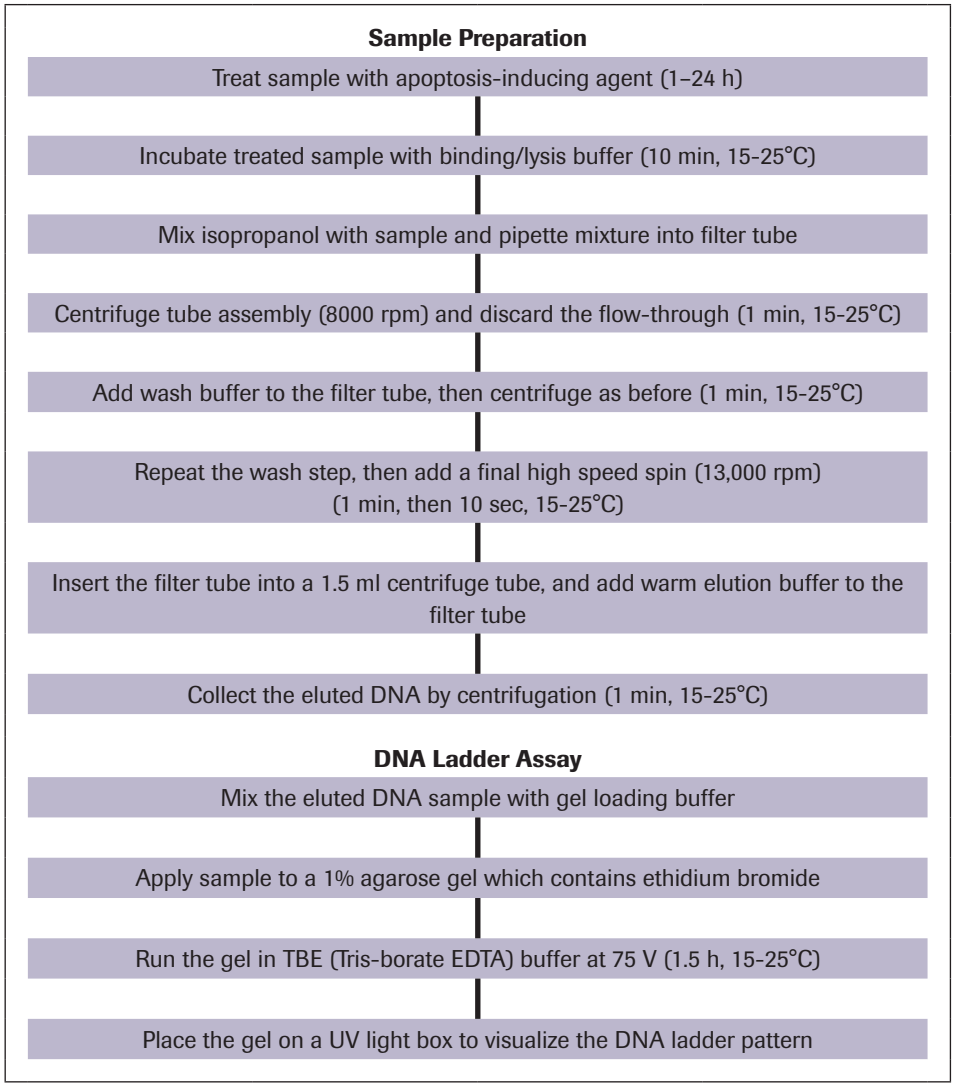
Type	DNA purification kit
Useful for	Preparation of apoptotic DNA fragments for display on electrophoretic gels
Sample material	Whole blood or cells in culture
Sample size	200 – 300 µl whole blood or cell suspension (for instance, 2 x 10^6 cells). The kit allows simultaneous processing of multiple samples.
Method	Cell lysis, followed by binding of cellular DNA on glass fiber, removal of impurities, and DNA recovery
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.
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.
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.
Time	DNA preparation: < 20 min (after induction of apoptosis)
Benefits	■ **Purify DNA** from cell samples in less than 20 minutes. ■ **Simplify DNA extraction** by eliminating organic extractions and DNA-precipitation steps. ■ **Compare your results to the kit's supplied control** (fragmented DNA purified from lyophilized apoptotic U-937 cells), simplifying data interpretation.

How to use the kit

I. Assay procedure overview

The procedure 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.



Yield

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

II. Kit content

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. 20 Glass fiber filter tubes, 700 µl capacity
5. 20 Polypropylene collection tubes, 2 ml (for washes)
6. Positive control, apoptotic U937 cells, lyophilized

III. Protocol for the use of the Apoptotic DNA Ladder Kit on tissue samples

The package insert for our Apoptotic DNA-Ladder Kit, Cat. No. 11 835 246 001, 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 weight 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 (step 4).

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.

Typical results with the kit

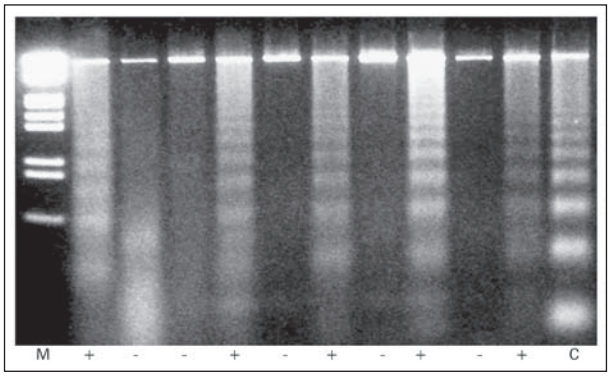


Figure 22: DNA ladder assayed with the Apoptotic DNA Ladder Kit

M = Size marker
– = Control cells without camptothecin
+ = Cells treated with camptothecin
C = Positive control from the kit

References

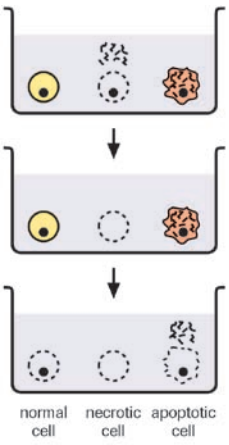
1. Carrageenan Induces Cell Cycle Arrest in Human Intestinal Epithelial Cells in Vitro
   Sumit Bhattacharyya, Alip Borthakur, Pradeep K. Dudeja, and Joanne K. Tobacman
   J. Nutr., Mar 2008; 138: 469 - 475
2. Assessment of Fat Specific Protein 27 (Fsp27) in the Adipocyte Lineage Suggests a Dual Role for Fsp27 in Adipocyte Metabolism and Cell Death
   Ji Young Kim, Kun Liu, Shengli Zhou, Kristin Tillison, Yu Wu, and Cynthia M. Smas
   Am J Physiol Endocrinol Metab, Jan 2008; 10.1152/ajpendo.00104.2007.
3. Glutathione Levels Modulate Domoic Acid–Induced Apoptosis in Mouse Cerebellar Granule Cells
   Gennaro Giordano, Collin C. White, Isaac Mohar, Terrance J. Kavanagh, and Lucio G. Costa
   Toxicol. Sci., Dec 2007; 100: 433 - 444.
4. Factors released from embryonic stem cells inhibit apoptosis of H9c2 cells
   Dinender K. Singla and Debbie E. McDonald
   Am J Physiol Heart Circ Physiol, Sep 2007; 293: H1590 - H1595
5. Direct Comparison of Antigen Production and Induction of Apoptosis by Canarypox Virus- and Modified Vaccinia Virus Ankara-Human Immunodeficiency Virus Vaccine Vectors
   Xiugen Zhang, Farah Cassis-Ghavami, Mike Eller, Jeff Currier, Bonnie M. Slike, Xuemin Chen, James Tartaglia, Mary Marovich, and Paul Spearman
   J. Virol., Jul 2007; 81: 7022 - 7033
6. Aloe vera leaf exudate induces a caspase-independent cell death in Leishmania donovani promastigotes
   Avijit Dutta, Suman Bandyopadhyay, Chitra Mandal, and Mitali Chatterjee
   J. Med. Microbiol., May 2007; 56: 629 - 636.
7. Sanguinarine-Dependent Induction of Apoptosis in Primary Effusion Lymphoma Cells
   Azhar R. Hussain, Naif A. Al-Jomah, Abdul K. Siraj, Pulicat Manogaran, Khalid Al-Hussein, Jehad Abubaker, Leonidas C. Platanias, Khawla S. Al-Kuraya, and Shahab Uddin
   Cancer Res., Apr 2007; 67: 3888 - 3897
8. In Vitro Toxicity of Tetrabromobisphenol-A on Cerebellar Granule Cells: Cell Death, Free Radical Formation, Calcium Influx and Extracellular Glutamate
   Trine Reistad, Espen Mariussen, Avi Ring, and Frode Fonnum
   Toxicol. Sci., Apr 2007; 96: 268 - 278.

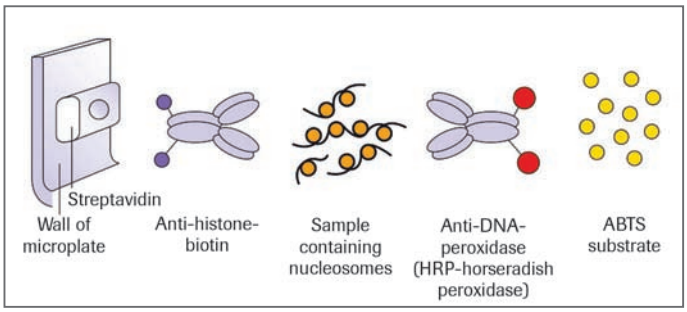
9. Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival
Shahab Uddin, Azhar R. Hussain, Abdul K. Siraj, Pulicat S. Manogaran, Naif A. Al-Jomah, Azadali Moorji, Valerie Atizado, Fouad Al-Dayel, Asim Belgaumi, Hassan El-Solh, Adnan Ezzat, Prashant Bavi, and Khawla S. Al-Kuraya
Blood, Dec 2006; 108: 4178 - 4186
10. Calcium-independent Phospholipase A2 Localizes in and Protects Mitochondria during Apoptotic Induction by Staurosporine
Konstantin Seleznev, Chunying Zhao, Xu Hannah Zhang, Keying Song, and Zhongmin Alex Ma
J. Biol. Chem., Aug 2006; 281: 22275 - 22288.

Cell Death Detection ELISA^{PLUS}

Cat. No. 11 774 425 001 96 tests
Cat. No. 11 920 685 001 10 x 96 tests

Type	One-step sandwich ELISA, colorimetric
Useful for	Relative quantification of apoptosis without cell labeling; differentiating apoptosis from necrosis
Sample material	Cell lysates, cell culture supernatants, serum, or plasma Adherent cells, cells in suspension
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)*
Test principle	**Sample preparation** After incubating cells with an apoptosis-inducing agent, pellet the cells by centrifugation. For the detection of necrosis retain a sample of the supernatant, which may contain necrotic DNA that leaked through the membrane during the incubation. Incubate cells with lysis buffer. Pellet the intact nuclei by centrifugation. Take an aliquot of the supernatant (cell lysate) and determine the amount of apoptotic nucleosomes present.





Significance of kit	Use this kit for relative quantification of 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.

HTS = Suitable for high-throughput screening.

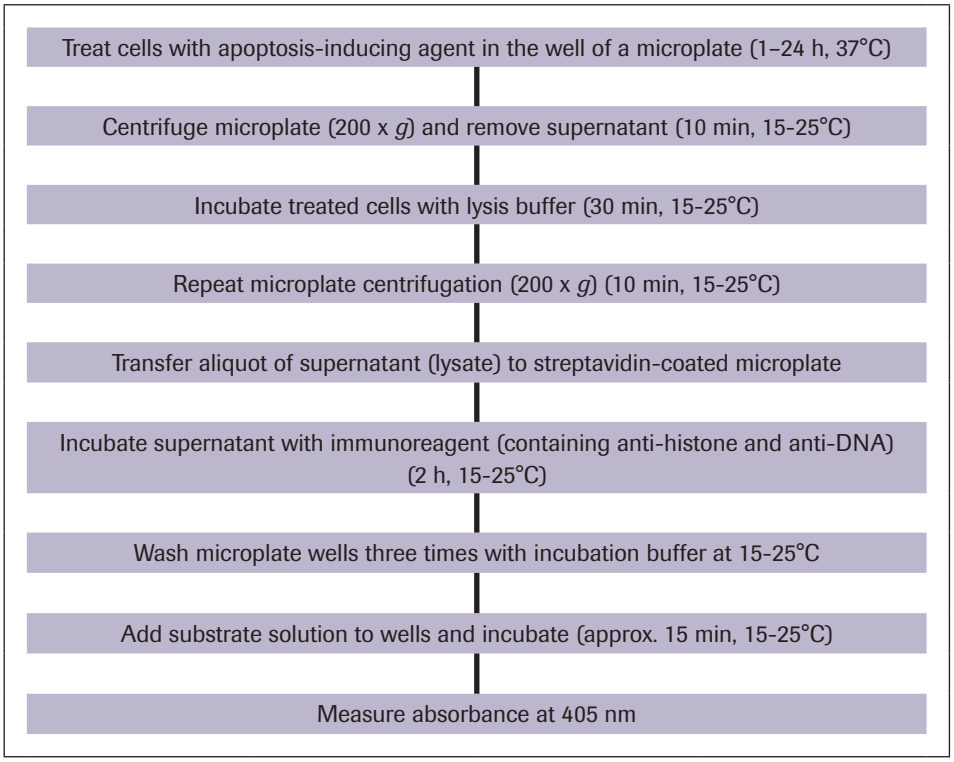
Sensitivity	In a model system, nucleosomes were detectable in as few as 600 campothecin-induced U937 cells (Figure 23). However, the lower limit for detecting dying/dead cells in a particular sample varies with the kinetics of the apoptotic process, the cytotoxic agent used, and the number of affected cells in the total cell population.
Specificity	The ELISA is specific for nucleosomes containing single- or double-stranded DNA (Figure 24). It is not species specific.
Time	Approx. 3 h (after induction of apoptosis)
Benefits	■ **Obtain reproducible relative quantification of cell death** that correlates well with the DNA ladder method. ■ **Perform high-throughput analysis of cell death with a one-step ELISA** that processes hundreds of cell samples in parallel, saving time and effort. ■ **Discriminate between cells undergoing apoptosis and those undergoing necrosis** using one sample. ■ **Achieve high sensitivity** by detecting apoptosis in as few as 600 cells. ■ **Choose the improved Cell Death Detection ELISA^{PLUS}** and obtain all the benefits of the Cell Death Detection ELISA with the added advantages of fewer steps, a shorter assay time, and an ABTS Stop Solution.

How to use the kit

I. Assay procedure overview

The assay uses an one-step sandwich immunoassay to detect nucleosomes. The procedure 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 (CAM)). 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 module (included in kit).
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.



Kit content

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. ABTS stop solution
9. Microplate modules 12 x 8 wells (streptavidin-coated)
10. Adhesive cover foils

Typical results with the kit

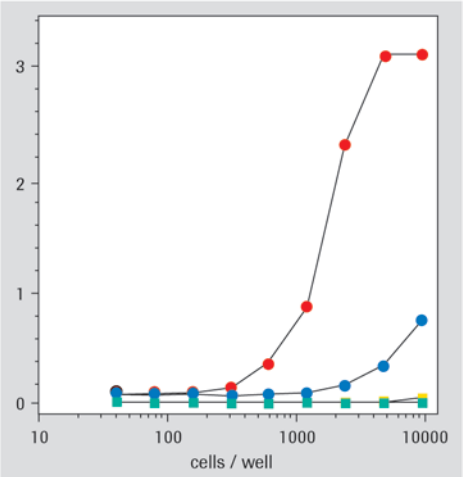


Figure 23: Sensitivity of Cell Death Detection ELISA^{PLUS}.
Different cell concentrations of U937 cells were incubated with camptothecin (CAM) (2 µg/ml) or without CAM for 4 h at 37°C. 20 µl of cell culture supernatant and cell lysates were analyzed in the ELISA. Substrate reaction time: 10 min. ● Lysate 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.

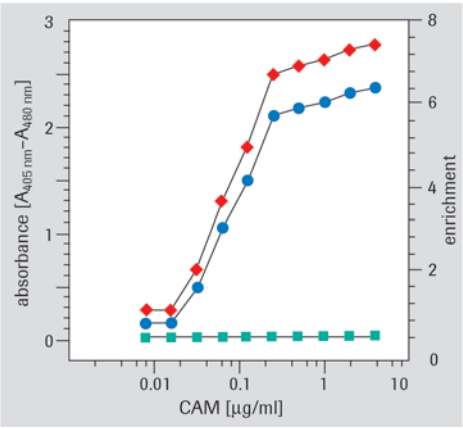


Figure 24: Dose-response experiment analyzed by the Cell Death Detection ELISA^{PLUS}.
U937 cells (10^4 cells/well, in 200 µl) were incubated with different concentrations of camptothecin (CAM) for 4 h at 37°C. Before and after lysis, cells were centrifuged and a 20 µl aliquot of the supernatant was 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.

References

Cell Death Detection ELISA^{PLUS}

1. Integrin-linked kinase localizes to the centrosome and regulates mitotic spindle organization
   Andrew B. Fielding, Iveta Dobreva, Paul C. McDonald, Leonard J. Foster, and Shoukat Dedhar
   J. Cell Biol., Feb 2008; 180: 681 - 689.
2. Interleukin-18 Suppresses Adiponectin Expression in 3T3-L1 Adipocytes via a Novel Signal Transduction Pathway Involving ERK1/2-dependent NFATc4 Phosphorylation
   Bysani Chandrasekar, Devang N. Patel, Srinivas Mummidi, Jae-woo Kim, Robert A. Clark, and Anthony J. Valente
   J. Biol. Chem., Feb 2008; 283: 4200 - 4209.
3. Testosterone exacerbates obstructive renal injury by stimulating TNF- production and increasing proapoptotic and profibrotic signaling
   Peter D. Metcalfe, Jeffrey A. Leslie, Matthew T. Campbell, Daniel R. Meldrum, Karen L. Hile, and Kirstan K. Meldrum
   Am J Physiol Endocrinol Metab, Feb 2008; 294: E435 - E443
4. TNF-/cycloheximide-induced apoptosis in intestinal epithelial cells requires Rac1-regulated reactive oxygen species
   Shi Jin, Ramesh M Ray, and Leonard R Johnson
   Am J Physiol Gastrointest Liver Physiol, Jan 2008; 10.1152/ajpgi.00219.2007.
5. Overexpression of TRPC3 increases apoptosis but not necrosis in response to ischemia/reperfusion in adult mouse cardiomyocytes
   Dan Shan, Richard B Marchase, and John C. Chatham
   Am J Physiol Cell Physiol, Jan 2008; 10.1152/ajpcell.00313.2007.
6. HIV Impairs TNF- Mediated Macrophage Apoptotic Response to Mycobacterium tuberculosis
   Naimish R. Patel, Jinping Zhu, Souvenir D. Tachado, Jianmin Zhang, Zhi Wan, Jussi Saukkonen, and Henry Koziel
   J. Immunol., Nov 2007; 179: 6973 - 6980
7. Dopamine induces apoptosis in young, but not in neonatal, neurons via Ca2+-dependent signal
   Kousaku Iwatsubo, Sayaka Suzuki, Chanxia Li, Takashi Tsunematsu, Fumi Nakamura, Satoshi Okumura, Motohiko Sato, Susumu Minamisawa, Yoshiyuki Toya, Satoshi Umemura, and Yoshihiro Ishikawa
   Am J Physiol Cell Physiol, Nov 2007; 293: C1498 - C1508.
8. Blocking heat shock protein-90 inhibits the invasive properties and hepatic growth of human colon cancer cells and improves the efficacy of oxaliplatin in p53-deficient colon cancer tumors in vivo
   Christian Moser, Sven A. Lang, Silvia Kainz, Andreas Gaumann, Stefan Fichtner-Feigl, Gudrun E. Koehl, Hans J. Schlitt, Edward K. Geissler, and Oliver Stoeltzing
   Mol. Cancer Ther., Nov 2007; 6: 2868 - 2878.
9. Phosphoinositide 3-Kinase-independent Non-genomic Signals Transit from the Androgen Receptor to Akt1 in Membrane Raft Microdomains
   Bekir Cinar, Nishit K. Mukhopadhyay, Gaoyuan Meng, and Michael R. Freeman
   J. Biol. Chem., Oct 2007; 282: 29584 - 29593.
10. Estrogen Signaling via a Linear Pathway Involving Insulin-Like Growth Factor I Receptor, Matrix Metalloproteinases, and Epidermal Growth Factor Receptor to Activate Mitogen-Activated Protein Kinase in MCF-7 Breast Cancer Cells
    Robert X.-D. Song, Zhenguo Zhang, Yucai Chen, Yongde Bao, and Richard J. Santen
    Endocrinology, Aug 2007; 148: 4091 - 4101

Cell Death Detection ELISA

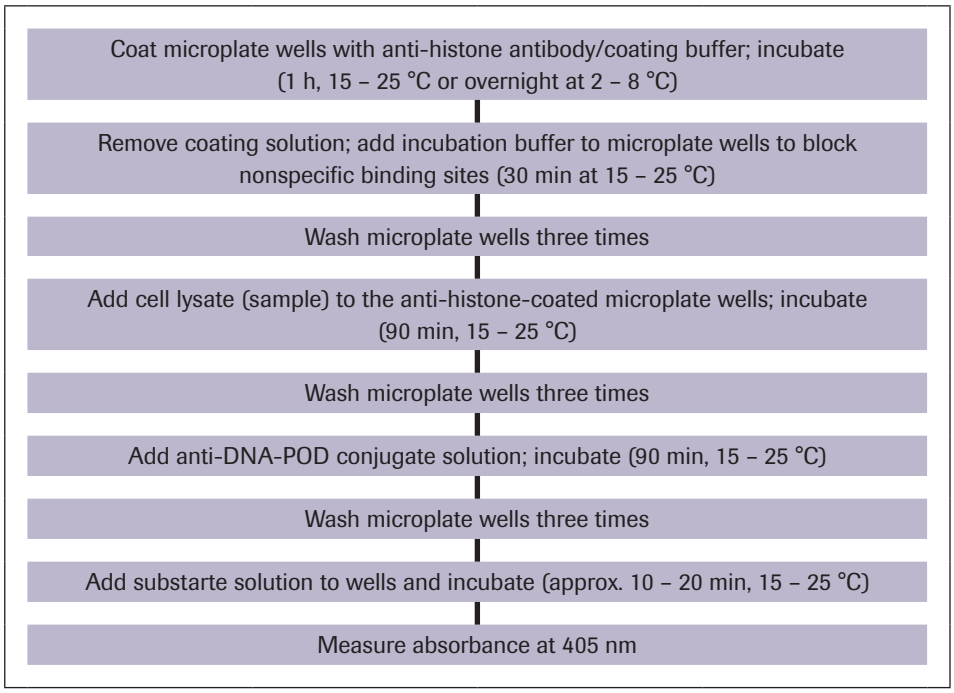
Cat. No. 11 544 675 001

Type	Three-step sandwhich ELISA, colorimetric
Useful for	Specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.
Sample material	Cytoplasmic fractions (lysates) of cell lines, cells ex vivo, or tissue homogenates
Method	Cell lysis, followed by immunochemical determination if histone-complexed DNA fragments in a microplate well
Time	5-6 h

How to use the kit

I. Assay procedure overview

The procedure involves:



Kit content

1. Anti-histone, (clone H11–4)
2. Anti-DNA-POD, (clone MCA-33)
3. Coating buffer
4. Washing buffer
5. Incubation buffer, ready-to-use solution
6. Substrate buffer, ready-to-use solution
7. ABTS substrate tablet
8. Microplate modules
9. Adhesive plate cover foils

References

Cell Death Detection ELISA

1. Mechanisms underlying p53 regulation of PIK3CA transcription in ovarian surface epithelium and in ovarian cancer
   Arezoo Astanehe, David Arenillas, Wyeth W. Wasserman, Peter C. K. Leung, Sandra E. Dunn, Barry R. Davies, Gordon B. Mills, and Nelly Auersperg
   J. Cell Sci., Mar 2008; 121: 664 - 674.
2. Methylseleninic Acid Enhances Taxane Drug Efficacy against Human Prostate Cancer and Down-Regulates Antiapoptotic Proteins Bcl-XL and Survivin
   Hongbo Hu, Guang-xun Li, Lei Wang, Jennifer Watts, Gerald F. Combs, Jr., and Junxuan Lü
   Clin. Cancer Res., Feb 2008; 14: 1150 - 1158.
3. FTY720 Induces Apoptosis in Hepatocellular Carcinoma Cells through Activation of Protein Kinase C Signaling
   Jui-Hsiang Hung, Yen-Shen Lu, Yu-Chieh Wang, Yi-Hui Ma, Da-Sheng Wang, Samuel K. Kulp, Natarajan Muthusamy, John C. Byrd, Ann-Lii Cheng, and Ching-Shih Chen
   Cancer Res., Feb 2008; 68: 1204 - 1212.
4. Anoikis effector Bit1 negatively regulates Erk activity
   Rania Kairouz-Wahbe, Hector Biliran, Xiuquan Luo, IngWei Khor, Miriam Wankell, Cynthia Besch-Williford, Jaime Pascual, Robert Oshima, and Erkki Ruoslahti
   PNAS, Feb 2008; 105: 1528 - 1532.
5. Effects of short-term GH supplementation and treadmill exercise training on physical performance and skeletal muscle apoptosis in old rats
   Emanuele Marzetti, Leanne Groban, Stephanie E. Wohlgemuth, Hazel A. Lees, Marina Lin, Harrison Jobe, Silvia Giovannini, Christiaan Leeuwenburgh, and Christy S. Carter
   Am J Physiol Regulatory Integrative Comp Physiol, Feb 2008; 294: R558 - R567
6. Natural and recombinant human glycodelin activates a proapoptotic gene cascade in monocyte cells
   Meng Kian Tee, Jean-Louis Vigne, Jie Yu, and Robert N. Taylor
   J. Leukoc. Biol., Jan 2008; 10.1189/jlb.0406291
7. Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic -cells
   Elida Lai, George Bikopoulos, Michael Wheeler, Maria Rozakis-Adcock, and Allen Volchuk
   Am J Physiol Endocrinol Metab, Jan 2008; 10.1152/ajpendo.00478.2007.
8. Transcription Factor FOXO3a Mediates Apoptosis in HIV-1-Infected Macrophages
   Min Cui, Yunlong Huang, Yong Zhao, and Jialin Zheng
   J. Immunol., Jan 2008; 180: 898 - 906.
9. Placental infection with human papillomavirus is associated with spontaneous preterm delivery
   L.M. Gomez, Y. Ma, C. Ho, C.M. McGrath, D.B. Nelson, and S. Parry
   Hum. Reprod., Jan 2008; 10.1093/humrep/dem404
10. The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes
    Chaoqian Xu, Yanjie Lu, Zhenwei Pan, Wenfeng Chu, Xiaobin Luo, Huixian Lin, Jiening Xiao, Hongli Shan, Zhiguo Wang, and Baofeng Yang
    J. Cell Sci., Sep 2007; 120: 3045 - 3052.

Apoptosis – DNA Fragmentation in Individual Cells

5

Assays that Measure DNA Fragmentation in Individual Cells

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 25). 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 (page 71 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 on page 49 of this guide).

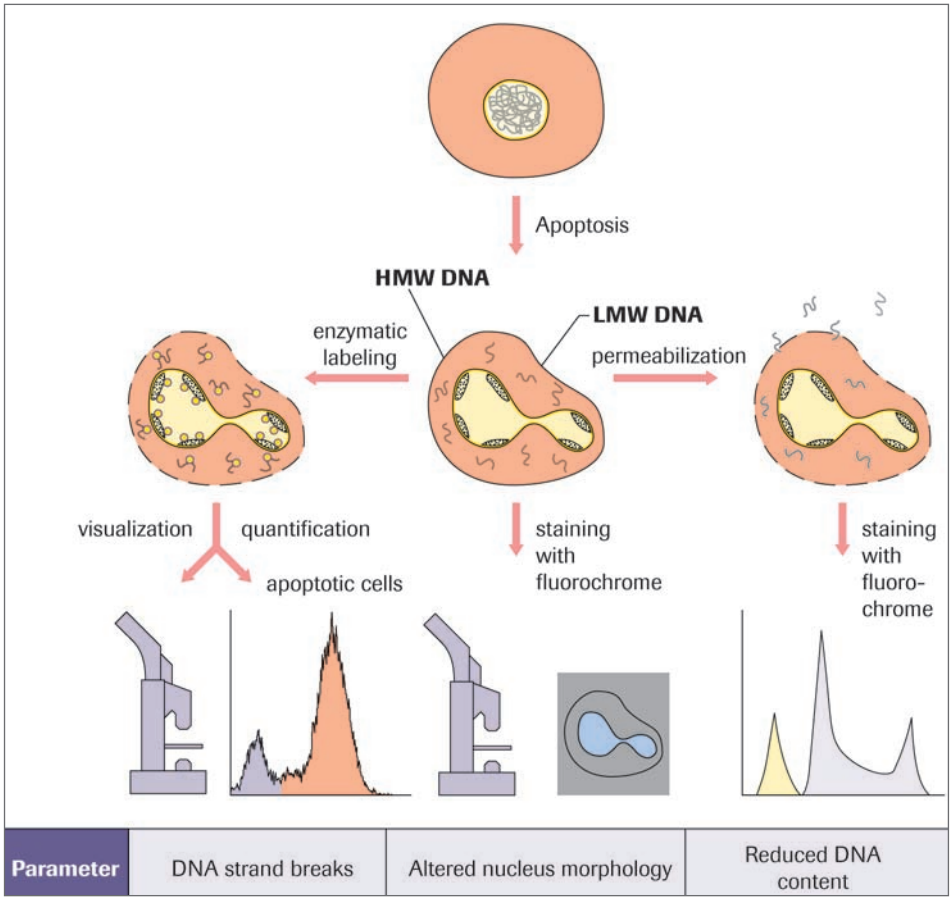


Figure 25: Schematic illustration of the two basic principles for detecting DNA fragmentation in single cells.

The TUNEL enzymatic labeling assay

Extensive DNA degradation is a characteristic event which occurs in the late stages of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks") in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides (X-dUTP, X = biotin, DIG or fluorescein). Suitable labeling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labeling) (Figure 26)

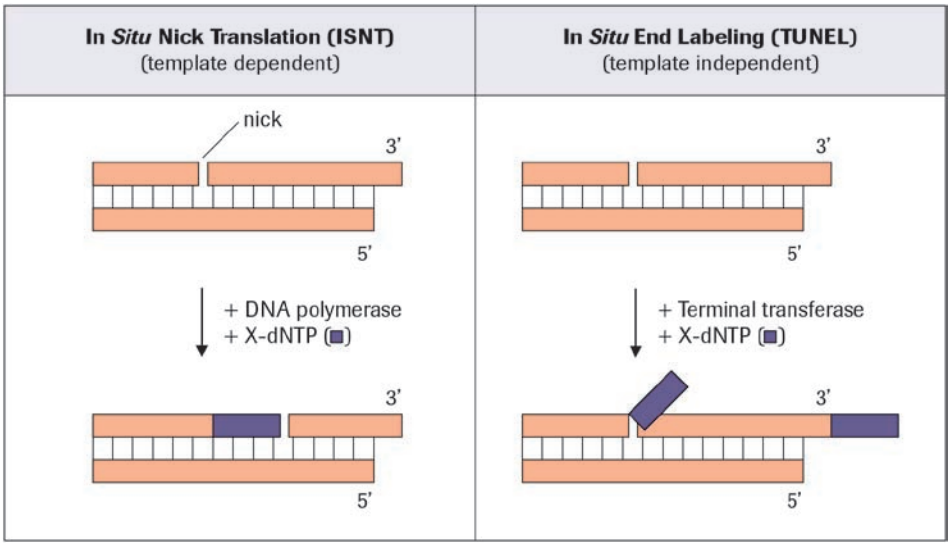


Figure 26: Schematic illustration of two enzymatic DNA labeling methods nick translation and end labeling.

Nick translation

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

End labeling

Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double-stranded DNA breaks independent of a template. The end-labeling method **TUNEL** (**T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abeling) enables the highly sensitive detection of apoptosis in tissue and single cells (Figure 27 and 28).

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

For a comparison of results with the TUNEL and ISNT methods, see Figure 27.

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

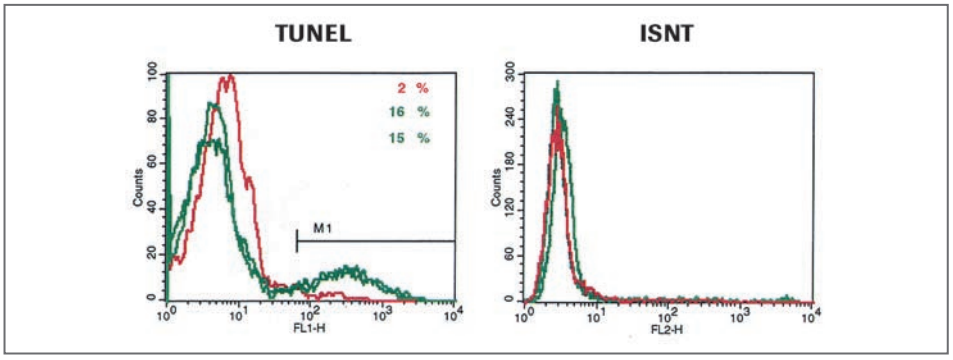


Figure 27: Comparison of TUNEL and ISNT methods for detecting apoptosis in CD8⁺ T cells from TcR transgenic mice. F5 mice are transgenic for a T cell receptor (TcR) specific for a peptide derived from a nucleoprotein of influenza virus ANT/1968. In this experiment, the nucleopeptide protein was injected 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.

5

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. Direct labeling with fluorescein-dUTP offers several other advantages. Direct labeling (Figure 28A) produces less nonspecific background with sensitivity equal to indirect labeling (Figure 28B) 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 7) 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.

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

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

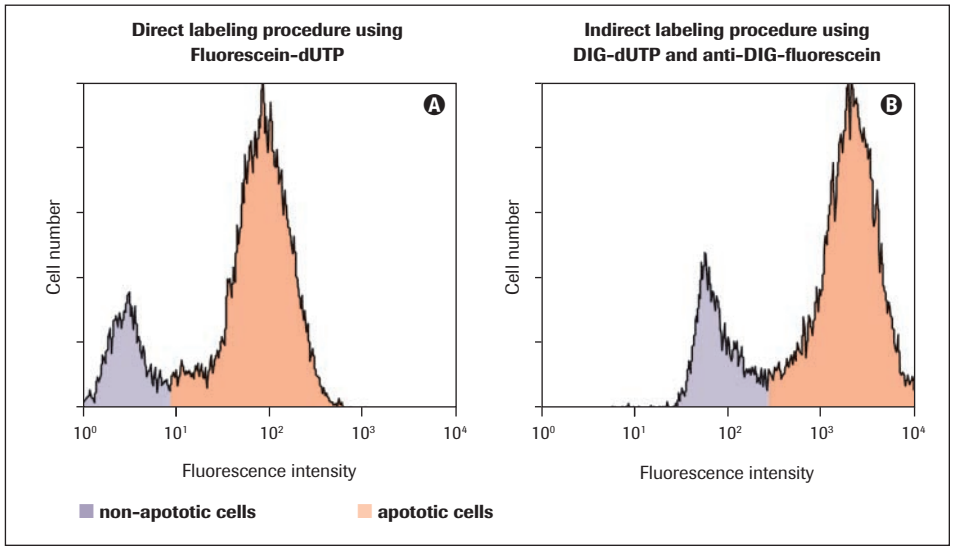


Figure 28: Comparison of direct and indirect labeling of DNA strand breaks in apoptotic cells. PBL were incubated with 1 µM dexamethasone for 24 h at 37°C and then labeled by TUNEL. Recordings were made at the same photomultiplier settings.
(Data were kindly provided by R. Sgonc, University of Innsbruck, Austria).
Result: Direct labeling is as sensitive as indirect labeling, but produces less non-specific background.

Method/RAS product	Label	Indirect (secondary) detection system	Analysis by	Page
***In Situ* Cell Death Detection Kit, Fluorescein**	Fluorescein-dUTP	None (direct detection)	Flow cytometry Fluorescence microscopy	74
***In Situ* Cell Death Detection Kit, TMR red**	TMR-dUTP	None (direct detection)	Fluorescence microscopy	74
***In Situ* Cell Death Detection Kit, AP**	Fluorescein-dUTP	Anti-Fluorescein-AP	Light microscopy	79
***In Situ* Cell Death Detection Kit, POD**	Fluorescein-dUTP	Anti-Fluorescein-POD	Light microscopy	79

Table 7: Four different kits for the enzymatic labeling of DNA and the secondary detection systems required.

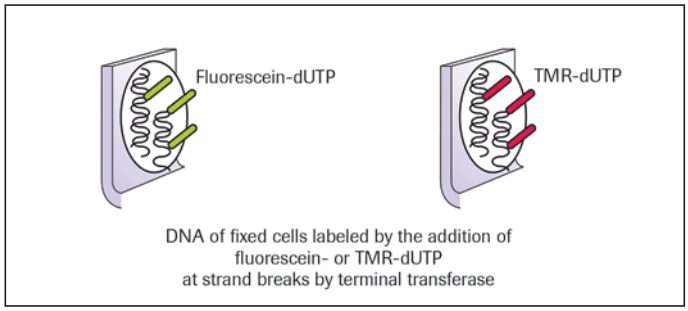
In Situ Cell Death Detection Kit, Fluorescein

Cat. No. 11 684 795 910 50 tests

In Situ Cell Death Detection Kit, TMR red

Cat. No. 12 156 792 910 50 tests

Type	Direct TUNEL labeling assay
Useful for	Detection of DNA strand breaks in apoptotic cells by flow cytometry or fluorescence microscopy
Sample material	Cells in suspension, adherent cells, cell smears, frozen or paraffin-embedded tissue sections
Test principle	The assays use an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP or TMR-dUTP.
Method	End-labeling of DNA with fluorescein-dUTP or tetramethylrhodamine-dUTP (TMR-dUTP), followed by direct analysis of fluorescent cells
Significance of the kit	This two *In Situ* Cell Death Detection Kits, measure and quantitate cell death (apoptosis) by labeling and detection of DNA strand breaks in individual cells by flow cytometry or fluorescence microscopy. The kits offer a direct TUNEL detection method, for maximum sensitivity and minimal background.
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. It detects early stage of DNA fragmentation in apoptotic cells. 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.
Time	1–2 h (+ sample preparation, permeabilization, etc.)
Benefits	■ **Easily obtain results** as no secondary detection system is required. ■ **Identify apoptosis at a molecular level** (DNA-strand breaks) and cells at the very early stages of apoptosis. ■ **Achieve maximum sensitivity** with minimal background.

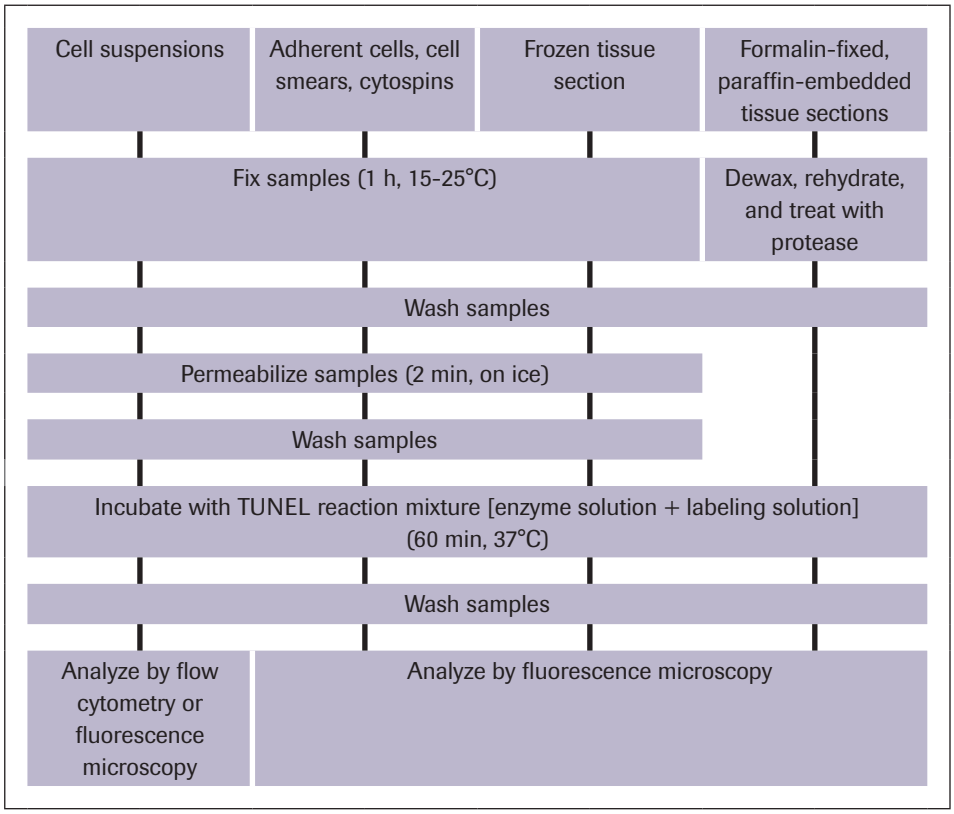


How to use the kits

I. Assay procedure overview

The procedure involves:

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



II. Kits content

In Situ Cell Death Detection Kit, Fluorescein

1. Enzyme solution (TdT), 5 tubes
2. Labeling solution (fluorescein-dUTP), 5 tubes

In Situ Cell Death Detection Kit, TMR red

1. Enzyme solution (TdT), 5 tubes
2. Label solution (TMR-dUTP), 5 vials

The TUNEL reaction mixture is prepared by mixing the Enzyme solution and the Label solution prior to use.

Typical results with the kit

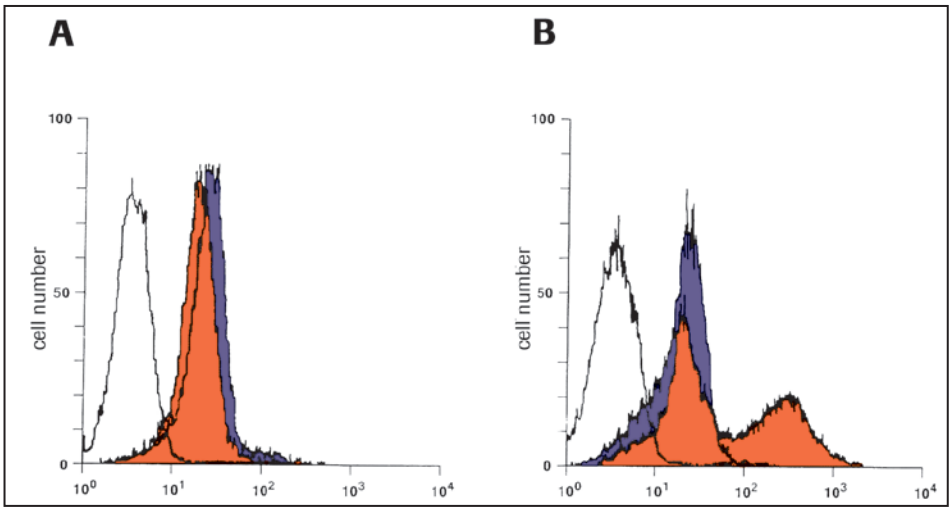


Figure 29: Detection of apoptotic cells by flow cytometry using the *In Situ* Cell Death Detection Kit, Fluorescein. HL60 cells were cultured in the absence (A) or presence (B) of 2 µg/ml Camptothecin for 3 h at 37°C. Cells were incubated either with TUNEL reaction mixture (■) or label solution as negative control (■) or PBS for autofluorescence (□).

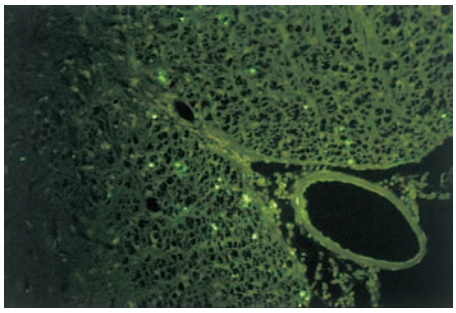


Figure 30: Detection of apoptotic cells (green) by fluorescence microscopy in a tissue section from rat. A tissue section from a rat spinal cord was prepared and assayed with the *In Situ* Cell Death Detection Kit, Fluorescein. The treated section was viewed under a fluorescence microscope. (Photomicrograph 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.

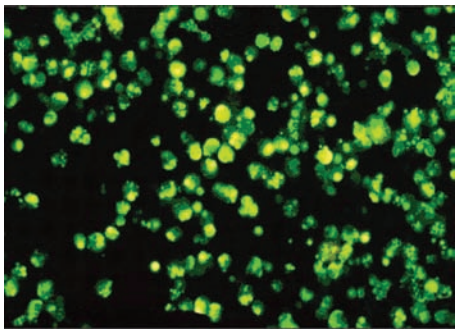


Figure 30a: Cell suspension stained with the *In Situ* Cell Death Detection Kit, Fluorescein. U-937 cells induced with 4 µg/ml camptothecin show positive staining of apoptotic nuclei.
Note: This figure shows a high number (>80%) of apoptotic cells. To avoid detecting cells that are undergoing secondary necrosis, analyze cells earlier in the process after induction of apoptosis.

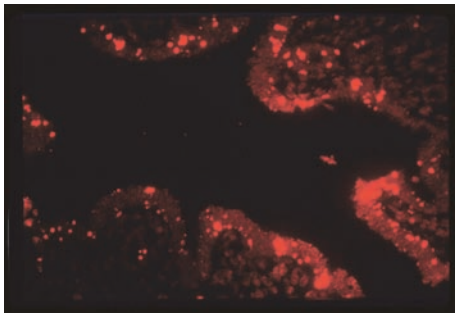


Figure 31: Use of the *In Situ* Cell Death Detection Kit, TMR red to detect apoptotic cells (red) by immunohistochemical staining. Tissue from rabbit endometrium was assayed with the kit and viewed under a fluorescence microscope. Apoptotic nuclei stain bright red; limited fluorescence is visible in background tissue.

References

***In Situ* Cell Death Detection ELISA, Fuorescein**

1. GPR30 Contributes to Estrogen-Induced Thymic Atrophy
   Chunhe Wang, Babak Dehghani, I. Jack Magrisso, Elizabeth A. Rick, Edna Bonhomme, David B. Cody, Laura A. Elenich, Sandhya Subramanian, Stephanie J. Murphy, Martin J. Kelly, Jan S. Rosenbaum, Arthur A. Vandenbark, and Halina Offner
   Mol. Endocrinol., Mar 2008; 22: 636 - 648
2. Dicer-dependent pathways regulate chondrocyte proliferation and differentiation
   Tatsuya Kobayashi, Jun Lu, Bradley S. Cobb, Stephen J. Rodda, Andrew P. McMahon, Ernestina Schipani, Matthias Merkenschlager, and Henry M. Kronenberg
   PNAS, Feb 2008; 105: 1949 - 1954.
3. BAX Inhibitor-1 Modulates Endoplasmic Reticulum Stress-mediated Programmed Cell Death in Arabidopsis
   Naohide Watanabe and Eric Lam
   J. Biol. Chem., Feb 2008; 283: 3200 - 3210.
4. The O-fucose glycan in the ligand-binding domain of Notch1 regulates embryogenesis and T cell development
   Changhui Ge and Pamela Stanley
   PNAS, Feb 2008; 105: 1539 - 1544.
5. The Mouse Polyubiquitin Gene Ubb Is Essential for Meiotic Progression
   Kwon-Yul Ryu, Shamim A. Sinnar, Laura G. Reinholdt, Sergio Vaccari, Susan Hall, Manuel A. Garcia, Tatiana S. Zaitseva, Donna M. Bouley, Kim Boekelheide, Mary Ann Handel, Marco Conti, and Ron R. Kopito
   Mol. Cell. Biol., Feb 2008; 28: 1136 - 1146
6. Parafibromin, a component of the human PAF complex, regulates growth factors and is required for embryonic development and survival in adult mice
   Pengfei Wang, Michael R. Bowl, Stephanie Bender, Jun Peng, Leslie Farber, Jindong Chen, Asif Ali, Jacob Zhang, Arthur S. Alberts, Rajesh V. Thakker, Ali Shilatifard, Bart O. Williams, and Bin Tean Teh
   Mol. Cell. Biol., Jan 2008; 10.1128/MCB.00654-07.
7. Blockade of Tumor Growth Due to Matrix Metalloproteinase-9 Inhibition Is Mediated by Sequential Activation of β1-Integrin, ERK, and NF-B
   Praveen Bhoopathi, Chandramu Chetty, Sateesh Kunigal, Sravan K. Vanamala, Jasti S. Rao, and Sajani S. Lakka
   J. Biol. Chem., Jan 2008; 283: 1545 - 1552.
8. TAT-mediated PRDX6 protein transduction protects against eye lens epithelial cell death and delays lens opacity
   Eri Kubo, Nigar Fatma, Yoshio Akagi, David R Beier, Sanjay P Singh, and Dhirendra P Singh
   Am J Physiol Cell Physiol, Jan 2008; 10.1152/ajpcell.00540.2007.
9. Conservation of the pro-apoptotic nuclease activity of endonuclease G in unicellular trypanosomatid parasites
   Sreenivas Gannavaram, Chetan Vedvyas, and Alain Debrabant
   J. Cell Sci., Jan 2008; 121: 99 - 109.
10. Copper and Manganese Induce Yeast Apoptosis via Different Pathways
    Qiuli Liang and Bing Zhou
    Mol. Biol. Cell, Dec 2007; 18: 4741 - 4749.

In Situ Cell Death Detection ELISA, TMR red

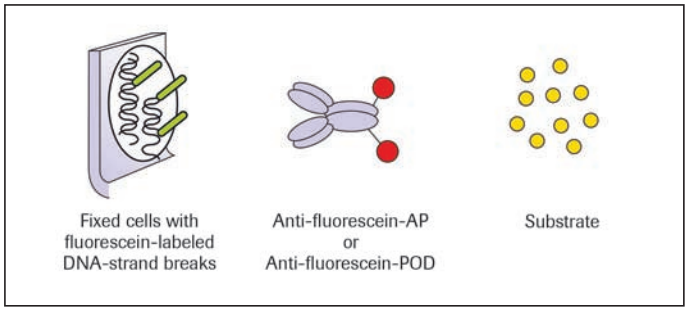
1. Early Embryonic Lethality of Mice Lacking ZO-2, but Not ZO-3, Reveals Critical and Nonredundant Roles for Individual Zonula Occludens Proteins in Mammalian Development
   Jianliang Xu, P. Jaya Kausalya, Dominic C. Y. Phua, Safiah Mohamed Ali, Zakir Hossain, and Walter Hunziker
   Mol. Cell. Biol., Mar 2008; 28: 1669 - 1678.
2. Exosomes As a Short-Range Mechanism to Spread Alloantigen between Dendritic Cells during T Cell Allorecognition
   Angela Montecalvo, William J. Shufesky, Donna Beer Stolz, Mara G. Sullivan, Zhiliang Wang, Sherrie J. Divito, Glenn D. Papworth, Simon C. Watkins, Paul D. Robbins, Adriana T. Larregina, and Adrian E. Morelli
   J. Immunol., Mar 2008; 180: 3081 - 3090.
3. Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1
   Eiji Furuta, Sudha K. Pai, Rui Zhan, Sucharita Bandyopadhyay, Misako Watabe, Yin-Yuan Mo, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Shuichi Kamada, Ken Saito, Megumi Iiizumi, Wen Liu, Johan Ericsson, and Kounosuke Watabe
   Cancer Res., Feb 2008; 68: 1003 - 1011.
4. RASSF7 is a Member of a New Family of RAS Association Domain-Containing Proteins and is Required for Completing Mitosis
   Victoria Sherwood, Ria Manbodh, Carol Sheppard, and Andrew D. Chalmers
   Mol. Biol. Cell, Feb 2008; 10.1091/mbc.E07-07-0652.
5. NF-B-inducing Kinase Phosphorylates and Blocks the Degradation of Down Syndrome Candidate Region 1
   Eun Jung Lee, Su Ryeon Seo, Ji Won Um, Joongkyu Park, Yohan Oh, and Kwang Chul Chung
   J. Biol. Chem., Feb 2008; 283: 3392 - 3400.
6. Developing Postmitotic Mammalian Neurons In Vivo Lacking Apaf-1 Undergo Programmed Cell Death by a Caspase-Independent, Nonapoptotic Pathway Involving Autophagy
   Ronald W. Oppenheim, Klas Blomgren, Douglas W. Ethell, Masato Koike, Masaaki Komatsu, David Prevette, Kevin A. Roth, Yasuo Uchiyama, Sharon Vinsant, and Changlian Zhu
   J. Neurosci., Feb 2008; 28: 1490 - 1497
7. Inhibition of Autophagy Prevents Hippocampal Pyramidal Neuron Death after Hypoxic-Ischemic Injury
   Masato Koike, Masahiro Shibata, Masao Tadakoshi, Kunihito Gotoh, Masaaki Komatsu, Satoshi Waguri, Nobutaka Kawahara, Keisuke Kuida, Shigekazu Nagata, Eiki Kominami, Keiji Tanaka, and Yasuo Uchiyama
   Am. J. Pathol., Feb 2008; 172: 454 - 469
8. The anti-inflammatory cytokine IL-1Ra protects from high fat diet-induced hyperglycemia
   Nadine S. Sauter, Fabienne T. Schulthess, Ryan Galasso, Lawrence W. Castellani, and Kathrin Maedler
   Endocrinology, Jan 2008; 10.1210/en.2007-1059.
9. B-Crystallin Protects Retinal Tissue During S. aureus-Induced Endophthalmitis
   Emily A. Whiston, Norito Sugi, Merideth C. Kamradt, Coralynn Sack, Susan Heimer, Michael Engelbert, Eric F. Wawrousek, Michael S. Gilmore, Bruce R. Ksander, and Meredith S. Gregory
   Infect. Immun., Jan 2008; 10.1128/IAI.01285-07
10. IFN- Mediates the Death of Th1 Cells in a Paracrine Manner
    Kathryn E. Foulds, Masashi J. Rotte, Michael A. Paley, Babu Singh, Daniel C. Douek, Brenna J. Hill, John J. O'Shea, Wendy T. Watford, Robert A. Seder, and Chang-You Wu
    J. Immunol., Jan 2008; 180: 842 - 849.

In Situ Cell Death Detection Kit, AP

Cat. No. 11 684 809 910 50 tests

In Situ Cell Death Detection Kit, POD

Cat. No. 11 684 817 910 50 tests

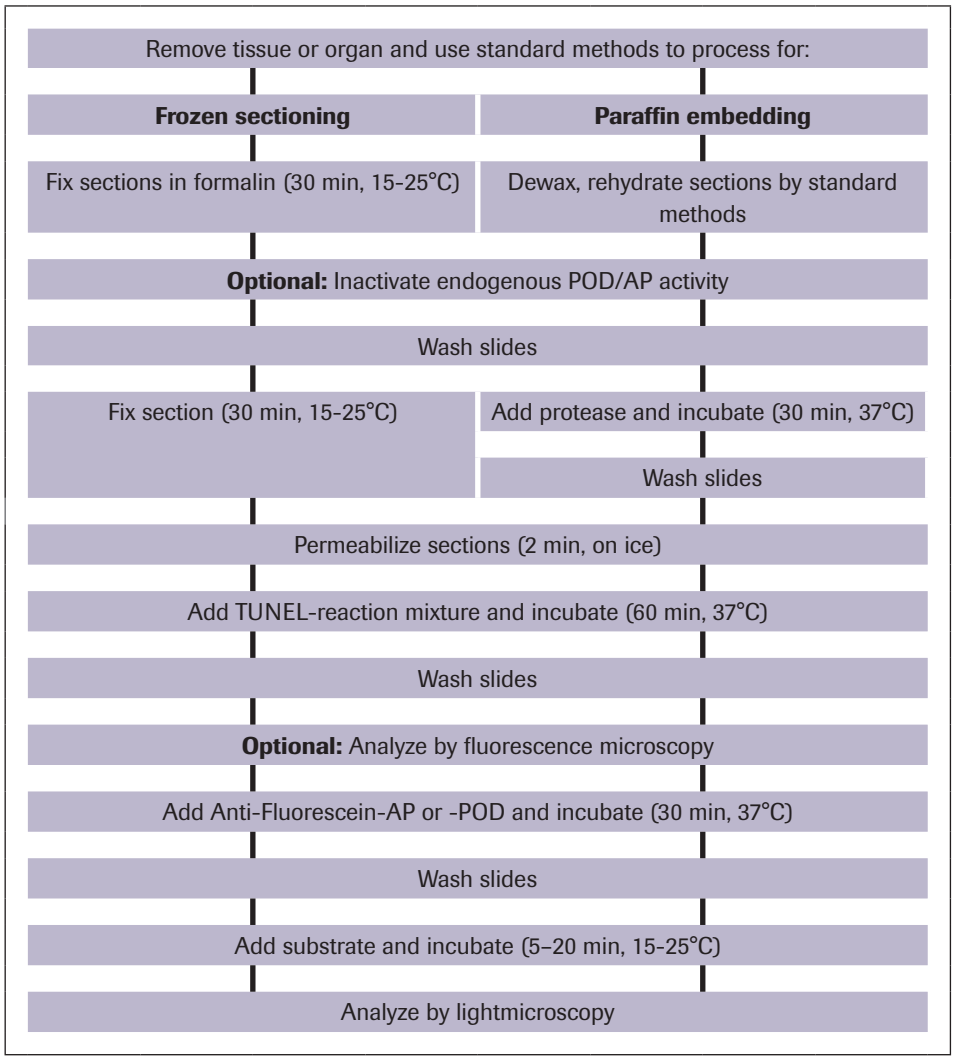
Type	Indirect TUNEL labeling assay
Useful for	Detection of DNA strand breaks in apoptotic cells under a light microscope
Sample material	Cell smears, adherent cells, cytospins, frozen or fixed tissue sections
Test principle	The assays use an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP. 
Method	End-labeling of DNA with fluorescein-dUTP, followed by detection of incorporated fluorescein with an antibody and visualization of the antibody
Significance of the kit	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.
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. It detects early stage of DNA fragmentation in apoptotic cells. 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.
Time	Approx. 3 h (+ sample preparation, permeabilization, etc.)
Benefits	■ **Achieve maximum sensitivity** - intensity of labeling (cell staining) of apoptotic cells is higher than the nick translation method. ■ **The direct labeling procedure using fluorescein-dUTP** allows verification of the efficiency of the TUNEL reaction during the assay procedure ■ **Identify apoptosis** at a molecular level (DNA-strand breaks) and at very early stages. ■ **No substrate included** - provides the opportunity to select the staining procedure of choice.

How to use the kit

I. Assay procedure overview

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.



II. Kits content

In Situ Cell Death Detection Kit, AP

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

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, page 84).

III. TUNEL protocol for tissues which tend to give false positive results

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. 11 684 817 910
DAB Substrate, Cat. No. 11 718 096 001

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). *DO NOT perform a proteinase K treatment!*
3	Immerse the slide(s) for 30 min at 15-25°C in a blocking solution containing 0.1 M Tris-HCl, 3% BSA, and 20% normal bovine serum, pH 7.5.
4	Rinse the slide(s) twice with PBS at 15-25°C. 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). *At this stage, you can evaluate the section under a fluorescence microscope.*
7	Block endogenous peroxidase activity by incubating slides for 10 min at 15-25°C 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 15-25°C for 5 min each.
11	Add 50 µl DAB substrate solution and incubate for 1–3 min at 15-25°C.
12	Wash slide(s) extensively in tap water and counterstain if needed.

IV. Troubleshooting

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. 11 966 006 001) 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 *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. 11 966 006 001) 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

To avoid this artifact	Which may be caused by	Try the following
Low TUNEL labeling	■ Ethanol and methanol fixation	■ Use buffered 4% paraformaldehyde as fixative
(low sensitivity)	■ 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) ! *To avoid possible nuclease contamination, use only Proteinase K from Roche Applied Science, Cat. No. 03 115 836 001* ■ 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) ! *To avoid possible nuclease contamination, use only Proteinase K from Roche Applied Science, Cat. No 03 115 836 001* ■ Immerse dewaxed tissue sections in 200 ml 0.01 M citrate buffer (pH 6.0) and treat with microwave irradiation (370 W, 5 min) ! *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

Typical results with the kit

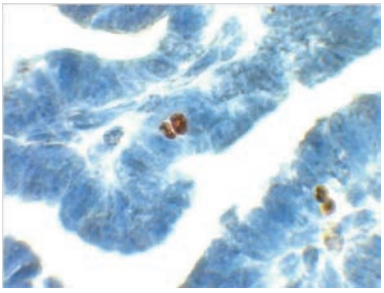


Figure 32: Detection of apoptotic cells by TUNEL and peroxidase staining in rabbit endometrium. A tissue section from rabbit endometrium was prepared and assayed with the *In Situ* Cell Death Detection Kit, POD. Slide was counterstained with hematoxylin and viewed under a light microscope.
Result: A subpopulation of apoptotic cells, scattered throughout the tissue section, are intensely stained (brown) by the TUNEL treatment and subsequent peroxidase immunostaining.

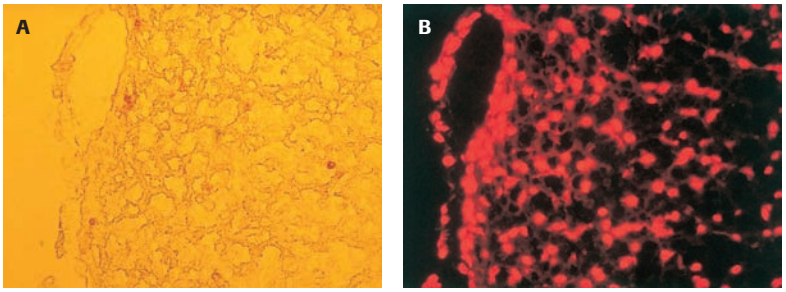


Figure 33: Detection of apoptotic cells by TUNEL and alkaline phosphatase staining in rat spinal cord. A tissue section from rat spinal cord was prepared and assayed with the *In Situ* Cell Death Detection Kit, AP. The slide was viewed under a light microscope (Panel A). After viewing, the same slide 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).

For your convenience, we offer a number of additional single reagents to optimize your TUNEL reaction (Table 8)

Product	Cat. No.	Pack Size
TUNEL Label Mix	11 767 291 910	3 x 550 µl (30 tests)
TUNEL Enzyme	11 767 305 001	2 x 50 µl (20 tests)
TUNEL POD (Anti-Fluorescein, POD conjugate)	11 772 465 001	3.5 ml (70 tests)
TUNEL AP (Anti-Fluorescein, AP conjugate)	11 772 457 001	3.5 ml (70 tests)
TUNEL Dilution Buffer	11 966 006 001	2 x 10 ml
DAB Substrate, metal enhanced, precipitating (Peroxidase (POD) substrate)	11 718 096 001	1 pack
NBT/BCIP Stock Solution (AP substrate)	11 681 451 001	8 ml
Fast Red Tablets (AP substrate)	11 496 549 001	20 tablets

Table 8: Single reagents available for the TUNEL technique.

References

In Situ Cell Death Detection ELISA, AP

1. Glucose and leptin induce apoptosis in human -cells and impair glucose-stimulated insulin secretion through activation of c-Jun N-terminal kinases
   Kathrin Maedler, Fabienne T. Schulthess, Christelle Bielman, Thierry Berney, Christophe Bonny, Marc Prentki, Marc Y. Donath, and Raphael Roduit
   FASEB J, Feb 2008; 10.1096/fj.07-101824.
2. Genotoxic Stress–Induced Expression of p53 and Apoptosis in Leukemic Clam Hemocytes with Cytoplasmically Sequestered p53
   Stefanie Böttger, Emily Jerszyk, Ben Low, and Charles Walker
   Cancer Res., Feb 2008; 68: 777 - 782.
3. Mitochondrial Bax Translocation Accelerates DNA Fragmentation and Cell Necrosis in a Murine Model of Acetaminophen Hepatotoxicity
   Mary Lynn Bajt, Anwar Farhood, John J. Lemasters, and Hartmut Jaeschke
   J. Pharmacol. Exp. Ther., Jan 2008; 324: 8 - 14.
4. Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation
   Cristina González-Estévez, Daniel A. Felix, Aziz A. Aboobaker, and Emili Saló
   PNAS, Aug 2007; 104: 13373 - 13378. Abstract
5. Developmental differences in the responses of IL-6 and IL-13 transgenic mice exposed to hyperoxia
   Rayman Choo-Wing, Jonathan H. Nedrelow, Robert J. Homer, Jack A. Elias, and Vineet Bhandari
   Am J Physiol Lung Cell Mol Physiol, Jul 2007; 293: L142 - L150.
6. Pure red cell aplasia associated with type I autoimmune polyglandular syndrome—successful response to treatment with mycophenolate mofetil: case report and review of literature
   Milena Bakrac, Vladimir Jurisic, Tanja Kostic, Vera Popovic, Sandra Pekic, Nada Kraguljac, and Milica Colovic
   J. Clin. Pathol., Jun 2007; 60: 717 - 720.
7. Semaphorin 7A plays a critical role in TGF-ß1–induced pulmonary fibrosis
   Hye-Ryun Kang, Chun Geun Lee, Robert J. Homer, and Jack A. Elias
   J. Exp. Med., May 2007; 204: 1083 - 1093.
8. Increased Hyperoxia-Induced Mortality and Acute Lung Injury in IL-13 Null Mice
   Vineet Bhandari, Rayman Choo-Wing, Robert J. Homer, and Jack A. Elias
   J. Immunol., Apr 2007; 178: 4993 - 5000.
9. Transforming Growth Factor (TGF)-1 Stimulates Pulmonary Fibrosis and Inflammation via a Bax-dependent, Bid-activated Pathway That Involves Matrix Metalloproteinase-12
   Hye-Ryun Kang, Soo Jung Cho, Chun Geun Lee, Robert J. Homer, and Jack A. Elias
   J. Biol. Chem., Mar 2007; 282: 7723 - 7732.
10. Ectopic Expression of Vascular Cell Adhesion Molecule-1 as a New Mechanism for Tumor Immune Evasion
    Ken-Yu Lin, Dan Lu, Chien-Fu Hung, Shiwen Peng, Lanqing Huang, Chunfa Jie, Francisco Murillo, Jesse Rowley, Ya-Chea Tsai, Liangmei He, Dae-Jin Kim, Elizabeth Jaffee, Drew Pardoll, and T.-C. Wu
    Cancer Res., Feb 2007; 67: 1832 - 1841.

In Situ Cell Death Detection ELISA, POD

1. Lipopolysaccharide-induced carotid body inflammation in cats: Functional manifestations, histopathology and involvement of tumour necrosis factor-
   Ricardo Fernandez, Sergio Gonzalez, Sergio Rey, Paula P Cortes, Kevin R Maisey, Edison-Pablo Reyes, Carolina Larrain, and Patricio Zapata
   Exp Physiol, Feb 2008; 10.1113/expphysiol.2007.041152.
2. Expression of Aire and the Early Wave of Apoptosis in Spermatogenesis
   Claudia E. Schaller, Clifford L. Wang, Gabriele Beck-Engeser, Lindsie Goss, Hamish S. Scott, Mark S. Anderson, and Matthias Wabl
   J. Immunol., Feb 2008; 180: 1338 - 1343.
3. Inhibition of Hypoxia Inducible Factor Hydroxylases Protects Against Renal Ischemia-Reperfusion Injury
   Peter Hill, Deepa Shukla, Maxine G.B. Tran, Julian Aragones, H. Terence Cook, Peter Carmeliet, and Patrick H. Maxwell
   J. Am. Soc. Nephrol., Jan 2008; 19: 39 - 46.
4. A-Raf and B-Raf Are Dispensable for Normal Endochondral Bone Development, and Parathyroid Hormone-Related Peptide Suppresses Extracellular Signal-Regulated Kinase Activation in Hypertrophic Chondrocytes
   Sylvain Provot, Gregory Nachtrab, Jennifer Paruch, Adele Pin Chen, Alcino Silva, and Henry M. Kronenberg
   Mol. Cell. Biol., Jan 2008; 28: 344 - 357.
5. The Anti-Apoptotic Role of Pregnane X Receptor in Human Colon Cancer Cells
   Jie Zhou, Mingjie Liu, Yonggong Zhai, and Wen Xie
   Mol. Endocrinol., Dec 2007; 10.1210/me.2007-0197.
6. Menstrual-like changes in mice are provoked through the pharmacologic withdrawal of progesterone using mifepristone following induction of decidualization
   X.B. Xu, B. He, and J.D. Wang
   Hum. Reprod., Dec 2007; 22: 3184 - 3191.
7. Comparative Protection against Liver Inflammation and Fibrosis by a Selective Cyclooxygenase-2 Inhibitor and a Nonredox-Type 5-Lipoxygenase Inhibitor
   Raquel Horrillo, Anna Planagumà, Ana González-Périz, Natàlia Ferré, Esther Titos, Rosa Miquel, Marta López-Parra, Jaime L. Masferrer, Vicente Arroyo, and Joan Clària
   J. Pharmacol. Exp. Ther., Dec 2007; 323: 778 - 786
8. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development
   Meritxell Gironella, Mylène Seux, Min-Jue Xie, Carla Cano, Richard Tomasini, Julien Gommeaux, Stephane Garcia, Jonathan Nowak, Man Lung Yeung, Kuan-Teh Jeang, Amandine Chaix, Ladan Fazli, Yoshiharu Motoo, Qing Wang, Palma Rocchi, Antonio Russo, Martin Gleave, Jean-Charles Dagorn, Juan L. Iovanna, Alice Carrier, Marie-Josèphe Pébusque, and Nelson J. Dusetti
   PNAS, Oct 2007; 104: 16170 - 16175.
9. Atherosclerosis and Vascular Aging as Modifiers of Tumor Progression, Angiogenesis, and Responsiveness to Therapy
   Halka Klement, Brad St. Croix, Chloe Milsom, Linda May, Qing Guo, Joanne L. Yu, Petr Klement, and Janusz Rak
   Am. J. Pathol., Oct 2007; 171: 1342 - 1351.
10. Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways
    Lavanya Reddivari, Jairam Vanamala, Sudhakar Chintharlapalli, Stephen H. Safe, and J.Creighton Miller, Jr
    Carcinogenesis, Oct 2007; 28: 2227 - 2235.

Cytotoxicity – Introduction

6

Relationship between Cytotoxicity, Apoptosis and Necrosis

As discussed in Chapter 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.

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.

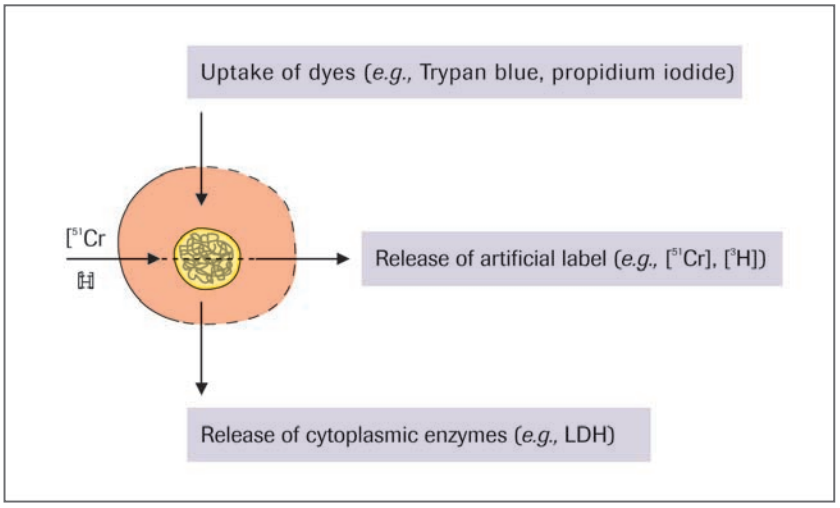


Figure 34: Schematic illustration of the three basic principles to assess plasma membrane leakage.

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. These two mechanisms are not mutually exclusive and, quite possibly, are complementary.

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 34) (see also on page 49 "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.

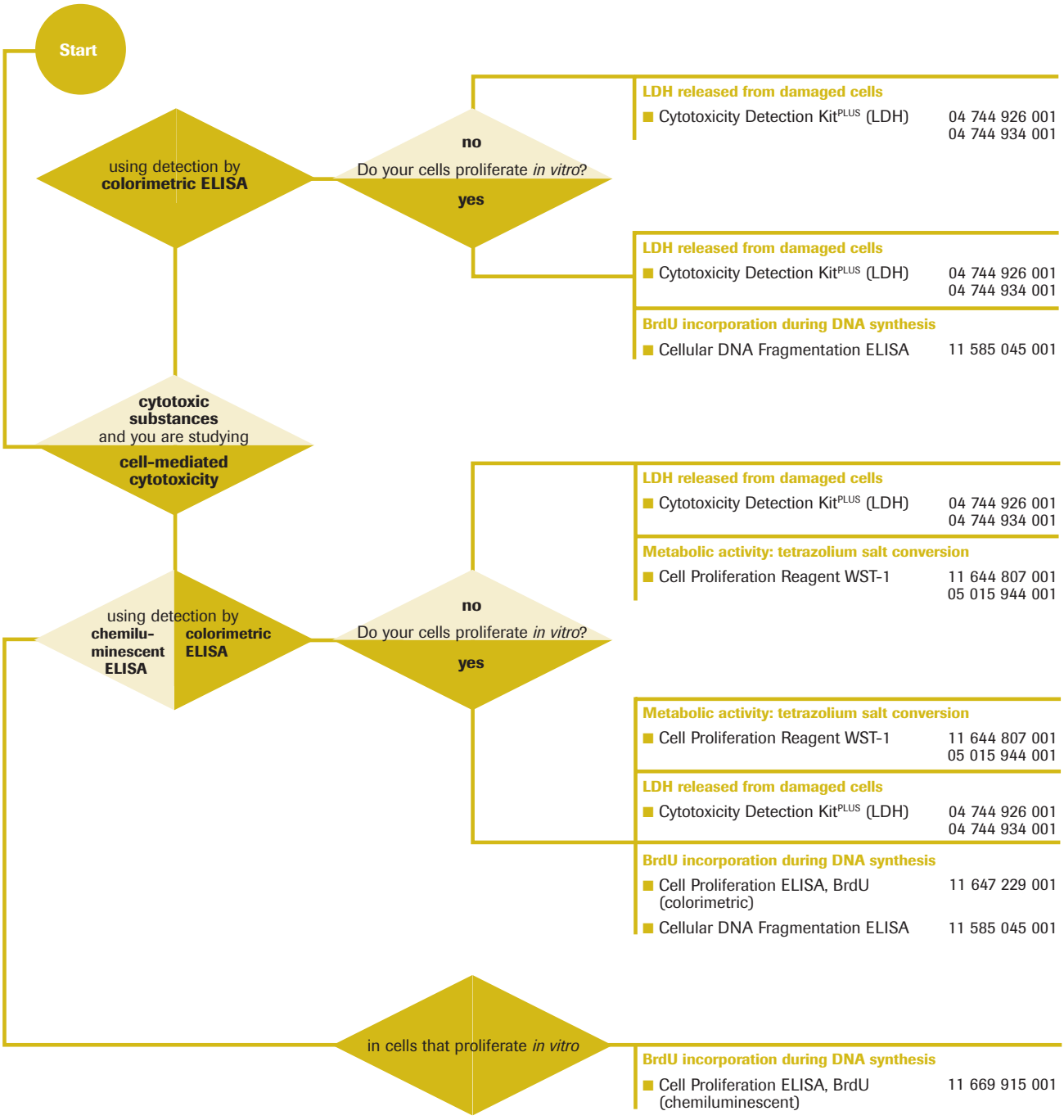
Alternatively, dead cells are unable to metabolize various tetrazolium salts (see also Chapter 9). This allows the use of the colorimetric assays MTT, XTT, or WST-1 to measure cell survival. Apoptosis, however, is an active mode of cell death requiring the metabolism of cells. Thus, like the permeability 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 cytotoxicity, 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.

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

Cytotoxicity Product Selection Guide

Follow the selection guide below to determine the appropriate Roche Applied Science product for the study of **cytotoxicity** to meet your needs. If you need additional help, please visit **www.roche-applied-science.com/apoptosis**



Cytotoxicity in Cell Populations

7

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. 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. 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 Kits (see page 94 and 98), 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 35).

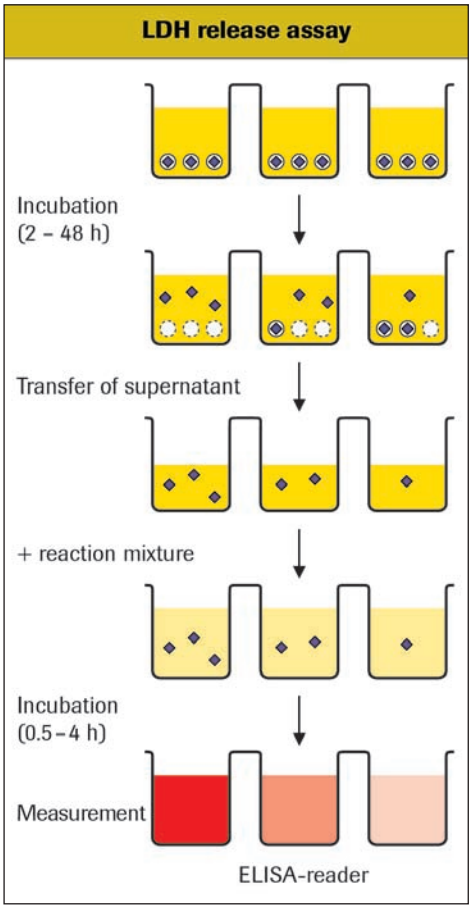


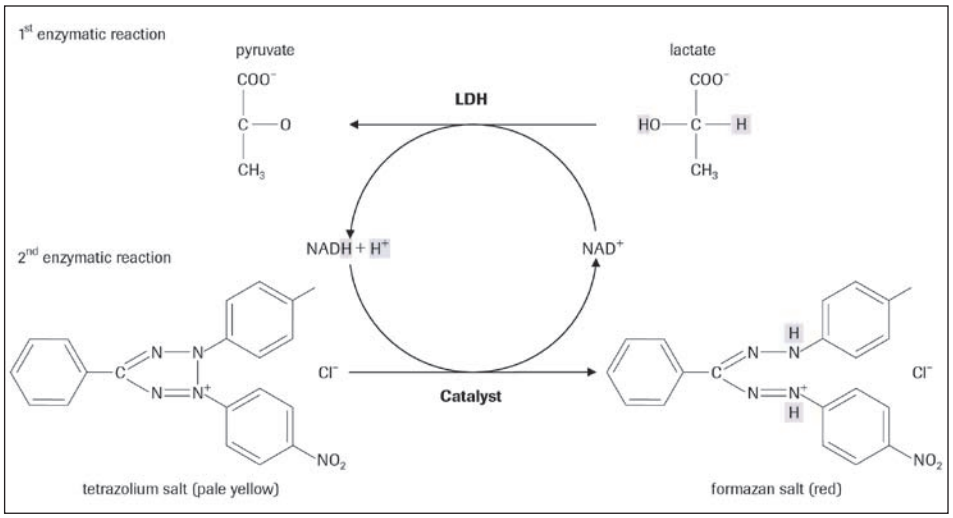
Figure 35: Measurement of LDH activity (◆) using the microplate format.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
Plasma Membrane Damage	Release of lactate dehydrogenase (LDH)	Colorimetric assay	**Cytotoxicity Detection Kit (LDH)**	94
			Cytotoxicity Detection Kit^{PLUS} (LDH)	98

Cytotoxicity Detection Kit (LDH)

Cat. No. 11 644 793 001 2,000 tests

Type	Colorimetric assay, microplate format
Useful for	Quantitation of LDH activity released from damaged/dying cells
Sample material	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
Test principle	The assay is based on the cleavage of a tetrazolium salt when LDH is present in the culture supernatant.



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

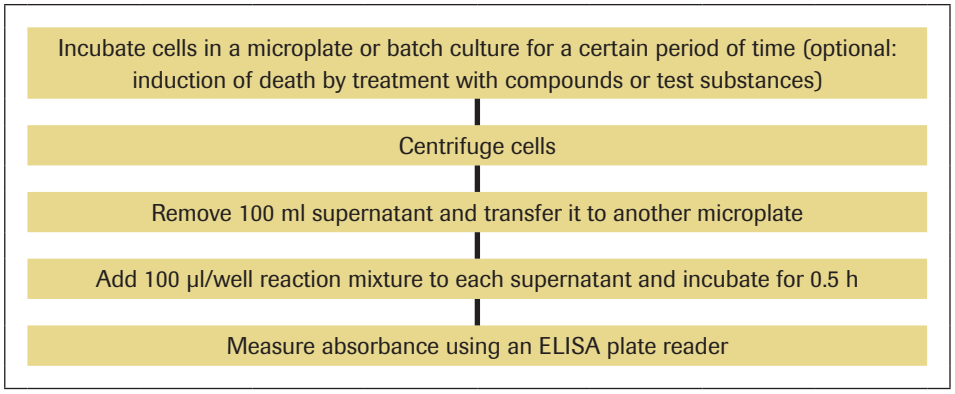
Significance of kit	The Cytotoxicity Detection Kit measures cytotoxicity and cell lysis by detecting LDH activity released from damaged cells. The assay is performed in a 96-well microplate. The kit can be used in many different *in vitro* cell systems where damage of the plasma membrane occurs. Examples 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.
Time	0.5–1 h (+ induction of cell death)
Benefits	■ **Increase safety** by eliminating the use of radioactive isotopes (such as [^{51}Cr] release assays). ■ **Obtain accurate assay results** that strongly correlate to the number of lysed cells. ■ **Detect low cell numbers** with high sensitivity.

How to use the kit

I. Assay procedure overview

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.



II. Kit content

1. Catalyst (Diaphorase/NAD^+ mixture)
2. Dye solution (INT and sodium lactate)

To prepare the reaction mixture, mix catalyst with dye solution prior to use.

Typical results with the kit

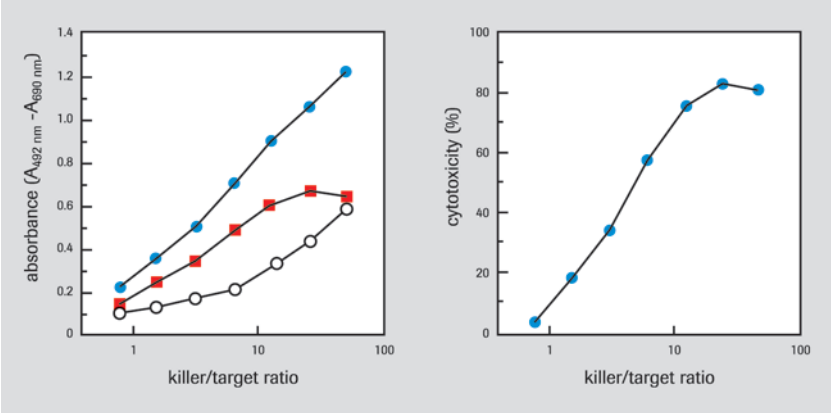


Figure 36: Determination of the cytolytic activity of allogen-stimulated cytotoxic T lymphocytes (CTLs) using the Cytotoxicity Detection Kit (LDH). A) Spleen cells of C57/Bl 6 mice (H-2b) were stimulated *in vitro* with P815 cells (H-2d). Viable CTLs were purified and titrated in the microplate as described 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 (■). B) Calculated percentage of cell-mediated lysis.

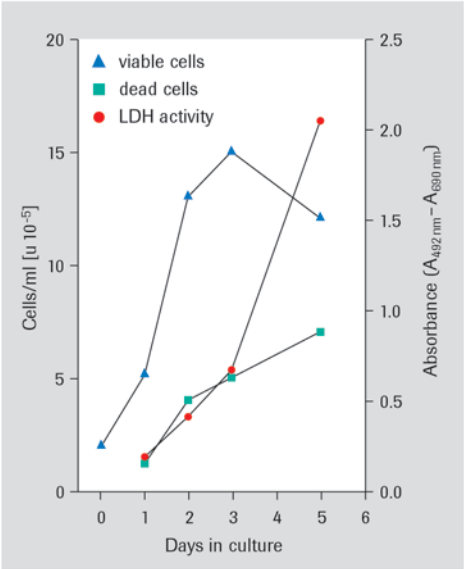


Figure 37: Correlation of cell death (defined by increased plasma membrane permeability) and LDH release. Ag8 cells (starting cell concentration: 2 x 10^5 /ml) were cultured and after 1, 2, 3 and 5 days, aliquots were removed. The amount of viable (▲) and dead (■) cells was determined by trypan blue 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.

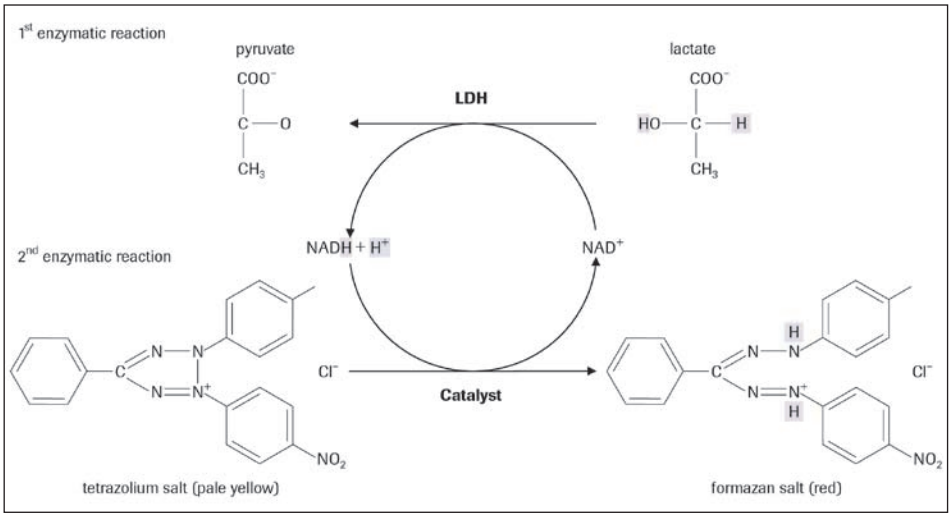
References

1. Single-particle characterization of iron-induced pore-forming -synuclein oligomers
   Marcus Kostka, Tobias Högen, Karin M. Danzer, Johannes Levin, Matthias Habeck, Andreas Wirth, Richard Wagner, Charles G. Glabe, Sabine Finger, Udo Heinzelmann, Patrick Garidel, Wenzhen Duan, Christopher A. Ross, Hans Kretzschmar, and Armin Giese
   J. Biol. Chem., Feb 2008; 10.1074/jbc.M709634200.
2. The Importance of Vibrio vulnificus rtxE for Virulence and Its Expression Induced by Exposure to Host Cell
   Byung Cheol Lee, Jeong Hyun Lee, Myung Won Kim, Byoung Sik Kim, Man Hwan Oh, Kun-Soo Kim, Tae Sung Kim, and Sang Ho Choi
   Infect. Immun., Feb 2008; 10.1128/IAI.01503-07
3. In Vitro Exposure of Jurkat T-Cells to Industrially Important Organic Solvents in Binary Combination: Interaction Analysis
   Catherine McDermott, Ashley Allshire, Frank van Pelt, and James J. A. Heffron
   Toxicol. Sci., Feb 2008; 101: 263 - 274
4. NF-B Activation during Acute Helicobacter pylori Infection in Mice
   Richard L. Ferrero, Patrick Avé, Delphine Ndiaye, Jean-Christophe Bambou, Michel R. Huerre, Dana J. Philpott, and Sylvie Mémet
   Infect. Immun., Feb 2008; 76: 551 - 561.
5. Role of the Sodium-Dependent Phosphate Cotransporters and Absorptive Endocytosis in the Uptake of Low Concentrations of Uranium and Its Toxicity at Higher Concentrations in LLC-PK1 Cells
   Dany S. Muller, Pascale Houpert, Jean Cambar, and Marie-Hélène Hengé-Napoli
   Toxicol. Sci., Feb 2008; 101: 254 - 262
6. Dietary flaxseed enhances antioxidant defenses and is protective in a mouse model of lung ischemia-reperfusion injury
   James C. Lee, Faiz Bhora, Jing Sun, Guanjun Cheng, Evguenia Arguiri, Charalambos C. Solomides, Shampa Chatterjee, and Melpo Christofidou-Solomidou
   Am J Physiol Lung Cell Mol Physiol, Feb 2008; 294: L255 - L265.
7. Glial Dysfunction in Parkin Null Mice: Effects of Aging
   Rosa M. Solano, Maria J. Casarejos, Jamie Menéndez-Cuervo, Jose A. Rodriguez-Navarro, Justo García de Yébenes, and Maria A. Mena
   J. Neurosci., Jan 2008; 28: 598 - 611.
8. Calreticulin expression in the clonal plasma cells of patients with systemic light-chain (AL-) amyloidosis is associated with response to high-dose melphalan
   Ping Zhou, Julie Teruya-Feldstein, Ping Lu, Martin Fleisher, Adam Olshen, and Raymond L Comenzo
   Blood, Jan 2008; 111: 549 - 557.
9. Biotin deficiency up-regulates TNF- production in murine macrophages
   Toshinobu Kuroishi, Yasuo Endo, Koji Muramoto, and Shunji Sugarawa
   J. Leukoc. Biol., Jan 2008; 10.1189/jlb.0607428.
10. IFN- induces barrier destabilization and apoptosis in renal proximal tubular epithelium
    Judith Lechner, Nadia Malloth, Thomas Seppi, Bea Beer, Paul Jennings, and Walter Pfaller
    Am J Physiol Cell Physiol, Jan 2008; 294: C153 - C160.

Cytotoxicity Detection Kit^{PLUS} (LDH)

Cat. No. 04 744 926 001 400 tests
Cat. No. 04 744 934 001 2,000 tests

Type	Colorimetric assay, microplate format
Useful for	Quantitation of LDH activity released from damaged/dying cells
Sample material	Cell cultures grown in 96- or 384-well plates can be measured directly. Aliquots from cultures grown in other formats can be transferred into 96- or 384-well plates for measurement without removing the cells.
Method	The Cytotoxicity Detection Kit^{PLUS} (LDH) can be performed in a homogeneous format and requires no transfer and centrifugation steps to separate the supernatant from the cells. The color reaction can be stopped for defined assay conditions.
Test principle	The culture is incubated with the Catalyst and Dye substrate mixture from the kit. For total cell number counting, the lysis solution is added prior to the substrate reaction. After the reaction, the assay can be terminated by adding the Stop Solution (optimal). The LDH activity is determined by a coupled enzymatic reaction, whereby the tetrazolium salt INT is reduced to formazan. The formazan dye formed is water soluble and shows a broad absorption maximum at approximately 500 nm.



Significance of kit	The Cytotoxicity Detection Kit^{PLUS} (LDH) is a fast, sensitive, and simple method to quantitate cytotoxicity/cytolysis based on the measurement of LDH activity released from damaged cells using the 96-well or 384-well plate format. The kit can be used in many different *in vitro* cell systems when damage to the plasma membrane occurs. For example: ■ 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.
Sensitivity	Less than 100 lysed cells can be detected in a 96-well plate.
Time	10 to 30 minutes for incubation.

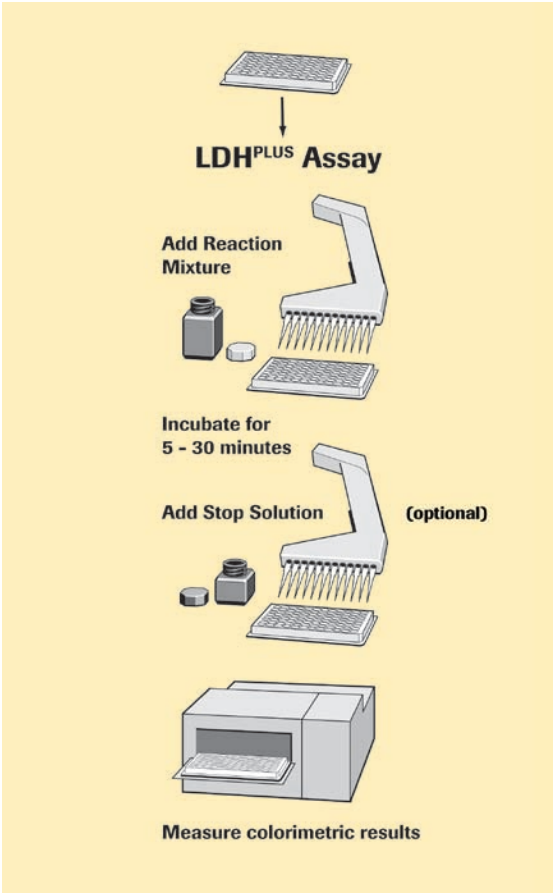
HTS = Suitable for high-throughput screening.

Benefits

- **Perform high-throughput analysis with minimal handling steps** – no transfer, centrifugation, or prelabeling steps are required.
- **Control assay conditions** by using the kit's Stop Solution to terminate the color reaction (optional).
- **Obtain accurate assay results** that strongly correlate to the number of lysed cells.
- **Detect low cell numbers** (<100 cells/well) with excellent linear range and high sensitivity.
- **Save time** by performing the assay in 96- or 384-well plates, enabling simultaneous processing of multiple samples using an ELISA reader.
- **Use the kit in combination with the WST-1 Assay** to obtain more information about cell status (Figure 40).

How to use the kit

I. Assay procedure overview



II. Kit content

1. Catalyst (Diaphorase/NAD^+ mixture)
2. Dye solution (INT and sodium lactate)
3. Lysis solution
4. Stop solution

Typical results with the kit

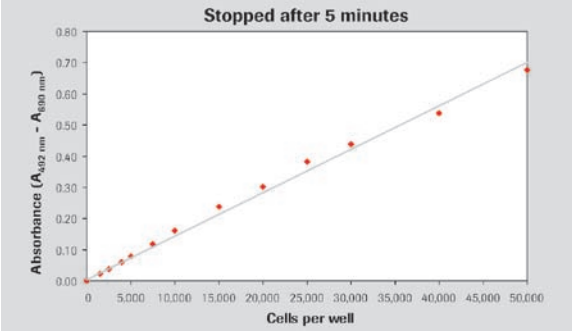


Figure 38: Linear range of the Cytotoxicity Detection Kit^{PLUS} (LDH). The kit was used to determine total LDH activity from different numbers of U-937 cells in whole cell cultures. This figure shows the values after a 5-minute incubation with the substrate mixture from the Cytotoxicity Detection Kit^{PLUS} (LDH).

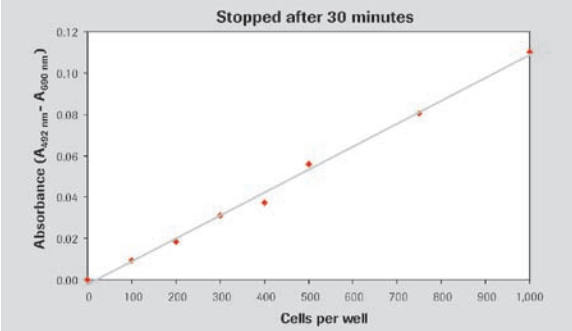


Figure 39: Sensitivity of the Cytotoxicity Detection Kit^{PLUS} (LDH). The kit was used to determine total LDH activity from different numbers of U-937 cells in whole cell cultures. This figure shows the values for cell numbers down to 100 cells per well after 30 minutes of incubation with the substrate.

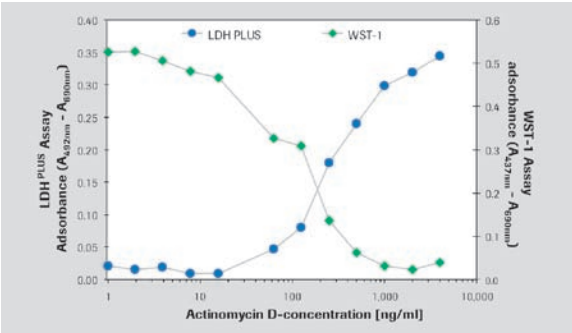


Figure 40: Use of the Cytotoxicity Detection Kit^{PLUS} (LDH) and the Cell Proliferation Reagent WST-1 to study dose response in U-937 cells. Different concentrations of Actinomycin D were added to the cells and cytotoxicity was measured in the culture using both kits.

References

1. Tumor Necrosis Factor Regulates Responses to Nerve Growth Factor, Promoting Neural Cell Survival but Suppressing Differentiation of Neuroblastoma Cells
   Yoshinori Takei and Ronald Laskey
   Mol. Biol. Cell, Mar 2008; 19: 855 - 864.
2. Cyclic Stretch Affects Pulmonary Endothelial Cell Control of Pulmonary Smooth Muscle Cell Growth
   Cristhiaan D Ochoa, Haven Baker, Stephen Hasak, Robina Matyal, Aleya Salam, Charles A Hales, William Hancock, and Deborah A Quinn
   Am. J. Respir. Cell Mol. Biol., Feb 2008; 10.1165/rcmb.2007-0283OC.
3. Utilization of Complement-Dependent Cytotoxicity To Measure Low Levels of Antibodies: Application to Nonstructural Protein 1 in a Model of Japanese Encephalitis Virus
   Eiji Konishi, Yoko Kitai, and Takashi Kondo
   Clin. Vaccine Immunol., Jan 2008; 15: 88 - 94.
4. A Critical Role for System A Amino Acid Transport in the Regulation of Dendritic Development by Brain-derived Neurotrophic Factor (BDNF)
   Julia Burkhalter, Hubert Fiumelli, Jeffrey D. Erickson, and Jean-Luc Martin
   J. Biol. Chem., Feb 2007; 282: 5152 - 5159.

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 41) 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).

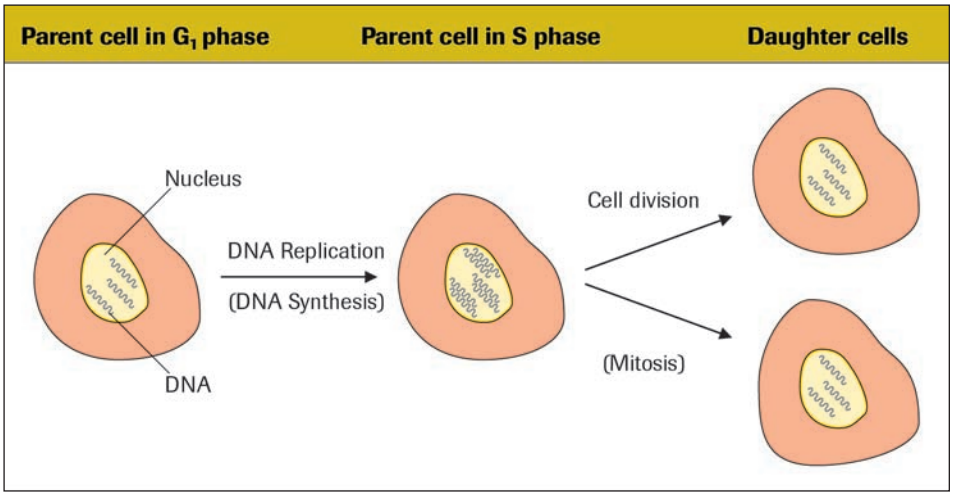


Figure 41: Cell proliferation, a close association between DNA synthesis and cell doubling.

Experiments have shown that the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) is incorporated into cellular DNA like thymidine (Figure 42). The incorporated BrdU could be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies directed against BrdU. 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. Because this method was relatively laborious, the entire BrdU-based procedure was adapted to a 96 well microplate. 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 43).

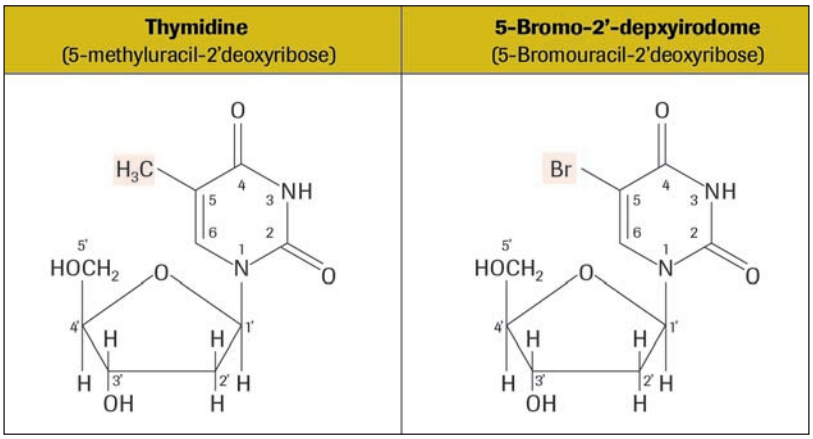


Figure 42: Molecular structure of thymidine and BrdU.

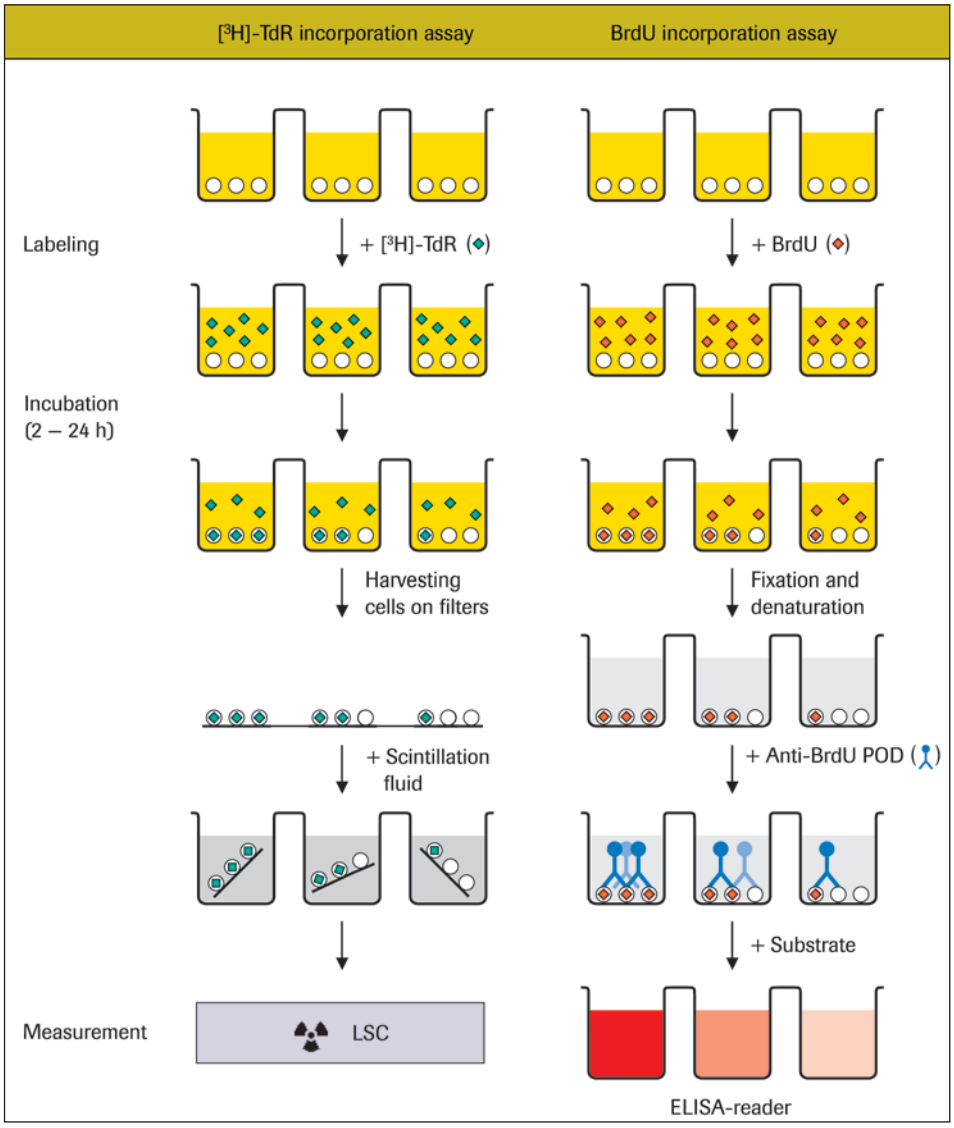


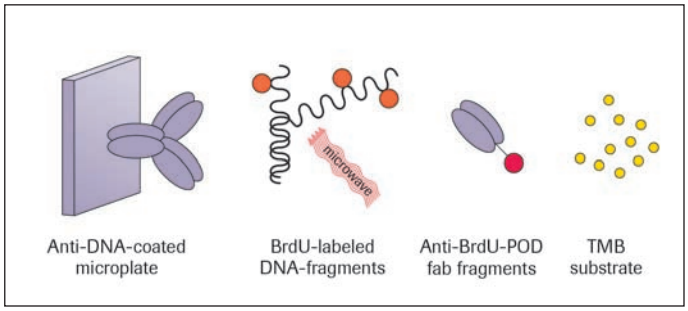
Figure 43: Measurement of DNA synthesis using modified nucleotides [³H]-TdR and BrdU.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
DNA Synthesis	BrdU incorporation	Colorimetric ELISA	**Cellular DNA Fragmentation ELISA**	103

Cellular DNA Fragmentation ELISA

Cat. No. 11 585 045 001 500 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
Sample material	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
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.



Significance of the 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.
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 44). **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. *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.*

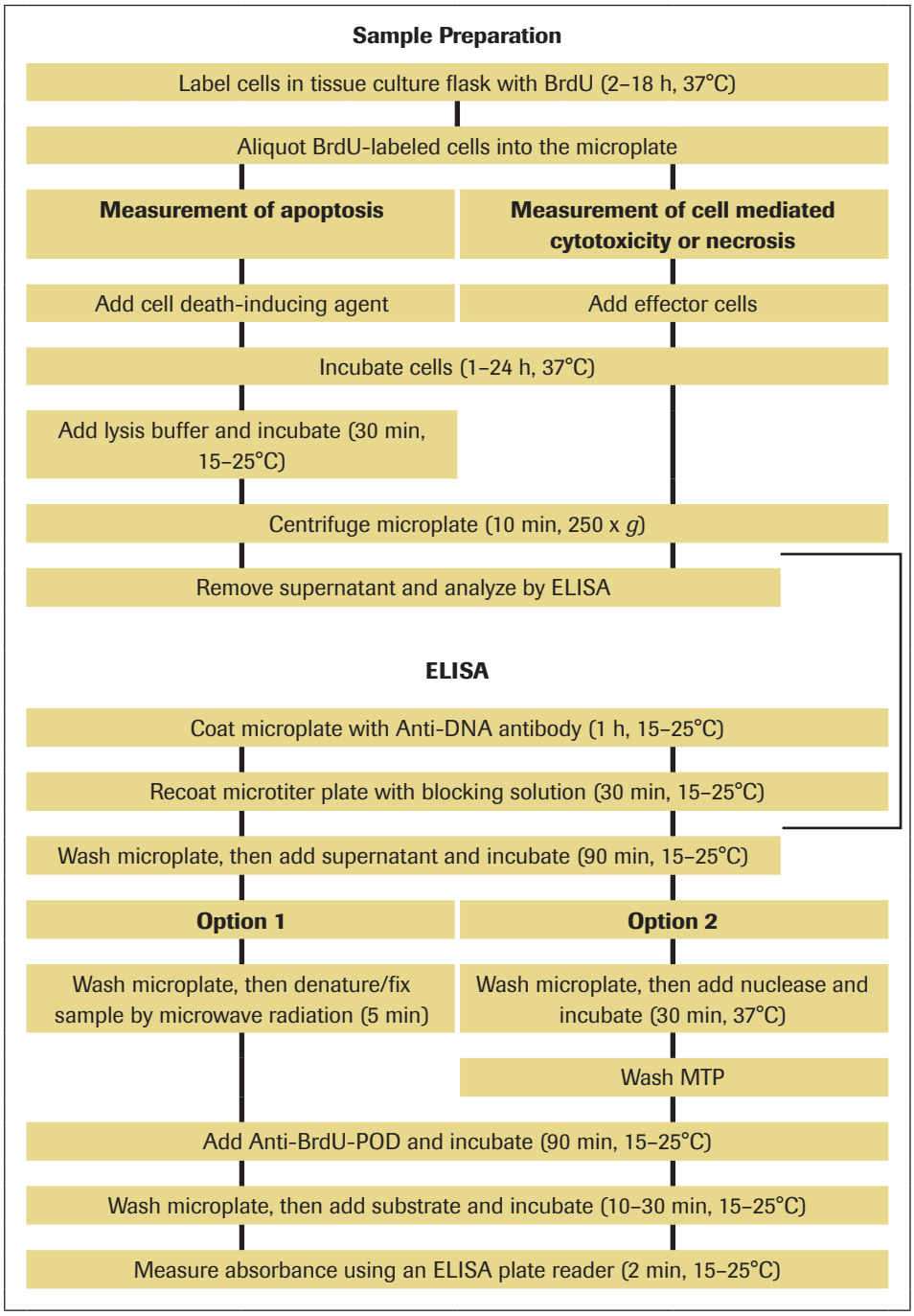
Specificity	■ The Anti-DNA antibody binds to single-and double-stranded DNA. It shows no cross-reactivity with BrdU. ■ The conjugated antibody (Anti-BrdU-POD, Fab fragments) will bind to BrdU-labeled DNA after the DNA is partially denatured. The antibody specifically recognizes 5-bromo-2'-deoxy-uridine. 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.
Time	4.5–5.5 h (+ BrdU labeling and induction of cell death)
Benefits	■ **Measure the amount of BrdU-labeled DNA fragments in cell lysates.** ■ **Quantify BrdU-labeled DNA** released into culture medium through necrosis or cell mediated cytotoxicity. ■ **Compare and quantify** the effects of apoptosis-inducing agents on a population of cells.

How to use the kit

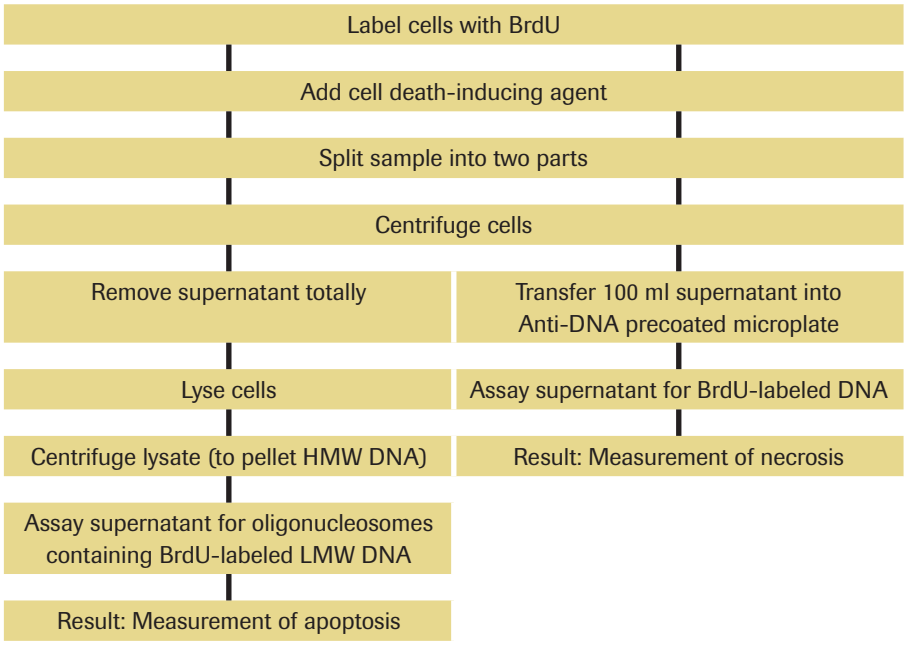
I. Assay procedure overview

The procedure 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.
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).


Sample Preparation
Label cells in tissue culture flask with BrdU (2–18 h, 37°C)
Aliquot BrdU-labeled cells into the microplate
Measurement of apoptosis
Measurement of cell mediated cytotoxicity or necrosis
Add cell death-inducing agent
Add effector cells
Incubate cells (1–24 h, 37°C)
Add lysis buffer and incubate (30 min, 15–25°C)
Centrifuge microplate (10 min, 250 x g)
Remove supernatant and analyze by ELISA
ELISA
Coat microplate with Anti-DNA antibody (1 h, 15–25°C)
Recoat microtiter plate with blocking solution (30 min, 15–25°C)
Wash microplate, then add supernatant and incubate (90 min, 15–25°C)
Option 1
Option 2
Wash microplate, then denature/fix sample by microwave radiation (5 min)
Wash microplate, then add nuclease and incubate (30 min, 37°C)
Wash MTP
Add Anti-BrdU-POD and incubate (90 min, 15–25°C)
Wash microplate, then add substrate and incubate (10–30 min, 15–25°C)
Measure absorbance using an ELISA plate reader (2 min, 15–25°C)

Simultaneous analysis of apoptosis and necrosis in the same sample with the Cellular DNA Fragmentation ELISA.



II. Kit content

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 with the kit

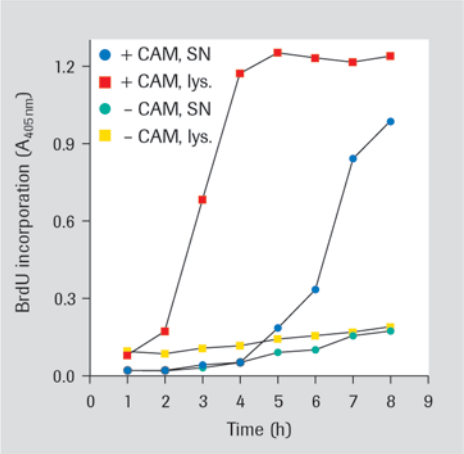


Figure 44: Kinetics of camptothecin (CAM) induced cell death in HL60 cells. Cells were prelabeled with BrdU overnight. Then, cells (1 x 10^4/well) were incubated either in the presence of 200 ng/ml CAM (●, ■) or without CAM (●, ■) for 1–8 h. Supernatant (100 µl/well) was removed, then cells were lysed 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.

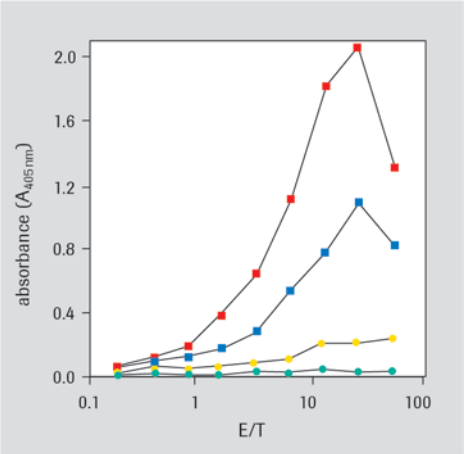


Figure 45: Kinetics of cytotoxic T lymphocyte (CTL)-mediated cytotoxicity in P815 target cells quantified with the Cellular DNA Fragmentation ELISA. 2 x 10^4 BrdU-labeled target cells/well were incubated with CTLs at different effector-to-target ratios (E/T) for varying times. After incubation for 1 h (●), 2 h (●), 4 h (■), and 6 h (■), culture supernatant samples (100 µl/well) were assayed for DNA fragments.

References

1. tPA Protects Renal Interstitial Fibroblasts and Myofibroblasts from Apoptosis
Kebin Hu, Ling Lin, Xiaoyue Tan, Junwei Yang, Guojun Bu, Wendy M. Mars, and Youhua Liu
J. Am. Soc. Nephrol., Mar 2008; 19: 503 - 514
2. Heterozygous disruption of the PTEN promotes intestinal neoplasia in APCmin/+ mouse: roles of osteopontin
Jinyi Shao, M.Kay Washington, Romil Saxena, and Hongmiao Sheng
Carcinogenesis, Dec 2007; 28: 2476 - 2483.
3. Involvement of Heat Shock Protein (Hsp)90 but Not Hsp90 in Antiapoptotic Effect of CpG-B Oligodeoxynucleotide
Cheng-Chin Kuo, Chi-Ming Liang, Chen-Yen Lai, and Shu-Mei Liang
J. Immunol., May 2007; 178: 6100 - 6108.
4. Pioglitazone Induces Apoptosis in Human Vascular Smooth Muscle Cells from Diabetic Patients Involving the Transforming Growth Factor-/Activin Receptor-Like Kinase-4/5/7/Smad2 Signaling Pathway
Emilio Ruiz, Santiago Redondo, Antonio Gordillo-Moscoso, and Teresa Tejerina
J. Pharmacol. Exp. Ther., May 2007; 321: 431 - 438.

5. CpG-B Oligodeoxynucleotide Promotes Cell Survival via Up-regulation of Hsp70 to Increase Bcl-xL and to Decrease Apoptosis-inducing Factor Translocation
Cheng-Chin Kuo, Shu-Mei Liang, and Chi-Ming Liang
J. Biol. Chem., Dec 2006; 281: 38200 - 38207.
6. Interaction of Vascular Endothelial Growth Factor 165 with Neuropilin-1 Protects Rheumatoid Synoviocytes from Apoptotic Death by Regulating Bcl-2 Expression and Bax Translocation
Wan-Uk Kim, Soon Suk Kang, Seung-Ah Yoo, Kyung-Hee Hong, Dong-Goo Bae, Mi-Sook Lee, Seung Woo Hong, Chi-Bom Chae, and Chul-Soo Cho
J. Immunol., Oct 2006; 177: 5727 - 5735.
7. Serum Amyloid A Binding to Formyl Peptide Receptor-Like 1 Induces Synovial Hyperplasia and Angiogenesis
Mi-Sook Lee, Seung-Ah Yoo, Chul-Soo Cho, Pann-Ghill Suh, Wan-Uk Kim, and Sung Ho Ryu
J. Immunol., Oct 2006; 177: 5585 - 5594.
8. Calcineurin Is Expressed and Plays a Critical Role in Inflammatory Arthritis
Seung-Ah Yoo, Bo-Hyoung Park, Gyeong-Sin Park, Hae-Seok Koh, Mi-Sook Lee, Sung Ho Ryu, Keiji Miyazawa, Sung-Hwan Park, Chul-Soo Cho, and Wan-Uk Kim
J. Immunol., Aug 2006; 177: 2681 - 2690.
9. Wood smoke extract induces oxidative stress-mediated caspase-independent apoptosis in human lung endothelial cells: role of AIF and EndoG
Po-Len Liu, Yuh-Lien Chen, Yung-Hsiang Chen, Shing-Jong Lin, and Yu Ru Kou
Am J Physiol Lung Cell Mol Physiol, Nov 2005; 289: L739 - L749.
10. Anti–Prostate Stem Cell Antigen Monoclonal Antibody 1G8 Induces Cell Death In vitro and Inhibits Tumor Growth In vivo via a Fc-Independent Mechanism
Zhennan Gu, Joyce Yamashiro, Evelyn Kono, and Robert E. Reiter
Cancer Res., Oct 2005; 65: 9495 - 9500.

Cell Proliferation – Introduction

Terminology of Cell Proliferation and Viability

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 anti-cancer 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.

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).

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 G0 phase (quiescence) of the cell cycle (Figure 46). The signals that induce cells to divide are diverse and trigger a large number of signal transduction cascades.

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

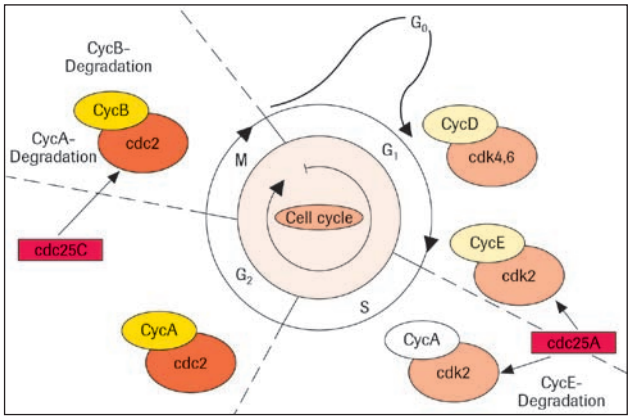


Figure 46: Cell cycle: A schematic overview.

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 exchange 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 46). 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.

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. 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 47). 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.

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 chapter 7 starting on page 92 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 (described in chapter 9 starting on page 116 of this guide).

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 (nonradioactive assays are described in chapter 7 starting on page 72 of this guide)

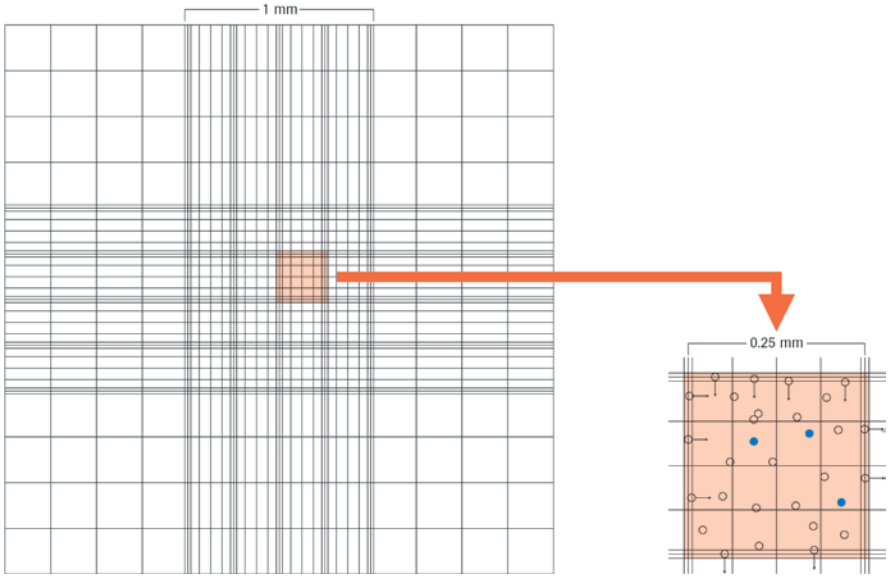
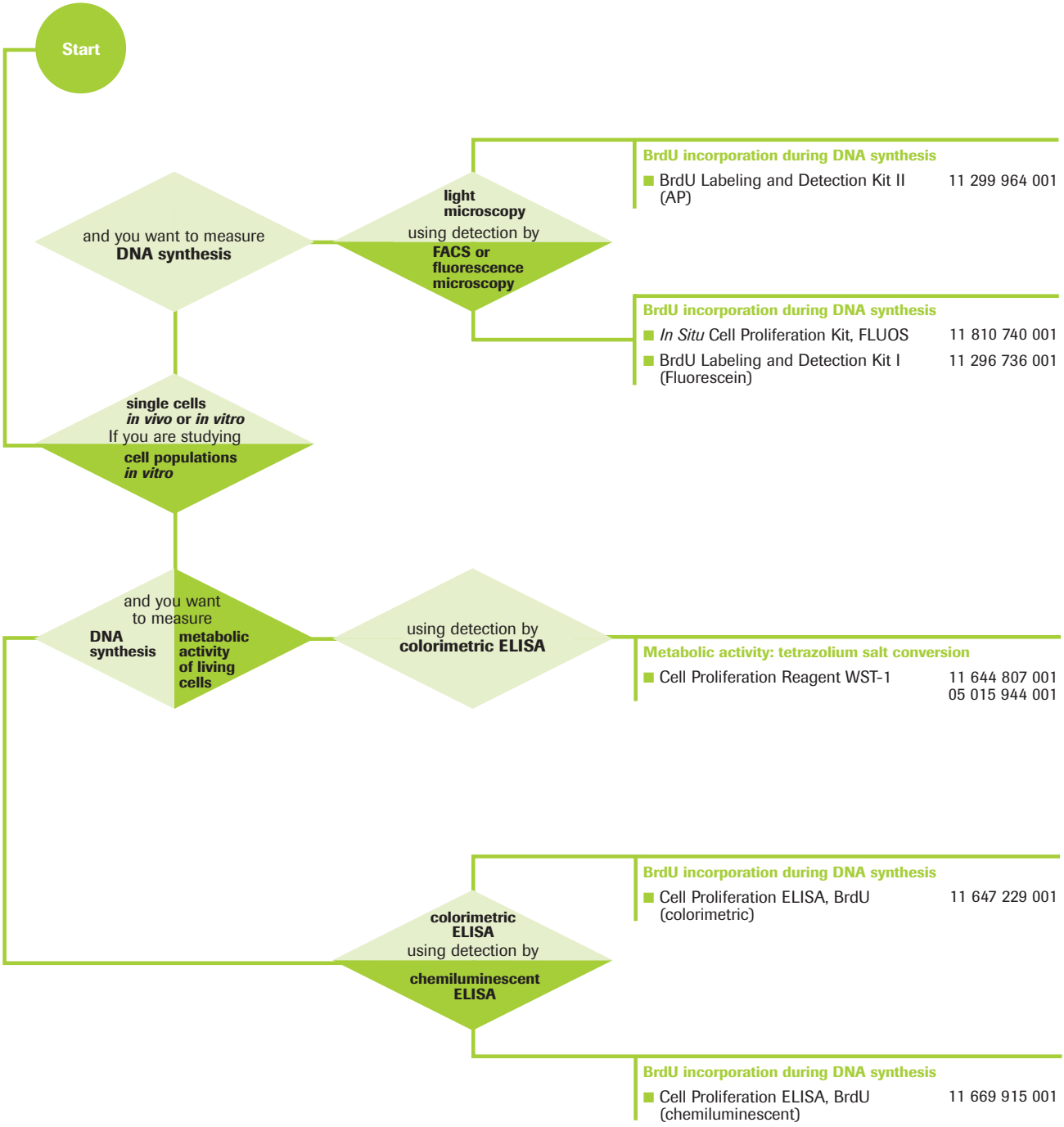


Figure 47: Measurement of proliferation by counting the cells with a hemocytometer. The addition of trypan blue helps to distinguish viable, unstained cells (○) from non-viable, blue-stained cells (●).

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*).

Cell Proliferation Product Selection Guide

Follow the selection guide below to determine the appropriate Roche Applied Science product for the study of **cell proliferation** to meet your needs. If you need additional help, please visit **www.roche-applied-science.com/apoptosis**



Cell Proliferation – Metabolic Activity

Methods for Studying Cell Proliferation and viability in Cell Populations

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 120-127 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 103 of this guide).

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

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.

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.]*

Modified tetrazolium salts like XTT, MTT, and WST-1 (Figure 48) have become available some times ago. The major advantage of these 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.

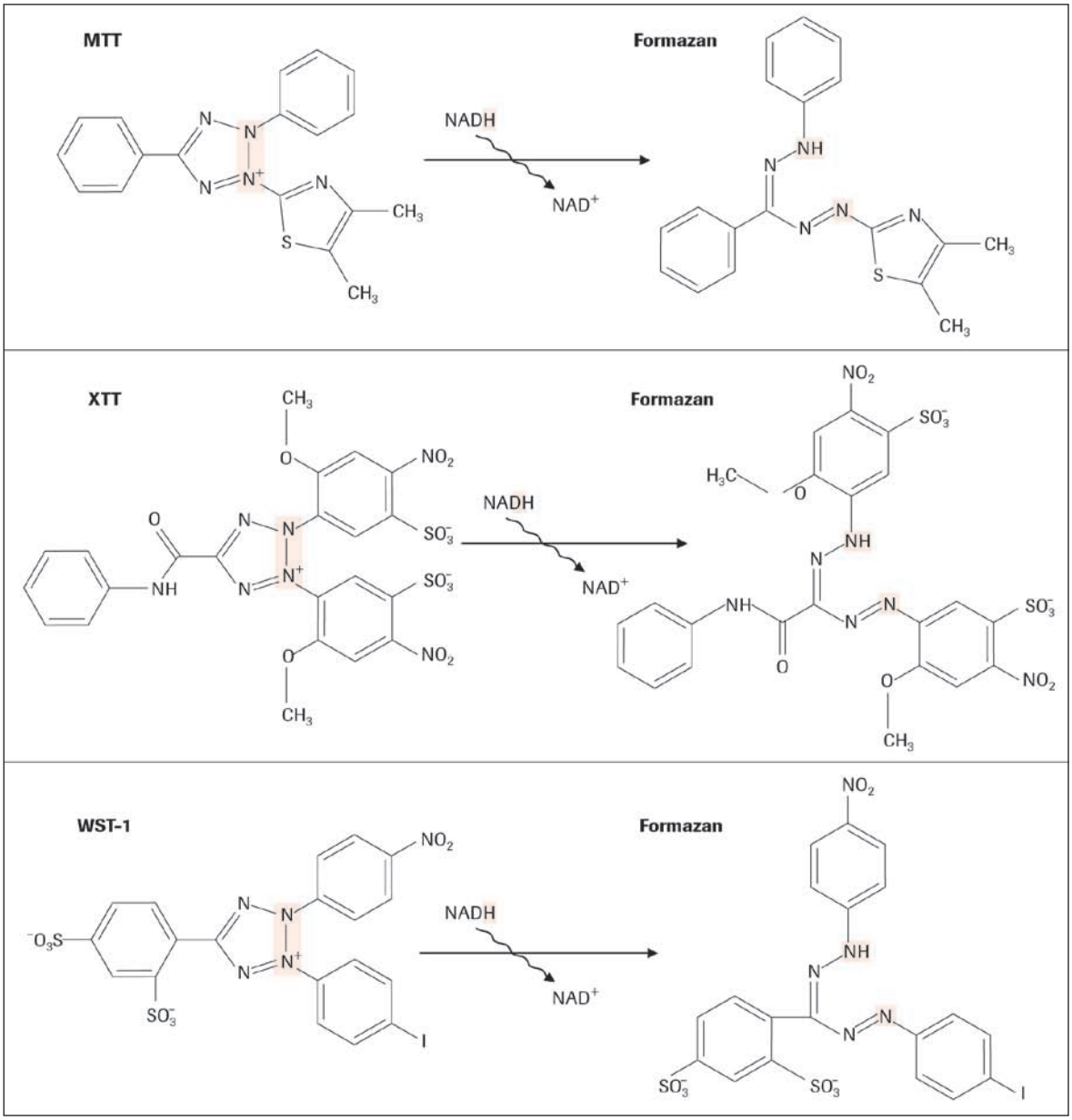


Figure 48: Molecular structure of MTT, XTT, WST-1 and their corresponding reaction products.

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 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).

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 a computer allows rapid and automated data processing (Figure 49).

Roche Applied Science offers three microplate-based assays. 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.

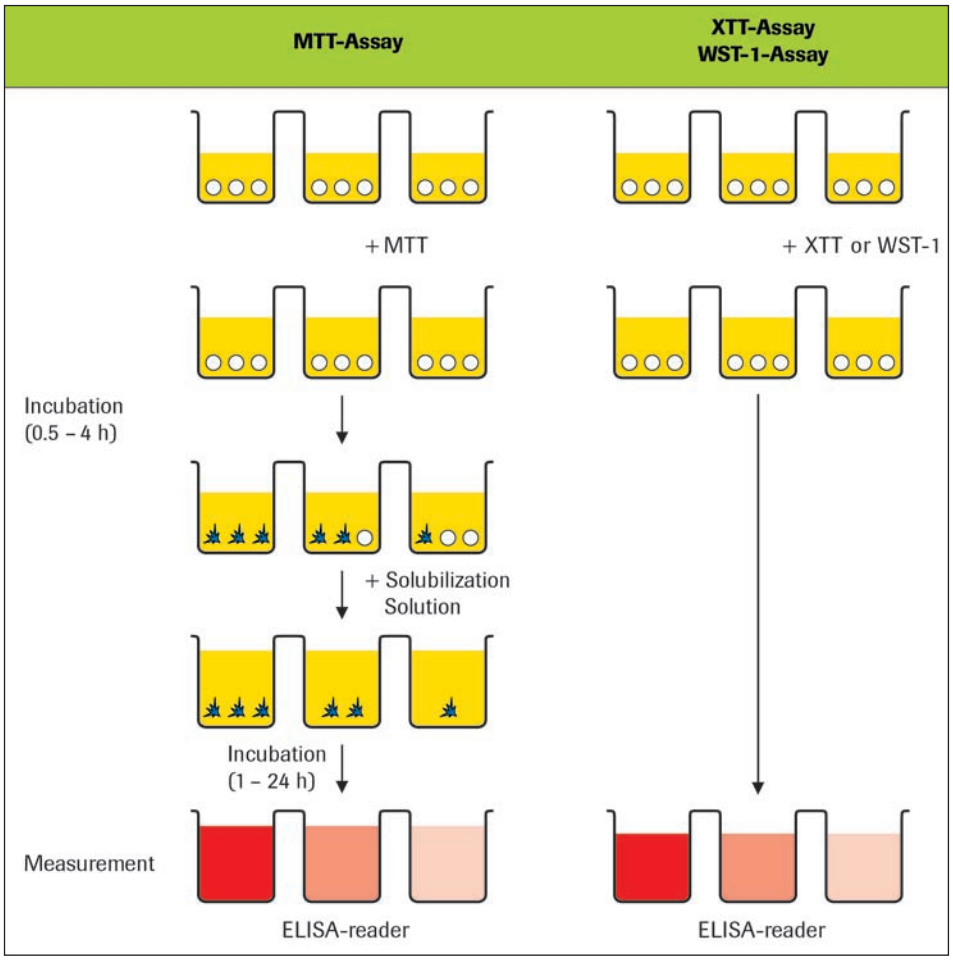
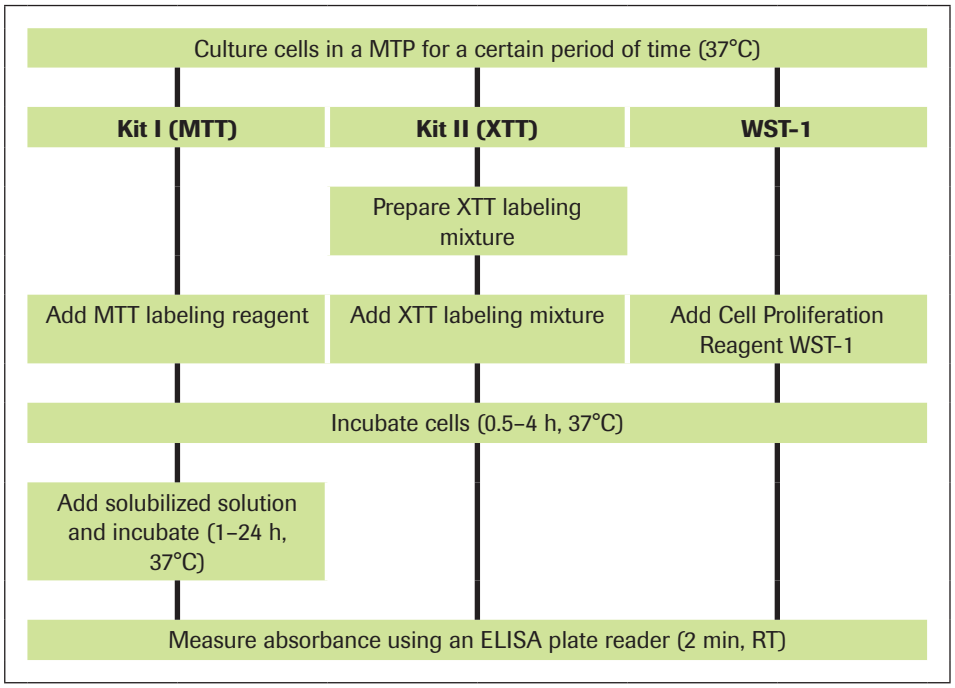


Figure 49: Measurement of metabolic activity using the tetrazolium salts MTT, XTT and WST-1.



Assay procedures for Cell Proliferation Kit I (MTT), Cell Proliferation Kit II (XTT), and Cell Proliferation Reagent WST-1.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
Metabolic Activity	Reduction of tetrazolium salts to formazan salts by viable cells	ELISA	**Cell Proliferation Reagent WST-1***	120
			Cell Proliferation Kit I (MTT)*	124
			Cell Proliferation Kit II (XTT)*	127
	ATP levels	Bioluminescence	**ATP Bioluminescence Assay Kit HS II**	130
			ATP Bioluminescence Assay Kit CLS II	130

* This product can also be used to study cytotoxicity.

Cell Proliferation Reagent WST-1

Cat. No. 05 015 944 001 800 tests
Cat. No. 11 644 807 001 2,500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability, proliferation, or cytotoxicity
Sample material	Adherent or suspension cell cultures
Test principle	The assay is based on the reduction of WST-1 by viable cells. The reaction produces a soluble formazan salt.
Method	Incubation of cells with WST-1, followed by spectrophotometric assay of colored product
Significance of reagent	The Cell Proliferation Reagent WST-1 is a ready-to-use substrate which 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.
Time	0.5-4 h
Benefits	■ **Accurately measure cell proliferation** – assay absorbance strongly correlates to the cell number. ■ **Detect low cell numbers** with higher sensitivity than with MTT and XTT (Figure 52b). ■ **Save time with a one-step assay and a ready-to-use reagent**; no washing steps or additional reagents are required. ■ **Normalize cell numbers** by using WST-1 in combination with reporter gene assays. ■ **Use the kit in combination with the Cytotoxicity Detection Kit^{PLUS} (LDH)** (Figure 40, page 100) to obtain more information.

How to use the reagent

I. Assay procedure overview

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.

II. Reagent content

Ready-to-use WST-1 reagent

III. Protocols for a cell proliferation and a cytotoxicity assay

IIIa. Determination of the cell proliferation activity of human interleukin-2 (IL-2) on the mouse T cell line CTLL-2 using the Cell Proliferation Reagent WST-1

(see Figure 50)

Additional materials needed

- Culture medium, *e.g.*, RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM Na-pyruvate, 1×non-essential amino acids, and 50µM 2-mercaptoethanol.
- Optionally, add Penicillin/Streptomycin or Gentamicin
- Human IL-2 (10,000 U/ml; 5 µg/ml), sterile filtered

1	Seed CTLL-2 cells at a concentration of 4×10^3 cells/well in 100 µl culture medium containing various amounts of IL-2 (final concentration *e.g.*, 0.005–25 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom)
2	Incubate cells for 48 h at 37°C and 5% CO_2.
3	Add 10 µl/well Cell Proliferation Reagent WST-1 and incubate for 4 h at 37°C and 5% CO_2.
4	Shake thoroughly for 1 min on a shaker.
5	Measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader. The wave-length for measuring the absorbance of the formazan product is between 420 – 480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader. The reference wavelength should be more than 600 nm.

IIIb. Determination of the cytotoxic effect of human tumor necrosis factor-α(TNF-α) on the mouse fibrosarcoma cell line WEHI-164 using the Cell Proliferation Reagent WST-1

(see Figure 51)

Additional materials needed

- Culture medium, *e.g.*, RPMI 1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, and actinomycin C1 (actinomycin D), 1µg/ml.
- Optionally, add Penicillin/Streptomycin or Gentamicin.
- Human TNF-α (10 µg/ml), sterile filtered.

1	Culture cells in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium in a humidified atmosphere (*e.g.*, 37°C, 5% CO_2).
2	Seed cells at a concentration of 5×10^4 cells/well in 100 µl culture medium containing actinomycin C1 (1 g/ml) and various amounts of TNF-α (final concentration *e.g.*, 0.001–0.5 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
3	Incubate cell cultures for 24 h at 37°C and 5% CO_2.
4	Add 10 µl Cell Proliferation Reagent WST-1 and incubate for 4 h at 37°C and 5% CO_2.
5	Shake thoroughly for 1 min on a shaker.
6	Measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader. The wave-length for measuring the absorbance of the formazan product is between 420 – 480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader. The reference wavelength should be more than 600 nm.

Typical results with the reagent

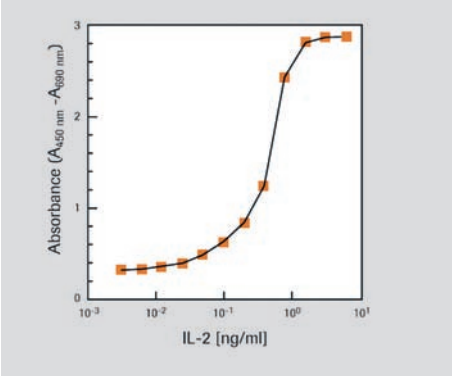


Figure 50: Use of WST-1 to measure proliferation of CTLL-2 cells in response to human interleukin-2 (hIL-2).

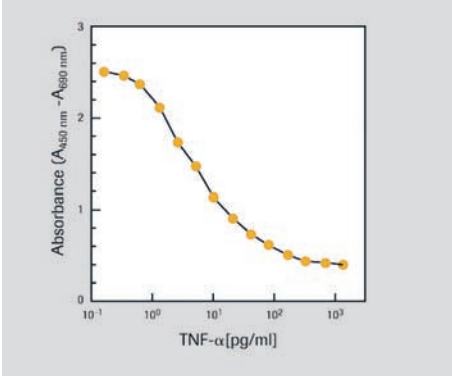


Figure 51: Use of WST-1 to determine the cytotoxic activity of human tumor necrosis factor-α (TNF-α) on WEHI-164 cells.

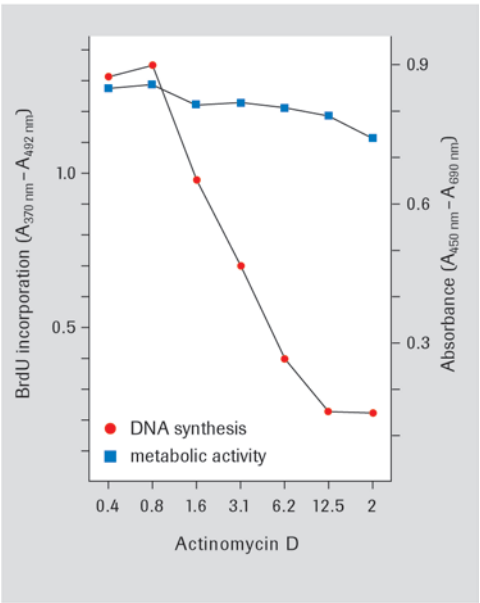


Figure 52a: Combined use of the Cell Proliferation Reagent WST-1 and the Cell Proliferation ELISA, BrdU (colorimetric) for the simultaneous measurement of cell viability and cell proliferation. A549 cells (1 x 10^4/well in 100 µl) were incubated in the presence of various amounts of actinomycin D for 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).

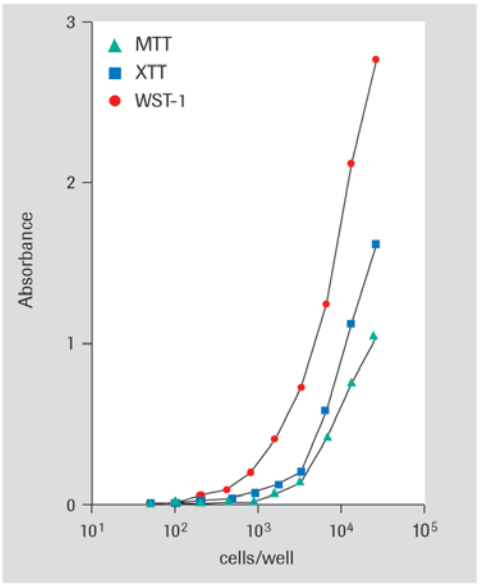


Figure 52b: Comparison of the sensitivity of various tetrazolium salts. P815 cells were preincubated at various concentrations for 20 h before MTT (▲), XTT (■) or Cell Proliferation Reagent WST-1 (●) was added. After 4 h substrate reaction, the absorbance was determined at the respective wavelength with an ELISA plate reader.

References

1. Extracellular Superoxide Dismutase Inhibits Inflammation by Preventing Oxidative Fragmentation of Hyaluronan
   Fei Gao, Jeffrey R. Koenitzer, Jacob M. Tobolewski, Dianhua Jiang, Jiurong Liang, Paul W. Noble, and Tim D. Oury
   J. Biol. Chem., Mar 2008; 283: 6058 - 6066.
2. Tumor Necrosis Factor Regulates Responses to Nerve Growth Factor, Promoting Neural Cell Survival but Suppressing Differentiation of Neuroblastoma Cells
   Yoshinori Takei and Ronald Laskey
   Mol. Biol. Cell, Mar 2008; 19: 855 - 864.
3. SLC24A5 Encodes a trans-Golgi Network Protein with Potassium-dependent Sodium-Calcium Exchange Activity That Regulates Human Epidermal Melanogenesis
   Rebecca S. Ginger, Sarah E. Askew, Richard M. Ogborne, Stephen Wilson, Dudley Ferdinando, Tony Dadd, Adrian M. Smith, Shubana Kazi, Robert T. Szerencsei, Robert J. Winkfein, Paul P. M. Schnetkamp, and Martin R. Green
   J. Biol. Chem., Feb 2008; 283: 5486 - 5495.
4. Targeting the p27 E3 ligase SCFSkp2 results in p27- and Skp2-mediated cell cycle arrest, and activation of autophagy
   Qing Chen, Weilin Xie, Deborah J. Kuhn, Peter M. Voorhees, Antonia Lopez-Girona, Derek Mendy, Laura G. Corral, Veronique Plantevin Krenitsky, Weiming Xu, Laure Moutouh-de Parseval, David R. Webb, Frank Mercurio, Keiichi I. Nakayama, Keiko Nakayama, and Robert Z Orlowski
   Blood, Feb 2008; 10.1182/blood-2007-09-112904.
5. Theiler's virus-induced intrinsic apoptosis in M1-D macrophages is Bax-mediated and restricts virus infectivity: A mechanism for persistence of a cytolytic virus
   Kyung-No Son, Bob Becker, Patricia Kallio, and Howard L. Lipton
   J. Virol., Feb 2008; 10.1128/JVI.02349-07.
6. The 44-kDa Pim-1 Kinase Phosphorylates BCRP/ABCG2 and Thereby Promotes Its Multimerization and Drug-resistant Activity in Human Prostate Cancer Cells
   Yingqiu Xie, Kexin Xu, Douglas E. Linn, Xi Yang, Zhiyong Guo, Hermela Shimelis, Takeo Nakanishi, Douglas D. Ross, Hegang Chen, Ladan Fazli, Martin E. Gleave, and Yun Qiu
   J. Biol. Chem., Feb 2008; 283: 3349 - 3356.
7. Krüppel-Like Factor 4 Is a Novel Mediator of Selenium in Growth Inhibition
   Shuang Liu, Haitao Zhang, Liyu Zhu, Lijuan Zhao, and Yan Dong
   Mol. Cancer Res., Feb 2008; 6: 306 - 313.
8. Identification of phosphorylation sites in mammalian mitochondrial ribosomal protein DAP3
   Jennifer L. Miller, Hasan Koc, and Emine C. Koc
   Protein Sci., Feb 2008; 17: 251 - 260.
9. Enhancement of cell-type specificity by quantitative modulation of a chimeric ligand
   Pablo Cironi, Ian A. Swinburne, and Pamela A. Silver
   J. Biol. Chem., Jan 2008; 10.1074/jbc.M708502200.
10. Cyclooxygenase-2–Derived Prostaglandin E2 Activates β-Catenin in Human Cholangiocarcinoma Cells: Evidence for Inhibition of These Signaling Pathways by 3 Polyunsaturated Fatty Acids
    Kyu Lim, Chang Han, Lihong Xu, Kumiko Isse, Anthony J. Demetris, and Tong Wu
    Cancer Res., Jan 2008; 68: 553 - 560.

Cell Proliferation Kit I (MTT)

Cat. No. 11 465 007 001 2,500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability, proliferation, or cytotoxicity
Sample material	Adherent or suspension cell cultures
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.
Method	Incubation of cells with MTT, followed by solubilization and spectrophotometric assay of colored product
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.
Time	5-28 h
Benefits	■ **Rapidly measure cell proliferation** in response to growth factors, cytokines, mitogens, and nutrients. ■ **Conserve resources** – no washing steps or additional reagents are required. ■ **Analyze cytotoxic and cytostatic compounds**, such as anti-cancer drugs and other pharmaceutical compounds. ■ **Assess growth-inhibitory antibodies and physiological mediators.** ■ **Save time** – perform the entire assay in one microplate.

How to use the kit

I. Assay procedure overview

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.

II. Kit content

1. MTT labeling reagent
2. Solubilization solution

III. Protocol for a cell proliferation assay

IIIa. Determination of human interleukin-6 (IL-6) activity on 7TD1 cells (mouse-mouse hybridoma) using the Cell Proliferation Kit I (MTT)

(see Figure 53)

Additional materials needed

- Culture medium, *e.g.* DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate, 50 µM 2-mercaptoethanol, HT-media supplement (1×), containing 0.1 mM hypoxanthine and 16 µM thymidine. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin
- Interleukin-6, human (hIL-6) (200000 U/ml, 2 µg/ml)

1	Seed 7TD1 cells at a concentration of 2 × 10^3 cells/well in 100 µl culture medium containing various amounts of IL-6 [final concentration *e.g.* 0.1–10 U/ml (0.001–0.1 ng/ml)] into microplates (tissue culture grade, 96 wells, flat bottom).
2	Incubate cell cultures for 4 days at 37°C and 6.5% CO_2.
3	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
4	Incubate the microplate for 4 h in a humidified atmosphere (*e.g.* 37°C, 6.5% CO_2).
5	Add 100 µl of the Solubilization solution into each well.
6	Allow the plate to stand overnight in the incuba-tor in a humidified atmosphere (*e.g.* 37°C, 6.5% CO_2).
7	Check for complete solubilization of the purple formazan crystals and measure the spectropho-tometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

Typical results with the kit

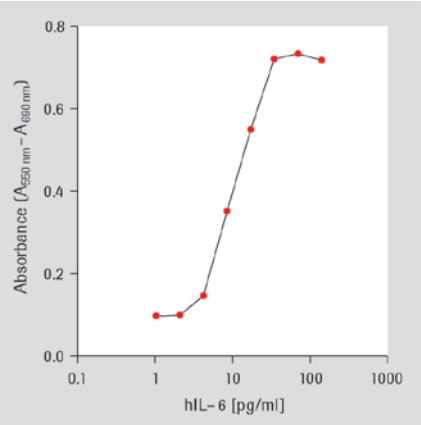


Figure 53: Use of the Cell Proliferation Kit I (MTT) to measure human Interleukin 6 (hIL-6) activity on the mouse hybridoma cell line 7TD1. Cells (2 x 10^3/well) were incubated in the presence of various amounts of hIL-6. After 4 days of incubation, cell proliferation was analyzed by Cell Proliferation Kit I (MTT).

References

1. Plitidepsin Has a Dual Effect Inhibiting Cell Cycle and Inducing Apoptosis via Rac1/c-Jun NH2-Terminal Kinase Activation in Human Melanoma Cells
   María J. Muñoz-Alonso, Laura González-Santiago, Natasha Zarich, Teresa Martínez, Enrique Alvarez, José María Rojas, and Alberto Muñoz
   J. Pharmacol. Exp. Ther., Mar 2008; 324: 1093 - 1101
2. JC Virus Agnoprotein Inhibits *In Vitro* Differentiation of Oligodendrocytes and Promotes Apoptosis
   Nana Merabova, Dorota Kaniowska, Rafal Kaminski, Satish L. Deshmane, Martyn K. White, Shohreh Amini, Armine Darbinyan, and Kamel Khalili
   J. Virol., Feb 2008; 82: 1558 - 1569.
3. Endosialin (Tem1) Is a Marker of Tumor-Associated Myofibroblasts and Tumor Vessel-Associated Mural Cells
   Sven Christian, Renate Winkler, Iris Helfrich, Anja M. Boos, Eva Besemfelder, Dirk Schadendorf, and Hellmut G. Augustin
   Am. J. Pathol., Feb 2008; 172: 486 - 494
4. Prostaglandin E2-Mediated Migration of Human Trophoblast Requires RAC1 and CDC42
   Catalin Nicola, Peeyush K. Lala, and Chandan Chakraborty
   Biol Reprod, Jan 2008; 10.1095/biolreprod.107.065433.
5. Glial Dysfunction in Parkin Null Mice: Effects of Aging
   Rosa M. Solano, Maria J. Casarejos, Jamie Menéndez-Cuervo, Jose A. Rodriguez-Navarro, Justo García de Yébenes, and Maria A. Mena
   J. Neurosci., Jan 2008; 28: 598 - 611.
6. Deficient TP53 Expression, Function, and Cisplatin Sensitivity Are Restored by Quinacrine in Head and Neck Cancer
   Jay Friedman, Liesl Nottingham, Praveen Duggal, Francisco G. Pernas, Bin Yan, Xin Ping Yang, Zhong Chen, and Carter Van Waes
   Clin. Cancer Res., Nov 2007; 13: 6568 - 6578.
7. Lymphoma Chemovirotherapy: CD20-Targeted and Convertase-Armed Measles Virus Can Synergize with Fludarabine
   Guy Ungerechts, Christoph Springfeld, Marie E. Frenzke, Johanna Lampe, Patrick B. Johnston, William B. Parker, Eric J. Sorscher, and Roberto Cattaneo
   Cancer Res., Nov 2007; 67: 10939 - 10947
8. Low-dose arsenic trioxide sensitizes glucocorticoid-resistant acute lymphoblastic leukemia cells to dexamethasone via an Akt-dependent pathway
   Beat C. Bornhauser, Laura Bonapace, Dan Lindholm, Rodrigo Martinez, Gunnar Cario, Martin Schrappe, Felix K. Niggli, Beat W. Schäfer, and Jean-Pierre Bourquin
   Blood, Sep 2007; 110: 2084 - 2091
9. Activation of Targeted Necrosis by a p53 Peptide: A NOVEL DEATH PATHWAY THAT CIRCUMVENTS APOPTOTIC RESISTANCE
   Richard D. Dinnen, Lisa Drew, Daniel P. Petrylak, Yuehua Mao, Nicholas Cassai, Joseph Szmulewicz, Paul Brandt-Rauf, and Robert L. Fine
   J. Biol. Chem., Sep 2007; 282: 26675 - 26686.
10. ALL1 fusion proteins induce deregulation of EphA7 and ERK phosphorylation in human acute leukemias
    Hiroshi Nakanishi, Tatsuya Nakamura, Eli Canaani, and Carlo M. Croce
    PNAS, Sep 2007; 104: 14442 - 14447

Cell Proliferation Kit II (XTT)

Cat. No. 11 465 015 001 2,500 tests

Type	Colorimetric, microplate format
Useful for	Quantitation of cell viability, proliferation, or cytotoxicity
Sample material	Adherent or suspension cell cultures
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.
Method	Incubation of cells with MTT, followed by solubilization and spectrophotometric assay of colored product
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.
Time	4 h
Benefits	■ **Rapidly measure cell proliferation** in response to growth factors, cytokines, mitogens, and nutrients. ■ **Conserve resources** – no washing steps or additional reagents are required. ■ **Analyze cytotoxic and cytostatic compounds**, such as anti-cancer drugs and other pharmaceutical compounds. ■ **Assess growth-inhibitory antibodies and physiological mediators.** ■ **Save time** – perform the entire assay in one microplate.

How to use the kit

I. Assay procedure overview

The procdure involves:

1	Culturing the cells in a 96-well microplate, then incubating them with XTT solution for 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.

II. Kit content

1. XTT Labeling reagent
2. Electron-coupling reagent

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

III. Protocol for a cytotoxicity assay

IIIa. Determination of the cytotoxic effect of human tumor necrosis factor-α (hTNF-α) on WEHI-164 cells (mouse fibrosarcoma) using the Cell Proliferation Kit II (XTT)

(see Figure 54)

Additional materials needed

- Culture medium, *e.g.*, RPMI 1640 containing 10% heat inactivated FCS (fetal calf serum), 2 mM Lglutamine, and actinomycin C1 (actinomycin D), 1 µg/ml. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin.
- Tumor necrosis-factor-α, human (hTNF-α) (10 µg/ml), sterile

1	Preincubate WEHI-164 cells at a concentration of 1× 10^6 cells/ml in culture medium with actino-mycin C1, 1 mg/ml for 3 h at 37°C and 6.5% CO_2.
2	Seed cells at a concentration of 5 × 10^4 cells/well in 100 µl culture medium containing actinomy-cin C1 (1 mg/ml) and various amounts of hTNF-α(final concentration *e.g.*, 0.001–0.5 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
3	Incubate cell cultures for 24 h at 37°C and 6.5% CO_2.
4	Add 50 µl XTT labeling mixture and incubate for 18 h at 37°C and 6.5% CO_2.
5	Measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 450 and 500 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

Typical results with the kit

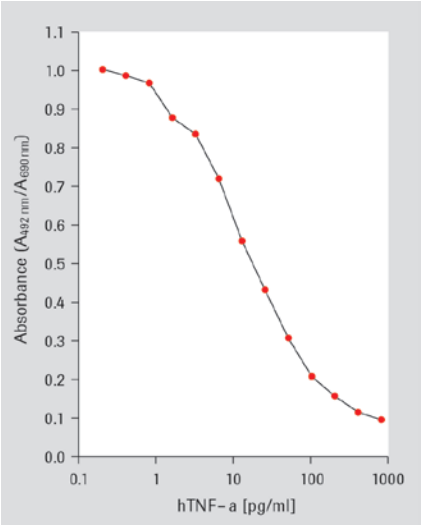


Figure 54: Use of the Cell Proliferation Kit II (XTT) to measure human tumor necrosis factor α (hTNF-α) activity on the mouse fibrosarcoma cell line WEHI-164. After preincubation of the cells (1 x 10^6/ml) with Actinomycin C (1 µg/ml) for 3 hours, cells (5 x 10^4/well) were incubated in the presence of Actinomycin C and various amounts of TNF-α for 24 hours. The cellular response was analyzed by Cell Proliferation Kit II (XTT).

References

1. Loss of CHK1 function impedes DNA damage-induced FANCD2 monoubiquitination but normalizes the abnormal G2 arrest in Fanconi anemia
Jean-Hugues Guervilly, Gaëtane Macé-Aimé, and Filippo Rosselli
Hum. Mol. Genet., Mar 2008; 17: 679 - 689.
2. Insulin-Like Growth Factor Type-I Receptor-Dependent Phosphorylation of Extracellular Signal-Regulated Kinase 1/2 but not Akt (Protein Kinase B) Can Be Induced by Picropodophyllin
Radu Vasilcanu, Daiana Vasilcanu, Bita Sehat, Shucheng Yin, Ada Girnita, Magnus Axelson, and Leonard Girnita
Mol. Pharmacol., Mar 2008; 73: 930 - 939
3. Heart failure drug digitoxin induces calcium uptake into cells by forming transmembrane calcium channels
Nelson Arispe, Juan Carlos Diaz, Olga Simakova, and Harvey B. Pollard
PNAS, Feb 2008; 105: 2610 - 2615.
4. Effect of Low Doses of Red Wine and Pure Resveratrol on Circulating Endothelial Progenitor Cells
Maria Luisa Balestrieri, Concetta Schiano, Francesca Felice, Amelia Casamassimi, Antonio Balestrieri, Lara Milone, Luigi Servillo, and Claudio Napoli
J. Biochem., Feb 2008; 143: 179 - 186.
5. Evaluation of Two Types of Sponges Used To Collect Cervical Secretions and Assessment of Antibody Extraction Protocols for Recovery of Neutralizing Anti-Human Papillomavirus Type 16 Antibodies
Troy J. Kemp, Allan Hildesheim, Roni T. Falk, John T. Schiller, Douglas R. Lowy, Ana Cecilia Rodriguez, and Ligia A. Pinto
Clin. Vaccine Immunol., Jan 2008; 15: 60 - 64
6. The Cell-Selective Neurotoxicity of the Alzheimer's Aβ Peptide Is Determined by Surface Phosphatidylserine and Cytosolic ATP Levels. Membrane Binding Is Required for Aβ Toxicity
Olga Simakova and Nelson J. Arispe
J. Neurosci., Dec 2007; 27: 13719 - 13729.
7. Anti-hepatitis C Virus Activity of Tamoxifen Reveals the Functional Association of Estrogen Receptor with Viral RNA Polymerase NS5B
Koichi Watashi, Daisuke Inoue, Makoto Hijikata, Kaku Goto, Hussein H. Aly, and Kunitada Shimotohno
J. Biol. Chem., Nov 2007; 282: 32765 - 32772
8. Identification of Acetylated, Tetrahalogenated Benzimidazole D-Ribonucleosides with Enhanced Activity against Human Cytomegalovirus
Jae-Seon Hwang, Oliver Kregler, Rita Schilf, Norbert Bannert, John C. Drach, Leroy B. Townsend, and Elke Bogner
J. Virol., Nov 2007; 81: 11604 - 11611.
9. Farnesol-Induced Apoptosis in Human Lung Carcinoma Cells Is Coupled to the Endoplasmic Reticulum Stress Response
Joung Hyuck Joo, Grace Liao, Jennifer B. Collins, Sherry F. Grissom, and Anton M. Jetten
Cancer Res., Aug 2007; 67: 7929 - 7936
10. Hypoxia-Inducible Factor Linked to Differential Kidney Cancer Risk Seen with Type 2A and Type 2B VHL Mutations
Lianjie Li, Liang Zhang, Xiaoping Zhang, Qin Yan, Yoji Andrew Minamishima, Aria F. Olumi, Mao Mao, Steven Bartz, and William G. Kaelin, Jr.
Mol. Cell. Biol., Aug 2007; 27: 5381 - 5392.

ATP Bioluminescence Assay Kit HS II

Cat. No. 11 699 709 001 1,000 assays (microplate), 500 assays (tube)

ATP Bioluminescence Assay Kit CLS II

Cat. No. 11 699 695 001 1,600 assays (microplate), 800 assays (tube)

Type	Tube and microplate format using a luminometer
Useful for	Sensitive and quantitative detection of ATP
Sample material	Eukaryotic and prokaryotic cells
Test principle	The luciferase from *Photinus pyralis* (American firefly) catalyzes the following reaction: ATP + D-luciferin + O_2 → oxyluciferin + PP_i + AMP + CO_2 + light. The quantum yield for this reaction is about 90%. The resulting green light has an emission maximum at 562 nm. The Michaelis equation has the following form: light intensity = $(V_{max} \times C_{ATP})/(K_m + C_{ATP})$. At low ATP concentrations ($C_{ATP} < K_m$), the formula is simplified to light intensity = $V_{max} \times C_{ATP}/K_m$. From this equation, it becomes obvious that the light output is directly proportional to the ATP concentration (C_{ATP}), and is dependent on the amount of luciferase (V_{max}) present in the assay. Therefore, for maximum sensitivity, the sample ATP must be in a minimum volume, and the luciferase reagent must not be diluted.
Method	Measurement of increased ATP level
Significance of kits	The **ATP Bioluminescence Assay Kit CLS II** is specially developed for highly sensitive and quantitative determination of ATP. Contains a cell lysis reagent and can be applied for the detection of microbial contamination. The **ATP Bioluminescence Assay Kit CLS II** exhibits a constant light signal that is sustained for several minutes. Therefore the kit is well suited for kinetic studies and ATP determinations in coupled enzymatic reactions.
Specificity	The **ATP Bioluminescence Assay Kit HS II** is specially developed for the highest-sensitivity detection of ATP. Due to the high concentration of luciferase in the assay, the reaction exhibits a peak kinetic (Figure 55 and 56). The **ATP Bioluminescence Assay Kit CLS II** is specially developed for applications in which constant light signals are required for kinetic studies of enzymes and metabolic studies, or if coupled enzymatic assays are applied. If ATP determinations are manually started, the CLS Kit provides high reproducibility due to the constant signal generation (Figure 55 and 56).
Benefits	■ **Measure cell proliferation using a well-established technique** – determination of ATP using bioluminescence. ■ **Choose from two formats** to meet your application needs. ■ **Detect extremely low concentrations of ATP.** ■ **Study adherent and suspension cells** cultured in 96-well microplates.

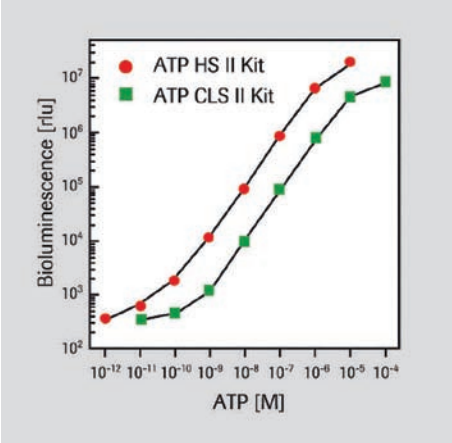


Figure 55: Sensitivity range of the ATP Bioluminescence Assay Kit HS II and the ATP Bioluminescence Assay Kit CLS II.

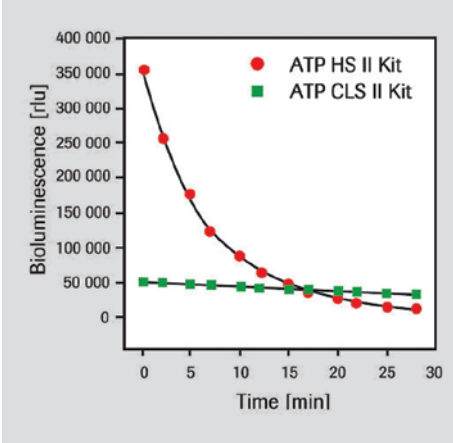


Figure 56: Kinetics of light generation of the ATP Bioluminescence Assay Kit HS II and the ATP Bioluminescence Assay Kit CLS II. ATP (10 pmol) in a volume of 50 µl was assayed with 50 µl luciferase reagent in a black microplate.

References

ATP Bioluminescence Assay Kit HS II

1. Human Immunodeficiency Virus Type 1 Vpr Inhibits Axonal Outgrowth through Induction of Mitochondrial Dysfunction
   Hiroko Kitayama, Yoshiharu Miura, Yoshinori Ando, Shigeki Hoshino, Yukihito Ishizaka, and Yoshio Koyanagi
   J. Virol., Mar 2008; 82: 2528 - 2542
2. Succinate Ameliorates Energy Deficits and Prevents Dysfunction of Complex I in Injured Renal Proximal Tubular Cells
   Grazyna Nowak, Ginger L. Clifton, and Diana Bakajsova
   J. Pharmacol. Exp. Ther., Mar 2008; 324: 1155 - 1162.
3. Loss of Macroautophagy Promotes or Prevents Fibroblast Apoptosis Depending on the Death Stimulus
   Yongjun Wang, Rajat Singh, Ashish C. Massey, Saul S. Kane, Susmita Kaushik, Taneisha Grant, Youqing Xiang, Ana Maria Cuervo, and Mark J. Czaja
   J. Biol. Chem., Feb 2008; 283: 4766 - 4777.
4. Mitochondrial ND5 Gene Variation Associated with Encephalomyopathy and Mitochondrial ATP Consumption
   Matthew McKenzie, Danae Liolitsa, Natalya Akinshina, Michelangelo Campanella, Sanjay Sisodiya, Ian Hargreaves, Niranjanan Nirmalananthan, Mary G. Sweeney, Patrick M. Abou-Sleiman, Nicholas W. Wood, Michael G. Hanna, and Michael R. Duchen
   J. Biol. Chem., Dec 2007; 282: 36845 - 36852.
5. Functional Analysis of the Mycoplasma genitalium MG312 Protein Reveals a Specific Requirement of the MG312 N-Terminal Domain for Gliding Motility
   Raul Burgos, Oscar Q. Pich, Enrique Querol, and Jaume Piñol
   J. Bacteriol., Oct 2007; 189: 7014 - 7023.
6. Targeted Deletion of Ectonucleoside Triphosphate Diphosphohydrolase 1/CD39 Leads to Desensitization of Pre- and Postsynaptic Purinergic P2 Receptors
   Ulrich Schaefer, Takuji Machida, M. Johan Broekman, Aaron J. Marcus, and Roberto Levi
   J. Pharmacol. Exp. Ther., Sep 2007; 322: 1269 - 1277.

7. A new promoterless reporter vector reveals antisense transcription in Mycoplasma genitalium
Maria Lluch-Senar, Miquel Vallmitjana, Enrique Querol, and Jaume Piñol
Microbiology, Aug 2007; 153: 2743 - 2752.
8. Modulation of Sympathetic Activity by Tissue Plasminogen Activator Is Independent of Plasminogen and Urokinase
Ulrich Schaefer, Sandra Vorlova, Takuji Machida, Jerry P. Melchor, Sidney Strickland, and Roberto Levi
J. Pharmacol. Exp. Ther., Jul 2007; 322: 265 - 273.
9. Ca2+-Dependent Interaction of S100A1 with F1-ATPase Leads to an Increased ATP Content in Cardiomyocytes
Melanie Boerries, Patrick Most, Jonathan R. Gledhill, John E. Walker, Hugo A. Katus, Walter J. Koch, Ueli Aebi, and Cora-Ann Schoenenberger
Mol. Cell. Biol., Jun 2007; 27: 4365 - 4373.
10. Activation of Peroxisome Proliferator-Activated Receptor Contributes to the Survival of T Lymphoma Cells by Affecting Cellular Metabolism
Chunyan Yang, Seung-Hee Jo, Balazs Csernus, Elizabeth Hyjek, Yifang Liu, Amy Chadburn, and Y. Lynn Wang
Am. J. Pathol., Feb 2007; 170: 722 - 732.

ATP Bioluminescence Assay Kit CLS II

1. The Mitochondrion Is a Site of Trypanocidal Action of the Aromatic Diamidine DB75 in Bloodstream Forms of Trypanosoma brucei
Charlotte A. Lanteri, Richard R. Tidwell, and Steven R. Meshnick
Antimicrob. Agents Chemother., Mar 2008; 52: 875 - 882.
2. Degradation of Glyoxylate and Glycolate with ATP Synthesis by a Thermophilic Anaerobic Bacterium, Moorella sp. Strain HUC22-1
Shinsuke Sakai, Kentaro Inokuma, Yutaka Nakashimada, and Naomichi Nishio
Appl. Envir. Microbiol., Mar 2008; 74: 1447 - 1452.
3. Cordycepin-hypersensitive growth links elevated polyphosphate levels to inhibition of poly(A) polymerase in Saccharomyces cerevisiae
Sandra Holbein, Florian M. Freimoser, Thomas P. Werner, Agnieszka Wengi, and Bernhard Dichtl
Nucleic Acids Res., Feb 2008; 36: 353 - 363.
4. Stable PNPase RNAi silencing: Its effect on the processing and adenylation of human mitochondrial RNA
Shimyn Slomovic and Gadi Schuster
RNA, Feb 2008; 14: 310 - 323.
5. Dodecamer rotor ring defines H+/ATP ratio for ATP synthesis of prokaryotic V-ATPase from Thermus thermophilus
Masashi Toei, Christoph Gerle, Masahiro Nakano, Kazutoshi Tani, Nobuhiko Gyobu, Masatada Tamakoshi, Nobuhito Sone, Masasuke Yoshida, Yoshinori Fujiyoshi, Kaoru Mitsuoka, and Ken Yokoyama
PNAS, Dec 2007; 104: 20256 - 20261.
6. The Cell-Selective Neurotoxicity of the Alzheimer's Aβ Peptide Is Determined by Surface Phosphatidylserine and Cytosolic ATP Levels. Membrane Binding Is Required for Aβ Toxicity
Olga Simakova and Nelson J. Arispe
J. Neurosci., Dec 2007; 27: 13719 - 13729.
7. Strain-dependent induction of epithelial cell oncosis by Campylobacter jejuni is correlated with invasion ability and is independent of cytolethal distending toxin
Lisa D. Kalischuk, G. Douglas Inglis, and Andre G. Buret
Microbiology, Sep 2007; 153: 2952 - 2963.

8. Third Activity of Bordetella Adenylate Cyclase (AC) Toxin-Hemolysin: membrane translocation of AC domain polypeptide promotes calcium influx into CD11b + monocytes independently of the catalytic and hemolytic activities
Radovan Fiser, Jirí Masín, Marek Basler, Jan Krusek, Veronika Spuláková, Ivo Konopásek, and Peter Sebo
J. Biol. Chem., Feb 2007; 282: 2808 - 2820.
9. Human Plasma ATP Concentration
Mark W. Gorman, Eric O. Feigl, and Charles W. Buffington
Clin. Chem., Feb 2007; 53: 318 - 325.
10. Absence of tektin 4 causes asthenozoospermia and subfertility in male mice
Angshumoy Roy, Yi-Nan Lin, Julio E. Agno, Francesco J. DeMayo, and Martin M. Matzuk
FASEB J, Apr 2007; 21: 1013 - 1025.

Cell Proliferation – DNA Synthesis in Cell Populations

10

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 57) 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).

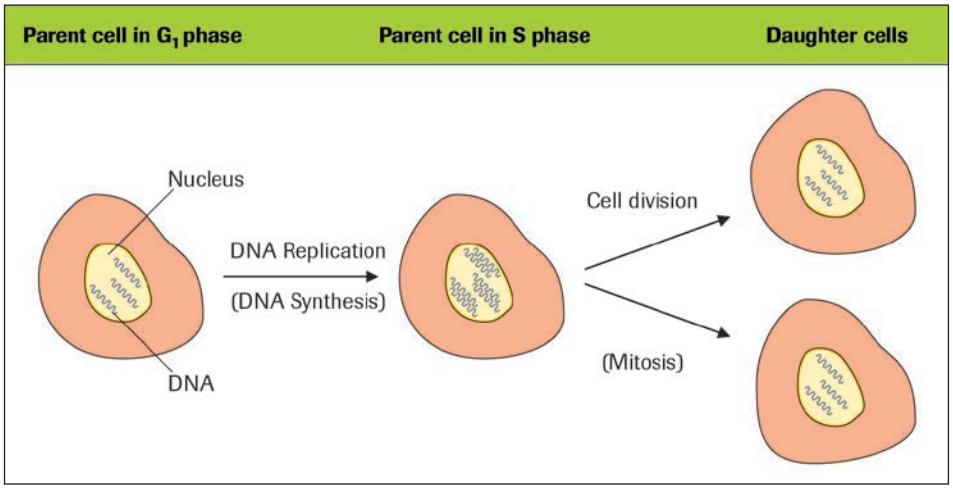


Figure 57: Cell proliferation, a close association between DNA synthesis and cell doubling.

Experiments have shown that the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) is incorporated into cellular DNA like thymidine (Figure 58). The incorporated BrdU could be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies directed against BrdU. 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. Because this method was relatively laborious, the entire BrdU-based procedure was adapted to a 96 well microplate. 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 59).

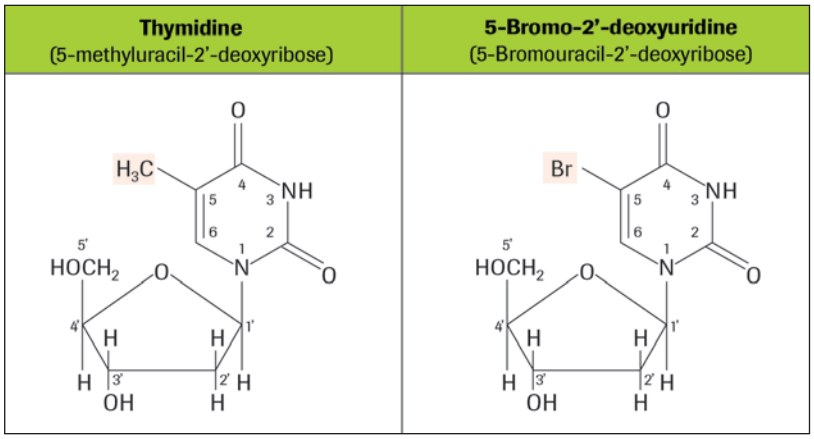


Figure 58: Molecular structure of thymidine and BrdU.

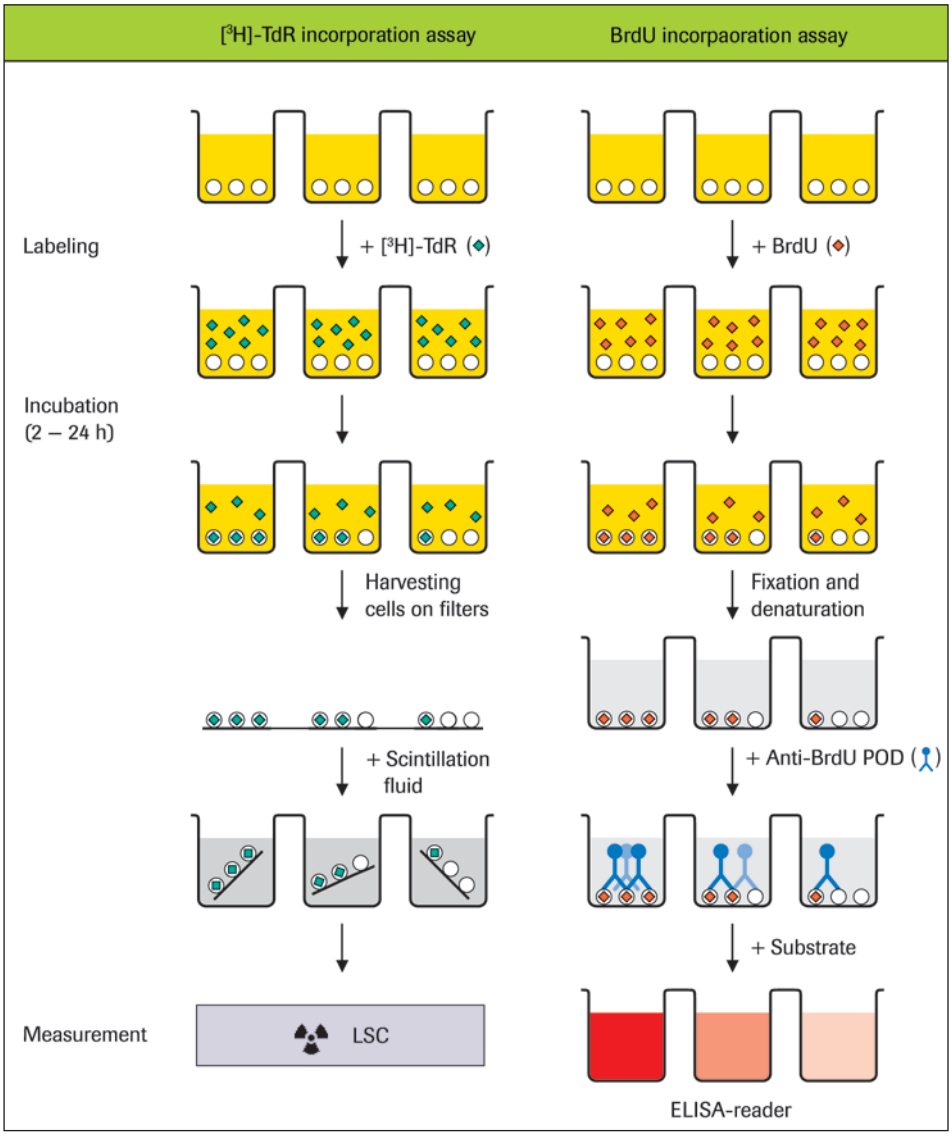


Figure 59: Measurement of DNA synthesis using modified nucleotides [^{3}H]-TdR and BrdU.

Roche Applied Science 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 9). These three kits are 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	–15 to –20°C: Fixation 15-25°C: Substrate reaction 37°C: Nuclease treatment 60°C: Air drying	For Cell Proliferation ELISA, BrdU (colorimetric) each step at 15-25°C
Measuring range	Absorbance: 0.1–2.5 U (factor 25)	Same as BrdU Kit III For Cell Proliferation ELISA, BrdU (chemiluminescence): rlu/s: 103–106 (factor 1000)
Sensitivity	Almost as sensitive as [^{3}H]-TdR	As sensitive as [^{3}H]-TdR

Table 9: Improvements of the assay procedure using the Cell Proliferation ELISA, BrdU (colorimetric) and Cell Proliferation ELISA, BrdU (chemiluminescence) described on the following pages.

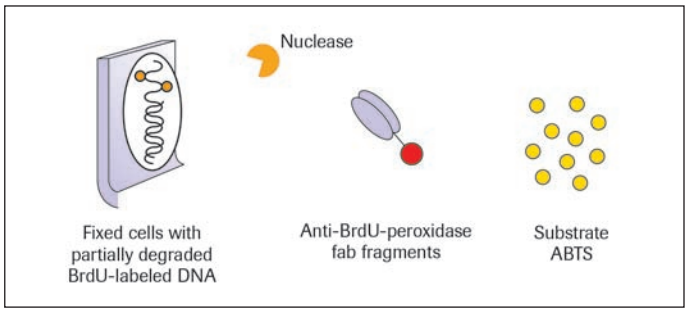
If you are studying…	and you wish to detect…	using detection by…	then use …	page
DNA Synthesis	BrdU incorporation	Colorimetric ELISA	**Cell Proliferation ELISA, BrdU (colorimetric)***	143
		Chemiluminescence ELISA	**Cell Proliferation ELISA, BrdU (chemiluminescent)***	143
		Colorimetric ELISA	**BrdU Labeling and Detection Kit III (POD)***	139

* This product can also be used to study cytotoxicity

10

5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit III (POD)

Cat. No. 11 444 611 001 1,000 tests

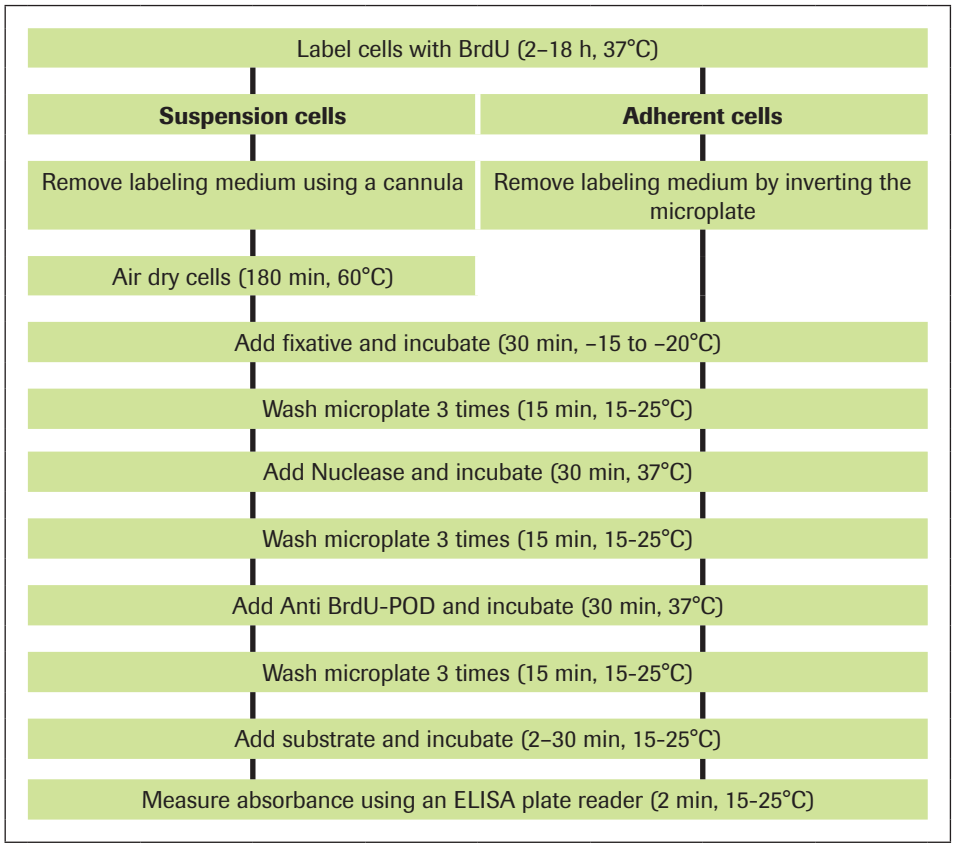
Type	1st generation ELISA with colorimetric detection
Useful for	Quantitation of DNA synthesis during cell activation and proliferation
Sample material	Adherent or suspension cell cultures
Method	Incubation of cells with BrdU, followed by partial digestion of DNA and immunodetection of incorporated BrdU label
Test principle	
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.
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 is conjugated to peroxidase and specifically recognizes 5-bromo-2'-deoxyuridine; it shows no cross-reactivity with any endogenous cellular components such as thymidine or uridine.
Time	2.5–6 h (+ cell labeling)
Benefits	■ **Improve safety** by avoiding the use of radioactive isotopes. ■ **Obtain accurate data** – results strongly correlate to those obtained with the [^{3}H]-thymidine method ■ **Achieve high sensitivity** using a nonradioactive assay as sensitive as [^{3}H]-thymidine ■ **Save time** by using of multiwell ELISA readers to process a large number of samples. ■ **Save money** with an assay that requires no expensive equipment or additional reagents, such as scintillation fluid.

How to use the kit

I. Assay procedure overview

The assay is a cellular immunoassay which uses a mouse monoclonal antibody directed against BrdU.

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 page 143).



II. Kit content

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

Typical results with the kit

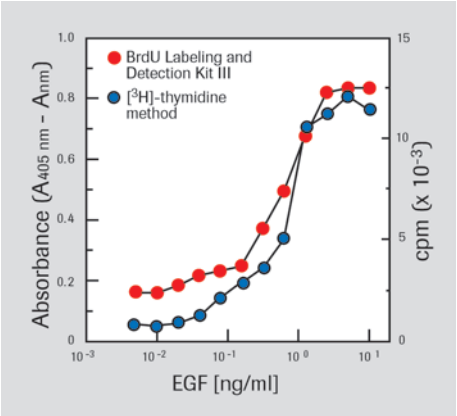


Figure 60: Proliferation of AKR-2B cells (mouse fibroblast cell line) in response to recombinant human epidermal growth factor (hEGF) using the BrdU Labeling and Detection Kit III (●), or the [3H]-thymidine method (●), respectively.

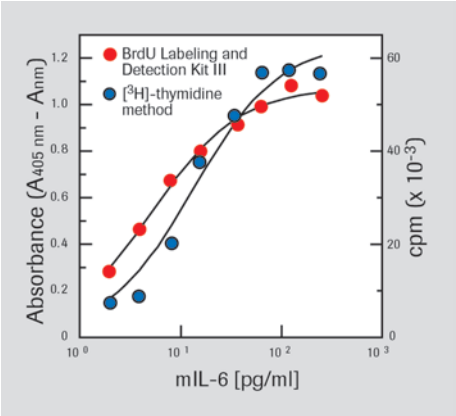


Figure 61: Proliferation of 7TD1 cells (mouse-mouse hybridoma) in response to recombinant mouse interleukin-6 (mIL-6) using the BrdU Labeling and Detection Kit III (●), or the [3H]-thymidine method (●), respectively.

Result: Figures 60 and 61 illustrate the equivalent sensitivity of the BrdU and [3H]-thymidine methods in measuring proliferation, as shown in hEGF and mIL-6 stimulation assays.

References

1. Carbon nanoparticle-induced lung epithelial cell proliferation is mediated by receptor-dependent Akt activation
   Klaus Unfried, Ulrich Sydlik, Katrin Bierhals, Alexander Weissenberg, and Josef Abel
   Am J Physiol Lung Cell Mol Physiol, Feb 2008; 294: L358 - L367.
2. Atorvastatin Promotes Presenilin-1 Expression and Notch1 Activity and Increases Neural Progenitor Cell Proliferation After Stroke
   Jieli Chen, Alex Zacharek, Ang Li, Xu Cui, Cynthia Roberts, Mei Lu, and Michael Chopp
   Stroke, Jan 2008; 39: 220 - 226.
3. Role of RhoA Inactivation in Reduced Cell Proliferation of Human Airway Smooth Muscle by Simvastatin
   Naoya Takeda, Masashi Kondo, Satoru Ito, Yasushi Ito, Kaoru Shimokata, and Hiroaki Kume
   Am. J. Respir. Cell Mol. Biol., Dec 2006; 35: 722 - 729.
4. Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R
   Ulrich Sydlik, Katrin Bierhals, Maria Soufi, Josef Abel, Roel P. F. Schins, and Klaus Unfried
   Am J Physiol Lung Cell Mol Physiol, Oct 2006; 291: L725 - L733.
5. Coordination of NF-B and NFAT Antagonism by the Forkhead Transcription Factor Foxd1
   Ling Lin and Stanford L. Peng
   J. Immunol., Apr 2006; 176: 4793 - 4803.
6. Killed Candida albicans Yeasts and Hyphae Inhibit Gamma Interferon Release by Murine Natural Killer Cells
   Celia Murciano, Eva Villamón, José-Enrique O'Connor, Daniel Gozalbo, and M. Luisa Gil
   Infect. Immun., Feb 2006; 74: 1403 - 1406.

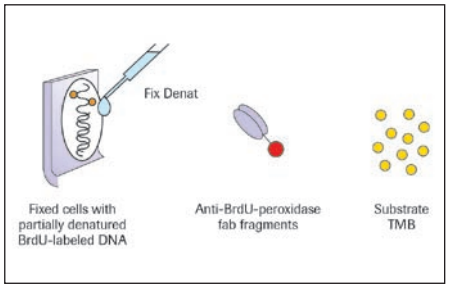
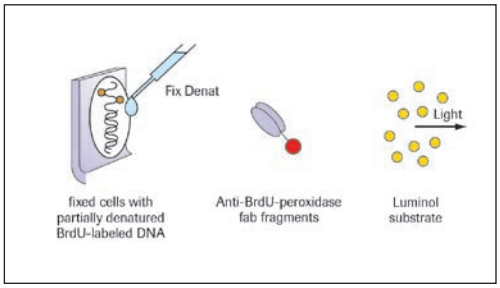
7. Expression and Potential Role of Fibroblast Growth Factor 2 and Its Receptors in Human Embryonic Stem Cells
Petr Dvorak, Dana Dvorakova, Stanislava Koskova, Martina Vodinska, Miroslava Najvirtova, Daniel Krekac, and Ales Hampl
Stem Cells, Sep 2005; 23: 1200 - 1211.
8. Mechanistic Studies on a Novel, Highly Potent Gold-Phosphole Inhibitor of Human Glutathione Reductase
Marcel Deponte, Sabine Urig, L. David Arscott, Karin Fritz-Wolf, Régis Réau, Christel Herold-Mende, Sasa Koncarevic, Markus Meyer, Elisabeth Davioud-Charvet, David P. Ballou, Charles H. Williams, Jr., and Katja Becker
J. Biol. Chem., May 2005; 280: 20628 - 20637.
9. Endothelial Nitric Oxide Synthase Regulates Brain-Derived Neurotrophic Factor Expression and Neurogenesis after Stroke in Mice
Jieli Chen, Alex Zacharek, Chunling Zhang, Hao Jiang, Yi Li, Cynthia Roberts, Mei Lu, Alissa Kapke, and Michael Chopp
J. Neurosci., Mar 2005; 25: 2366 - 2375.
10. Sonic Hedgehog Is Produced by Follicular Dendritic Cells and Protects Germinal Center B Cells from Apoptosis
Rosa Sacedón, Blanca Díez, Vanesa Nuñez, Carmen Hernández-López, Cruz Gutierr-ez-Frías, Teresa Cejalvo, Susan V. Outram, Tessa Crompton, Agustín G. Zapata, Angeles Vicente, and Alberto Varas
J. Immunol., Feb 2005; 174: 1456 - 1461.

Cell Proliferation ELISA, BrdU (colorimetric)

Cat. No. 11 647 229 001 1,000 tests

Cell Proliferation ELISA, BrdU (chemiluminescence)

Cat. No. 11 669 915 001 1,000 tests

Type	2nd generation ELISAs with colorimetric or chemiluminescent detection
Useful for	Quantitation of DNA synthesis during cell activation and proliferation
Sample material	Adherent or suspension cell cultures
Method	Incubation of cells with BrdU, followed by immunodetection of incorporated BrdU label
Test principle	 **Principle of the Cell Proliferation ELISA, BrdU (colorimetric).**  **Principle of the Cell Proliferation ELISA, BrdU (chemiluminescent).**
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.
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. *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 anti-BrdU antibody peroxidase-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.
Time	1.5–2.5 h (+ cell labeling)

Benefits

- **Obtain results that strongly correlate to the number of proliferating cells** (low mean deviation).
- **Avoid the use of hazardous radioactive isotopes by using a nonradioactive method** that is at least as sensitive as the [^{3}H]-thymidine incorporation assay, with measurement over a large logarithmic range.
- **Fix and denature cells in a single step** using the kit's supplied Fix Denat reagent, then perform only one washing and two incubation steps.
- **Benefit from the convenience of stable and optimized reagents**; perform the entire assay in one microplate.
- **Preserve cell morphology** with mild fixation and DNA denaturation methods.

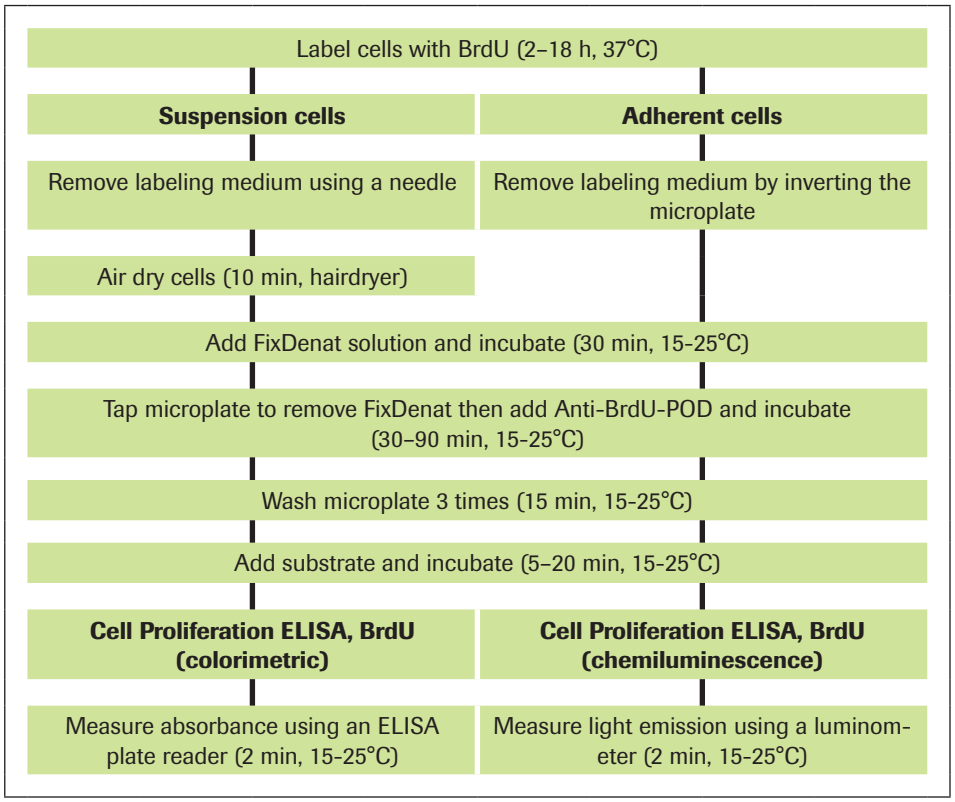
How to use the kit

I. Assay procedure overview

The assay is a cellular immunoassay which uses a mouse monoclonal antibody directed against BrdU. The procedure involves:

These two kits belong to the second, improved generation of kits for measuring DNA synthesis.

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).



II. Kits content

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)

Typical results with the kit

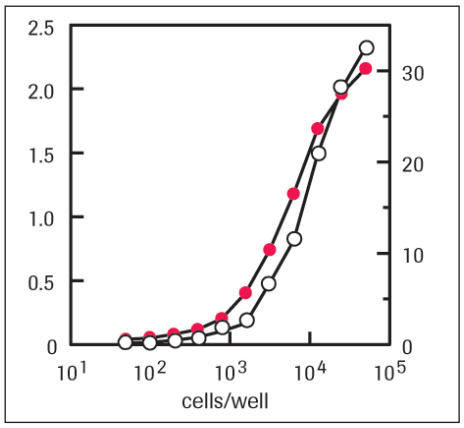


Figure 62: Comparison of the sensitivity of the Cell Proliferation ELISA, BrdU (colorimetric) and the radioactive thymidine incorporation assay for measuring proliferation in various concentrations of cells. Various concentrations of L929 cells were cultured in the wells of a microtiter plate. Duplicate 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.

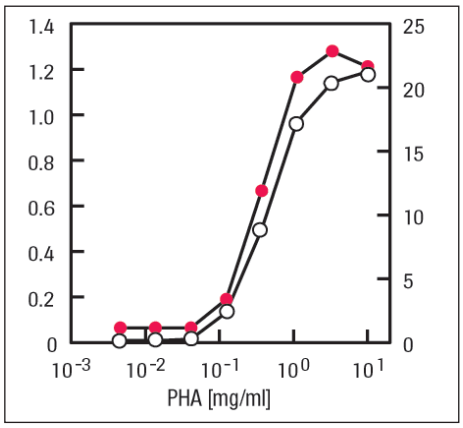


Figure 63: Comparison of the Cell Proliferation ELISA, BrdU (colorimetric) and the radioactive thymidine incorporation assay for measuring stimulation of various concentrations of mitogen. Human peripheral blood lymphocytes were cultured in the presence of varying concentrations of phytohemagglutinin (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.

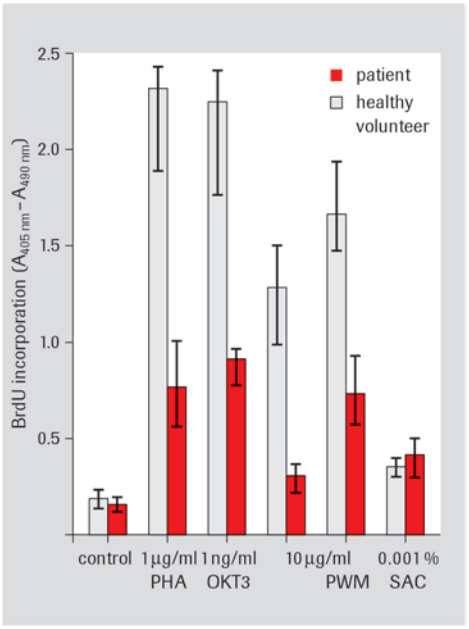


Figure 64: Reduced PBL proliferation of an immunosuppressed patient in response to various mitogens. Cells (1 x 10^5/well) from a healthy volunteer (■) or an immunosuppressed individual (■) were incubated in the presence of various mitogens for 56 h. Cells were labeled with BrdU for 16 h, then cell proliferation 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.

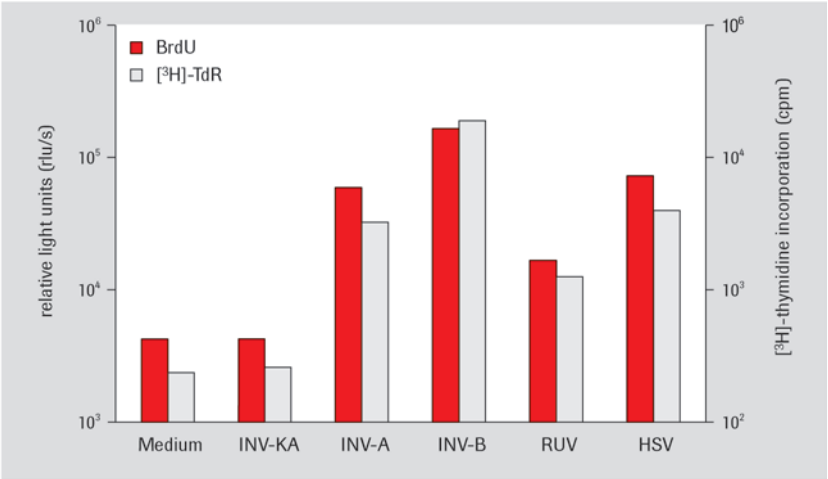


Figure 65: Measurement of the proliferation of antigen-activated PBL. Cells (1 x 10^5/well) were incubated in the presence of various viral antigens on culture medium alone for 5 days. After labeling with BrdU (■) or [^{3}H]-TdR (■) for 16 h, cell proliferation was analyzed by Cell Proliferation ELISA 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.

References

Cell Proliferation ELISA, BrdU (colorimetric)

1. Differential Effects of Interleukin-2 and Interleukin-15 versus Interleukin-21 on CD4+ Cutaneous T-Cell Lymphoma Cells
   Michal Marzec, Krzysztof Halasa, Monika Kasprzycka, Maria Wysocka, Xiaobin Liu, John W. Tobias, Donald Baldwin, Qian Zhang, Niels Odum, Alain H. Rook, and Mariusz A. Wasik
   Cancer Res., Feb 2008; 68: 1083 - 1091.
2. Evidence of key role of CDK-2 overexpression in pemphigus vulgaris
   Alessandro Lanza, Nicola Cirillo, Raffaele Rossiello, Monica Rienzo, Luisa Cutillo, Amelia Casamassimi, Fiolomena de Nigris, Concetta Schiano, Luigi Rossiello, Felice Femiano, Fernando Gombos, and Claudio Napoli
   J. Biol. Chem., Jan 2008; 10.1074/jbc.M702186200.
3. Overexpression of Cellular Iron Import Proteins Is Associated with Malignant Progression of Esophageal Adenocarcinoma
   Jessica Boult, Keith Roberts, Matthew J. Brookes, Sharon Hughes, Jonathan P. Bury, Simon S. Cross, Gregory J. Anderson, Robert Spychal, Tariq Iqbal, and Chris Tselepis
   Clin. Cancer Res., Jan 2008; 14: 379 - 387.
4. NADPH oxidase plays a crucial role in the activation of pancreatic stellate cells
   Atsushi Masamune, Takashi Watanabe, Kazuhiro Kikuta, Kennichi Satoh, and Tooru Shimosegawa
   Am J Physiol Gastrointest Liver Physiol, Jan 2008; 294: G99 - G108.
5. Differential Sensitivity of Malignant Glioma Cells to Methylating and Chloroethylating Anticancer Drugs: p53 Determines the Switch by Regulating xpc, ddb2, and DNA Double-Strand Breaks
   Luís F.Z. Batista, Wynand P. Roos, Markus Christmann, Carlos F.M. Menck, and Bernd Kaina
   Cancer Res., Dec 2007; 67: 11886 - 11895
6. Development of a Challenge-Protective Vaccine Concept by Modification of the Viral RNA-Dependent RNA Polymerase of Canine Distemper Virus
   D. Silin, O. Lyubomska, M. Ludlow, W. P. Duprex, and B. K. Rima
   J. Virol., Dec 2007; 81: 13649 - 13658.

7. Effective Proliferation of Human Regulatory T Cells Requires a Strong Costimulatory CD28 Signal That Cannot Be Substituted by IL-2
Andreas A. Hombach, David Kofler, Anja Hombach, Gunter Rappl, and Hinrich Abken
J. Immunol., Dec 2007; 179: 7924 - 7931
8. CUB Domain-Containing Protein 1 Is a Novel Regulator of Anoikis Resistance in Lung Adenocarcinoma
Takamasa Uekita, Lin Jia, Mako Narisawa-Saito, Jun Yokota, Tohru Kiyono, and Ryuichi Sakai
Mol. Cell. Biol., Nov 2007; 27: 7649 - 7660.
9. Response of human dendritic cells to different immunomodulatory polysaccharides derived from mushroom and barley
Wing Keung Chan, Helen Ka Wai Law, Zhi-Bin Lin, Yu Lung Lau, and Godfrey Chi-Fung Chan
Int. Immunol., Jul 2007; 19: 891 - 899.
10. Intercellular Adhesion Molecule-1 Deficiency Attenuates the Development of Skin Fibrosis in Tight-Skin Mice
Yukiyo Matsushita, Minoru Hasegawa, Takashi Matsushita, Manabu Fujimoto, Mayuka Horikawa, Tomoyuki Fujita, Ayako Kawasuji, Fumihide Ogawa, Douglas A. Steeber, Thomas F. Tedder, Kazuhiko Takehara, and Shinichi Sato
J. Immunol., Jul 2007; 179: 698 - 707.

Cell Proliferation ELISA, BrdU (chemiluminescent)

1. Characterization of Pulmonary T Cell Response to Helper-Dependent Adenoviral Vectors following Intranasal Delivery
Rahul Kushwah, Huibi Cao, and Jim Hu
J. Immunol., Mar 2008; 180: 4098 - 4108.
2. A Non-Tumor Suppressor Role for Basal p19ARF in Maintaining Nucleolar Structure and Function
Anthony J. Apicelli, Leonard B. Maggi, Jr., Angela C. Hirbe, Alexander P. Miceli, Mary E. Olanich, Crystal L. Schulte-Winkeler, Anthony J. Saporita, Michael Kuchenreuther, José Sanchez, Katherine Weilbaecher, and Jason D. Weber
Mol. Cell. Biol., Feb 2008; 28: 1068 - 1080.
3. AZD6244 and doxorubicin induce growth suppression and apoptosis in mouse models of hepatocellular carcinoma
Hung Huynh, Pierce K.H. Chow, and Khee-Chee Soo
Mol. Cancer Ther., Sep 2007; 6: 2468 - 2476.
4. Disruption of CXCR4 enhances osteoclastogenesis and tumor growth in bone
Angela C. Hirbe, Jessica Rubin, Özge Uluçkan, Elizabeth A. Morgan, Mark C. Eagleton, Julie L. Prior, David Piwnica-Worms, and Katherine N. Weilbaecher
PNAS, Aug 2007; 104: 14062 - 14067.
5. Secreted Frizzled-Related Protein 1 Loss Contributes to Tumor Phenotype of Clear Cell Renal Cell Carcinoma
Michelle L. Gumz, Hongzhi Zou, Pamela A. Kreinest, April C. Childs, Leandra S. Belmonte, Shauna N. LeGrand, Kevin J. Wu, Bruce A. Luxon, Mala Sinha, Alexander S. Parker, L-Z. Sun, David A. Ahlquist, Christopher G. Wood, and John A. Copland
Clin. Cancer Res., Aug 2007; 13: 4740 - 4749.

Cell Proliferation – DNA Synthesis in Individual Cells

11

Assays that Measure DNA Synthesis in Individual Cells

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.

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" 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 Applied Science offers several kits and reagents for measuring proliferating cells by BrdU incorporation. These products are described on the following pages.

If you are studying…	and you wish to detect…	using detection by…	then use …	page
DNA Synthesis	BrdU incorporation	Fluorescence microscopy or flow cytometry	**BrdU Labeling and Detection Kit I (Fluorescein)**	151
		Light microscopy	**BrdU Labeling and Detection Kit II (AP)**	151
		Fluorescence microscopy or flow cytometry	***In Situ* Cell Proliferation Kit, FLUOS**	156
		Multiple methods	**Anti-BrdU Antibodies**	160

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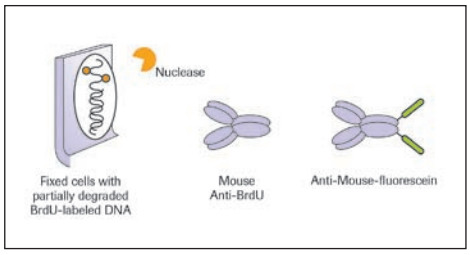
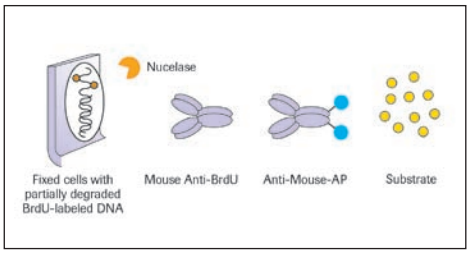
5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit I

Cat. No. 11 296 736 001 100 tests

5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit II

Cat. No. 11 299 964 001 100 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
Sample material	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
Test principle	 **Principle of the BrdU Labeling and Detection Kit I (Fluorescein).**  **Principle of the BrdU Labeling and Detection Kit II (AP).**
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). *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, see page 156).*
Specificity	Anti-BrdU monoclonal antibody specifically binds to 5-bromo-2'-deoxy-uridine, and shows crossreactivity with 5-iodo-2'-deoxy-uridine (10%). Anti-BrdU shows no crossreactivity with 5'-fluoro-2'-deoxy-uridine or any endogenous cellular component, such as thymidine or uridine.
Time	approx. 2–3 h (+ BrdU labeling)

Benefits

- **Perform simultaneous detection of other markers** (double staining).
- **Label cells *in vitro* and *in vivo*.**
- **Measure cell proliferation without using hazardous and inconvenient radioisotopes** ([^{3}H]-thymidine).

How to use the kits

I. Assay procedure overview

The procedure for the **5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I** involves:

1. Cells, tissue explants or organ cultures are incubated with BrdU, 10 µmol, for a short period of time (approx. 30 min). The addition of 5´-fluoro-2´-deoxy-uridine (FdU), being described to enhance BrdU incorporation has no advantage within short incubation periods and BrdU concentrations of 10 µM..
2. Fixation of samples with ethanol.
3. Incubation with anti-BrdU monoclonal antibody.
   The monoclonal antibody binds to BrdU incorporated into cellular DNA.
4. Incubation with anti-mouse-Ig-fluorescein.
5. Bound anti-BrdU monoclonal antibody is visualized by immunofluorescence microscopy.

The procedure for the **5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II** involves:

1. Incorporation of BrdU in DNA.
2. Binding of anti-BrdU antibody to the in DNA incorporated BrdU.
3. Binding of the AP conjugated anti-mouse-antibody to the anti-BrdU antibody.
4. Color reaction of NBT/BCIP with the alkaline phosphatase and detection by light microscopy.

II. Kits content

5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit I

1. BrdU labeling reagent, 1,000 x conc., sterile
2. Washing buffer concentrate, 10 x
3. Incubation buffer
4. Anti-BrdU, (contains nucleases for DNA denaturation)
5. Anti-mouse-Ig-fluorescein

5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit II

1. BrdU labeling reagent, 1,000 x conc., sterile
2. Washing buffer concentrate, 10 x
3. Incubation Buffer
4. Anti-BrdU, containing nucleases for DNA denaturation
5. Anti-mouse Ig-alkaline phosphatase
6. NBT
7. BCIP

Typical results with the kits

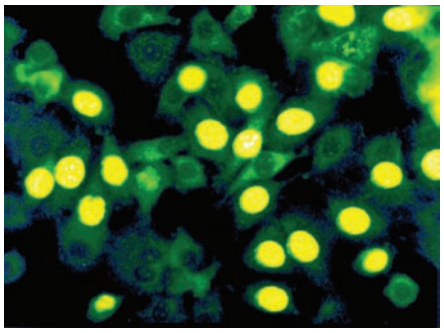


Figure 66: Fluorescence microscopy-based detection of cells undergoing DNA synthesis. BrdU incorporation was detected using the BrdU Labeling and Detection Kit I. Bright green fluorescence clearly indicates proliferating cells.

References

BrdU Labeling and Detection Kit I (Fluorescein)

1. Myoblasts and macrophages share molecular components that contribute to cell–cell fusion
   Kostandin V. Pajcini, Jason H. Pomerantz, Ozan Alkan, Regis Doyonnas, and Helen M. Blau
   J. Cell Biol., Mar 2008; 180: 1005 - 1019.
2. Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development
   Peer Heine, Eva Dohle, Keely Bumsted-O'Brien, Dieter Engelkamp, and Dorothea Schulte
   Development, Mar 2008; 135: 805 - 811.
3. Increasing GLP-1–Induced β-Cell Proliferation by Silencing the Negative Regulators of Signaling cAMP Response Element Modulator- and DUSP14
   Sonia Klinger, Carine Poussin, Marie-Bernard Debril, Wanda Dolci, Philippe A. Halban, and Bernard Thorens
   Diabetes, Mar 2008; 57: 584 - 593.
4. Human Chorionic Gonadotropin Stimulates Trophoblast Invasion through Extracellularly Regulated Kinase and AKT Signaling
   Johanna Prast, Leila Saleh, Heinrich Husslein, Stefan Sonderegger, Hanns Helmer, and Martin Knöfler
   Endocrinology, Mar 2008; 149: 979 - 987.
5. Stem Cells Derived from Goiters in Adults Form Spheres in Response to Intense Growth Stimulation and Require Thyrotropin for Differentiation into Thyrocytes
   Ling Lan, Dai Cui, Kathrin Nowka, and Michael Derwahl
   J. Clin. Endocrinol. Metab., Sep 2007; 92: 3681 - 3688.

6. Chondroitin sulfate glycosaminoglycans control proliferation, radial glia cell differentiation and neurogenesis in neural stem/progenitor cells
Swetlana Sirko, Alexander von Holst, Andrea Wizenmann, Magdalena Götz, and Andreas Faissner
Development, Aug 2007; 134: 2727 - 2738.
7. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells
Pietro Laneve, Lucia Di Marcotullio, Ubaldo Gioia, Micol E. Fiori, Elisabetta Ferretti, Alberto Gulino, Irene Bozzoni, and Elisa Caffarelli
PNAS, May 2007; 104: 7957 - 7962.
8. Forkhead Box F1 Is Essential for Migration of Mesenchymal Cells and Directly Induces Integrin-Beta3 Expression
Dmitriy Malin, Il-Man Kim, Evan Boetticher, Tanya V. Kalin, Sneha Ramakrishna, Lucille Meliton, Vladimir Ustiyan, Xiangdong Zhu, and Vladimir V. Kalinichenko
Mol. Cell. Biol., Apr 2007; 27: 2486 - 2498.
9. Active tissue-specific DNA demethylation conferred by somatic cell nuclei in stable heterokaryons
Fan Zhang, Jason H. Pomerantz, George Sen, Adam T. Palermo, and Helen M. Blau
PNAS, Mar 2007; 104: 4395 - 4400.
10. Stimulation of embryo hatching and implantation by prostacyclin and peroxisome proliferator-activated receptor activation: implication in IVF
J.-C. Huang, W.-S.A. Wun, J.S. Goldsby, I.C. Wun, D. Noorhasan, and K.K. Wu
Hum. Reprod., Mar 2007; 22: 807 - 814.

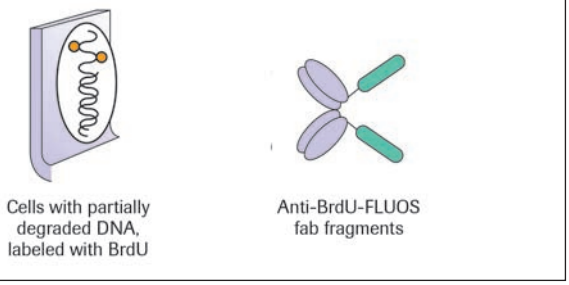
BrdU Labeling and Detection Kit II (AP)

1. The mutation ROR2W749X, linked to human BDB, is a recessive mutation in the mouse, causing brachydactyly, mediating patterning of joints and modeling recessive Robinow syndrome
Regina Raz, Sigmar Stricker, Elizabetta Gazzerro, Julie L. Clor, Florian Witte, Harakiran Nistala, Stefanie Zabski, Renata C. Pereira, Lisa Stadmeyer, Xiangmin Wang, Lori Gowen, Mark W. Sleeman, George D. Yancopoulos, Ernesto Canalis, Stefan Mundlos, David M. Valenzuela, and Aris N. Economides
Development, May 2008; 135: 1713 - 1723.
2. SAG/ROC2/RBX2 E3 ligase promotes UVB-induced skin hyperplasia, but not skin tumors, by simultaneously targeting c-Jun/AP-1 and p27
Hongbin He, Qingyang Gu, Min Zheng, Daniel Normolle, and Yi Sun
Carcinogenesis, Apr 2008; 29: 858 - 865.
3. Hypoxia and podocyte-specific Vhlh deletion confer risk of glomerular disease
Kirsten Brukamp, Belinda Jim, Marcus J. Moeller, and Volker H. Haase
Am J Physiol Renal Physiol, Oct 2007; 293: F1397 - F1407.
4. Hyaluronan and CD44 antagonize mitogen-dependent cyclin D1 expression in mesenchymal cells
Devashish Kothapalli, Liang Zhao, Elizabeth A. Hawthorne, Yan Cheng, Eric Lee, Ellen Puré, and Richard K. Assoian
J. Cell Biol., Feb 2007; 176: 535 - 544.
5. Disruption of Phospholipase C4 Gene Modulates the Liver Regeneration in Cooperation with Nuclear Protein Kinase C
Atsushi Akutagawa, Kiyoko Fukami, Yoshiko Banno, Tadaomi Takenawa, Reiji Kannagi, Yukihiro Yokoyama, Koji Oda, Masato Nagino, Yuji Nimura, Shonen Yoshida, and Keiko Tamiya-Koizumi
J. Biochem., Nov 2006; 140: 619 - 625.
6. Individual Cell-Based Models of Tumor-Environment Interactions: Multiple Effects of CD97 on Tumor Invasion
Joerg Galle, Doreen Sittig, Isabelle Hanisch, Manja Wobus, Elke Wandel, Markus Loeffler, and Gabriela Aust
Am. J. Pathol., Nov 2006; 169: 1802 - 1811.

7. Taste Receptor Cells Express Voltage-Dependent Potassium Channels in a Cell Age–Specific Manner
Makoto Ohmoto, Ichiro Matsumoto, Takumi Misaka, and Keiko Abe
Chem Senses, Oct 2006; 31: 739 - 746.
8. Conserved Functions of Ikaros in Vertebrate Lymphocyte Development: Genetic Evidence for Distinct Larval and Adult Phases of T Cell Development and Two Lineages of B Cells in Zebrafish
Michael Schorpp, Mike Bialecki, Dagmar Diekhoff, Brigitte Walderich, Jörg Odenthal, Hans-Martin Maischein, Agustin G. Zapata, Tübingen 2000 Screen Consortium, Freiburg Screening Group, and Thomas Boehm
J. Immunol., Aug 2006; 177: 2463 - 2476.
9. GLUT8 Is Dispensable for Embryonic Development but Influences Hippocampal Neurogenesis and Heart Function
Mathieu Membrez, Edith Hummler, Friedrich Beermann, Jacques-Antoine Haefliger, Rebecca Savioz, Thierry Pedrazzini, and Bernard Thorens
Mol. Cell. Biol., Jun 2006; 26: 4268 - 4276.
10. Skin lesion development in a mouse model of incontinentia pigmenti is triggered by NEMO deficiency in epidermal keratinocytes and requires TNF signaling
Arianna Nenci, Marion Huth, Alfred Funteh, Marc Schmidt-Supprian, Wilhelm Bloch, Daniel Metzger, Pierre Chambon, Klaus Rajewsky, Thomas Krieg, Ingo Haase, and Manolis Pasparakis
Hum. Mol. Genet., Feb 2006; 15: 531 - 542.

In Situ Cell Proliferation Kit, FLUOS

Cat. No. 11 810 740 001 100 tests

Type	Direct immunofluorescence staining for flow cytometry or fluorescence microscopy
Useful for	Detection of BrdU-labeled DNA in proliferating individual cells
Sample material	Cultured or freshly isolated cells, tissue explants labeled with BrdU *in vitro*. Frozen or paraffin-embedded tissue sections from animals labeled with BrdU *in vivo*
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
Test principle	
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.
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.
Time	approx. 2 h (+ 0.5–4 h BrdU labeling)
Benefits	■ **Avoid the hazards of radioactivity** by using this nonradioactive alternative to tissue autoradiography. ■ **Achieve high specificity** – no cross-reactivity with endogenous immunoglobulins. ■ **Save time** – the kit's direct antibody conjugate eliminates the need for a secondary detection system. ■ **Follow a standard immunohistochemistry protocol.** ■ **Assess cell proliferation** using FACS analysis or fluorescence microscopy.

11

How to use the kit

I. Assay procedure overview

The BrdU solution and fluorescein-conjugated anti-BrdU antibody supplied in the kit allow BrdU labeling and detection of proliferating cells. The procedure 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.
   ! *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.

II. Kit content

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

Typical result

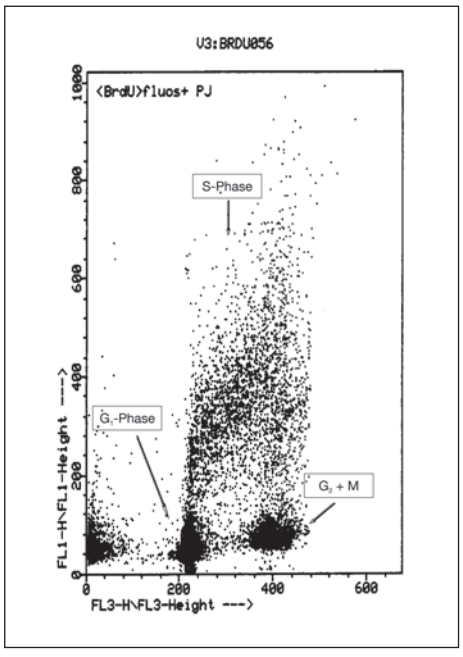


Figure 67: Flow cytometric measurement of total DNA and incorporated BrdU with the *In Situ* Cell Proliferation Kit, FLUOS. Exponentially growing U937 cells were incubated with BrdU for 30 min. Incorporated BrdU was measured flow cytometrically with the fluorescein-conjugated anti-BrdU antibody (<BrdU>fluos) 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.

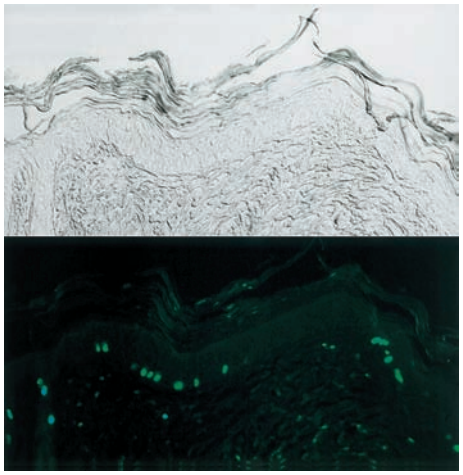


Figure 68: *In vivo* labeling and analysis of dorsal, hyperproliferative epidermis tissue from mouse with the *In Situ* Cell Proliferation Kit, FLUOS. Undiluted BrdU labeling solution from the kit was injected intraperitoneally into a mouse (1 ml BrdU solution/100 g body weight). After 2 h of *in vivo* 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.

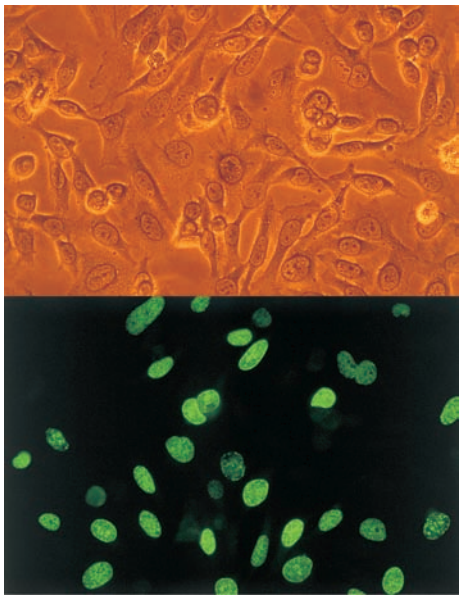


Figure 69: *In vitro* labeling and analysis of proliferating HeLa cells with the *In Situ* Cell Proliferation Kit, FLUOS. HeLa cells in culture were labeled with BrdU and the BrdU-labeled DNA detected with anti-BrdU-fluorescein, according to the package insert of the *In Situ* Cell Proliferation Kit, 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.

References

1. Micropatterning of single endothelial cell shape reveals a tight coupling between nuclear volume in G1 and proliferation
   Pere Roca-Cusachs, Jordi Alcaraz, Raimon Sunyer, Josep Samitier, Ramon Farré, and Daniel Navajas
   Biophys. J., Mar 2008; 10.1529/biophysj.107.116863.
2. Early Embryonic Lethality of Mice Lacking ZO-2, but Not ZO-3, Reveals Critical and Nonredundant Roles for Individual Zonula Occludens Proteins in Mammalian Development
   Jianliang Xu, P. Jaya Kausalya, Dominic C. Y. Phua, Safiah Mohamed Ali, Zakir Hossain, and Walter Hunziker
   Mol. Cell. Biol., Mar 2008; 28: 1669 - 1678.
3. Gene expression in mouse brain following chronic hypoxia: role of sarcospan in glial cell death
   Dan Zhou, Jiyi Wang, Matthew A. Zapala, Jin Xue, Nicholas J. Schork, and Gabriel G. Haddad
   Physiol Genomics, Feb 2008; 32: 370 - 379.
4. JAK1–STAT1–STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts
   Luguo Sun, Kewei Ma, Haixia Wang, Fang Xiao, Yan Gao, Wei Zhang, Kepeng Wang, Xiang Gao, Nancy Ip, and Zhenguo Wu
   J. Cell Biol., Oct 2007; 179: 129 - 138.
5. Splicing Regulator SC35 Is Essential for Genomic Stability and Cell Proliferation during Mammalian Organogenesis
   Ran Xiao, Ye Sun, Jian-Hua Ding, Shengrong Lin, Dave W. Rose, Michael G. Rosenfeld, Xiang-Dong Fu, and Xue Li
   Mol. Cell. Biol., Aug 2007; 27: 5393 - 5402.
6. Increased apoptosis, p53 up-regulation, and cerebellar neuronal degeneration in repair-deficient Cockayne syndrome mice
   R. R. Laposa, E. J. Huang, and J. E. Cleaver
   PNAS, Jan 2007; 104: 1389 - 1394.
7. Epidermal and craniofacial defects in mice overexpressing Klf5 in the basal layer of the epidermis
   Inderpreet Sur, Björn Rozell, Viljar Jaks, Åsa Bergström, and Rune Toftgård
   J. Cell Sci., Sep 2006; 119: 3593 - 3601.
8. The Aurora Kinase Inhibitor VX-680 Induces Endoreduplication and Apoptosis Preferentially in Cells with Compromised p53-Dependent Postmitotic Checkpoint Function
   Farid Gizatullin, Yao Yao, Victor Kung, Matthew W. Harding, Massimo Loda, and Geoffrey I. Shapiro
   Cancer Res., Aug 2006; 66: 7668 - 7677.
9. Disruption of G1-phase phospholipid turnover by inhibition of Ca2+-independent phospholipase A2 induces a p53-dependent cell-cycle arrest in G1 phase
   Xu Hannah Zhang, Chunying Zhao, Konstantin Seleznev, Keying Song, James J. Manfredi, and Zhongmin Alex Ma
   J. Cell Sci., Mar 2006; 119: 1005 - 1015.
10. Runx2 and MYC Collaborate in Lymphoma Development by Suppressing Apoptotic and Growth Arrest Pathways In vivo
    Karen Blyth, François Vaillant, Linda Hanlon, Nancy Mackay, Margaret Bell, Alma Jenkins, James C. Neil, and Ewan R. Cameron
    Cancer Res., Feb 2006; 66: 2195 - 2201.

Anti-Bromodeoxyuridine, formalin grade

Cat. No. 11 170 376 001 50 µg

Anti-Bromodeoxyuridine-Fluorescein

Cat. No. 11 202 693 001 50 µg

Anti-Bromodeoxyuridine-Peroxidase, Fab fragment

Cat. No. 11 585 860 001 15 units

Type	Monoclonal antibodies, from mouse
Useful for	Detection of BrdU-labeled DNA in proliferating individual cells
Sample material	Cultured or freshly isolated cells, tissue explants labeled with BrdU *in vitro*. Frozen or paraffin-embedded tissue sections from animals labeled with BrdU *in vivo*
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
Test Principle	The anti-BrdU antibodies may be used to detect BrdU-labeled DNA in proliferating cells.
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.
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.
Time	Variable (depending on sample and antibody used)
Benefits	■ **Select from several preparations** to best meet your needs. ■ **Achieve high specificity** – the antibodies demonstrate no cross-reactivity with other cellular components. ■ **Detect BrdU-labeled DNA** in proliferating individual cells.

How to use the reagents

I. Assay procedure overview

The procedure 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.
   ! *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.
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.

***In vitro* labeling of proliferating cells with BrdU**

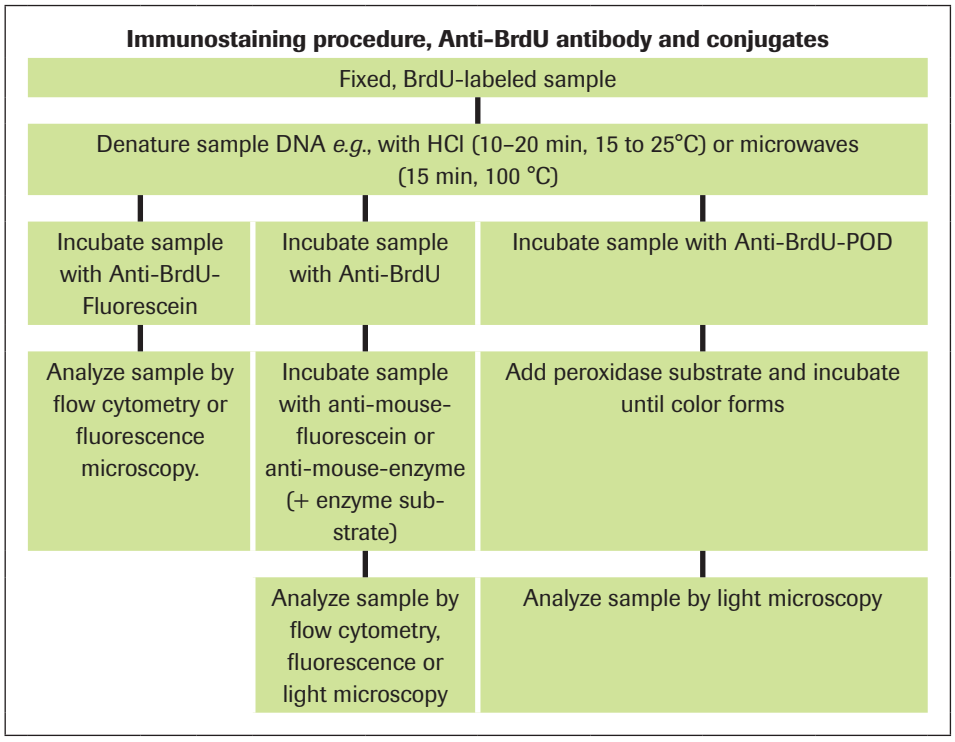
Inject the animal with BrdU labeling reagent intraperitoneally

Sacrifice the animal (approx. 1–4 h later) and remove tissue samples or organ

Process tissue samples or organ for:

Frozen sectioning	**Paraffin embedding**
Freeze tissue samples/organ immediately after removal	Fix tissue samples/organ in formalin immediately after removal
Store sample frozen until required for sectioning	Use standard dehydration and paraffin embedding procedures to process fixed sample
Cut sections of frozen sample in a cryostat	Cut sections of embedded sample on a microtome
Transfer sections onto a glass slide and fix	Transfer sections onto a glass slide and use standard procedures to dewax and rehydrate sections

Proceed with the immunostaining procedure



Typical result with the antibodies

The anti-BrdU antibody has been used to determine the cell cycle position of apoptotic cells.

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).

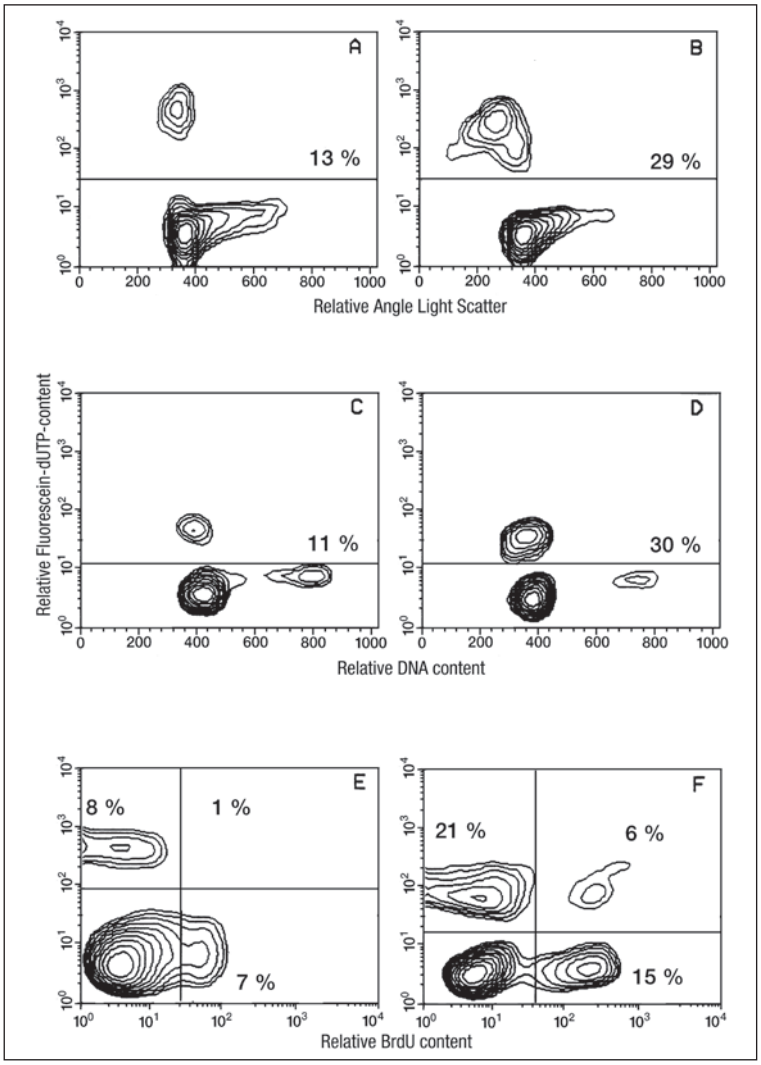


Figure 70: Concomitant flow cytometric analysis of apoptosis and cell cycle position with the anti-BrdU antibody, propidium iodide, and the *In Situ* Cell Death Detection Kit, Fluorescein. Cultured mouse thymocytes were treated with ionomycin (2 h or 12 h) to induce apoptosis. After treatment, the cells 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 G0/G1 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 G0/G1 (that is, were negative for BrdU).

References

Anti-Bromodeoxyuridine formalin grade

1. Effects of level and source of dietary selenium on maternal and fetal body weight, visceral organ mass, cellularity estimates, and jejunal vascularity in pregnant ewe lambs
   T. L. Neville, M. A. Ward, J. J. Reed, S. A. Soto-Navarro, S. L. Julius, P. P. Borowicz, J. B. Taylor, D. A. Redmer, L. P. Reynolds, and J. S. Caton
   J Anim Sci, Apr 2008; 86: 890 - 901.
2. Transrepression Function of the Glucocorticoid Receptor Regulates Eyelid Development and Keratinocyte Proliferation but Is Not Sufficient to Prevent Skin Chronic Inflammation
   Eva Donet, Pilar Bosch, Ana Sanchis, Pilar Bayo, Angel Ramírez, José L. Cascallana, Ana Bravo, and Paloma Pérez
   Mol. Endocrinol., Apr 2008; 22: 799 - 812.
3. Zinc Supplementation Enhances Hepatic Regeneration by Preserving Hepatocyte Nuclear Factor-4 in Mice Subjected to Long-Term Ethanol Administration
   Xinqin Kang, Zhenyuan Song, Craig J. McClain, Y. James Kang, and Zhanxiang Zhou
   Am. J. Pathol., Apr 2008; 172: 916 - 925.
4. Leptin promotes adult hippocampal neurogenesis in vivo and in vitro
   Jacob C Garza, Ming Guo, Wei Zhang, and Xin-Yun Lu
   J. Biol. Chem., Mar 2008; 10.1074/jbc.M800053200.
5. Misplacement of Purkinje Cells during Postnatal Development in Bax Knock-Out Mice: A Novel Role for Programmed Cell Death in the Nervous System?
   A-rong Jung, Tae Woo Kim, Im Joo Rhyu, Hyun Kim, Young Don Lee, Sharon Vinsant, Ronald W. Oppenheim, and Woong Sun
   J. Neurosci., Mar 2008; 28: 2941 - 2948.
6. Pathophysiology and fate of hepatocytes in a mouse model of mitochondrial hepatopathies
   F Diaz, S Garcia, D Hernandez, A Regev, A Rebelo, J Oca-Cossio, and C T Moraes
   Gut, Feb 2008; 57: 232 - 242.
7. Direct Repression of Cyclin D1 by SIP1 Attenuates Cell Cycle Progression in Cells Undergoing an Epithelial Mesenchymal Transition
   Jakob Mejlvang, Marina Kriajevska, Cindy Vandewalle, Tatyana Chernova, A. Emre Sayan, Geert Berx, J. Kilian Mellon, and Eugene Tulchinsky
   Mol. Biol. Cell, Nov 2007; 18: 4615 - 4624.
8. Irradiation Causes Acute and Long-Term Spermatogonial Depletion in Cultured and Xenotransplanted Testicular Tissue from Juvenile Nonhuman Primates
   Kirsi Jahnukainen, Jens Ehmcke, Mirja Nurmio, and Stefan Schlatt
   Endocrinology, Nov 2007; 148: 5541 - 5548.
9. Cooperation between p27 and p107 during Endochondral Ossification Suggests a Genetic Pathway Controlled by p27 and p130
   Nancy Yeh, Jeffrey P. Miller, Tripti Gaur, Terence D. Capellini, Janko Nikolich-Zugich, Carmen de la Hoz, Licia Selleri, Timothy G. Bromage, Andre J. van Wijnen, Gary S. Stein, Jane B. Lian, Anxo Vidal, and Andrew Koff
   Mol. Cell. Biol., Jul 2007; 27: 5161 - 5171.
10. Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation
    Amaya Miquelajauregui, Tom Van de Putte, Alexander Polyakov, Anjana Nityanandam, Sridhar Boppana, Eve Seuntjens, Anton Karabinos, Yujiro Higashi, Danny Huylebroeck, and Victor Tarabykin
    PNAS, Jul 2007; 104: 12919 - 12924.

Anti-Bromodeoxyuridine-Fluorescein formalin grade

1. Nuclear envelope defects cause stem cell dysfunction in premature-aging mice
   Jesús Espada, Ignacio Varela, Ignacio Flores, Alejandro P. Ugalde, Juan Cadiñanos, Alberto M. Pendás, Colin L. Stewart, Karl Tryggvason, María A. Blasco, José M.P. Freije, and Carlos López-Otín
   J. Cell Biol., Apr 2008; 181: 27 - 35.
2. Mitotic functions for SNAP45, a subunit of the small nuclear RNA activating protein complex SNAPc
   Mayilvahanan Shanmugam and Nouria Hernandez
   J. Biol. Chem., Mar 2008; 10.1074/jbc.M800833200.
3. Loss of the BH3-only protein Bmf impairs B cell homeostasis and accelerates irradiation–induced thymic lymphoma development
   Verena Labi, Miriam Erlacher, Stephan Kiessling, Claudia Manzl, Anna Frenzel, Lorraine O'Reilly, Andreas Strasser, and Andreas Villunger
   J. Exp. Med., Mar 2008; 205: 641 - 655.
4. Ku is involved in cell growth, DNA replication and G1-S transition
   Emmanouil Rampakakis, Domenic Di Paola, and Maria Zannis-Hadjopoulos
   J. Cell Sci., Mar 2008; 121: 590 - 600.
5. Oxygen regulation of arterial smooth muscle cell proliferation and survival
   Julie Basu Ray, Sara Arab, Yupu Deng, Peter Liu, Linda Penn, David W. Courtman, and Michael E. Ward
   Am J Physiol Heart Circ Physiol, Feb 2008; 294: H839 - H852.
6. Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin
   Melissa M. Rhodes, Prapaporn Kopsombut, Maurice C. Bondurant, James O. Price, and Mark J. Koury
   Blood, Feb 2008; 111: 1700 - 1708.
7. Neurotrophic Factor Neurotrophin-4 Regulates Ameloblastin Expression via Full-length TrkB
   Keigo Yoshizaki, Shinya Yamamoto, Aya Yamada, Kenji Yuasa, Tsutomu Iwamoto, Emiko Fukumoto, Hidemitsu Harada, Masahiro Saito, Akihiko Nakasima, Kazuaki Nonaka, Yoshihiko Yamada, and Satoshi Fukumoto
   J. Biol. Chem., Feb 2008; 283: 3385 - 3391.
8. Retinoblastoma tumor suppressor protein–dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit
   Alexandre Blais, Chris J.C. van Oevelen, Raphaël Margueron, Diego Acosta-Alvear, and Brian David Dynlacht
   J. Cell Biol., Dec 2007; 179: 1399 - 1412.
9. Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres
   Irene Siegl-Cachedenier, Ignacio Flores, Peter Klatt, and Maria A. Blasco
   J. Cell Biol., Oct 2007; 179: 277 - 290.
10. Hypoxia-Inducible Factor Linked to Differential Kidney Cancer Risk Seen with Type 2A and Type 2B VHL Mutations
    Lianjie Li, Liang Zhang, Xiaoping Zhang, Qin Yan, Yoji Andrew Minamishima, Aria F. Olumi, Mao Mao, Steven Bartz, and William G. Kaelin, Jr.
    Mol. Cell. Biol., Aug 2007; 27: 5381 - 5392.

Anti-Bromodeoxyuridine-Peroxidase, Fab fragments formalin grade

1. Fibroblast Growth Factor-2 Regulates Myocardial Infarct Repair: Effects on Cell Proliferation, Scar Contraction, and Ventricular Function
   Jitka A.I. Virag, Marsha L. Rolle, Julia Reece, Sandrine Hardouin, Eric O. Feigl, and Charles E. Murry
   Am. J. Pathol., Nov 2007; 171: 1431 - 1440.
2. Poly(ADP-ribose) polymerase-1 (PARP-1) controls lung cell proliferation and repair after hyperoxia-induced lung damage
   Alessandra Pagano, Isabelle Métrailler-Ruchonnet, Michel Aurrand-Lions, Monica Lucattelli, Yves Donati, and Constance Barazzone Argiroffo
   Am J Physiol Lung Cell Mol Physiol, Sep 2007; 293: L619 - L629.
3. Cyclosporine A induces senescence in renal tubular epithelial cells
   Paul Jennings, Christian Koppelstaetter, Sonia Aydin, Thomas Abberger, Anna Maria Wolf, Gert Mayer, and Walter Pfaller
   Am J Physiol Renal Physiol, Sep 2007; 293: F831 - F838.
4. Rotary Suspension Culture Enhances the Efficiency, Yield, and Homogeneity of Embryoid Body Differentiation
   Richard L. Carpenedo, Carolyn Y. Sargent, and Todd C. McDevitt
   Stem Cells, Sep 2007; 25: 2224 - 2234.
5. LIM-homeodomain proteins Lhx1 and Lhx5, and their cofactor Ldb1, control Purkinje cell differentiation in the developing cerebellum
   Yangu Zhao, Kin-Ming Kwan, Christina M. Mailloux, Woon-Kyu Lee, Alexander Grinberg, Wolfgang Wurst, Richard R. Behringer, and Heiner Westphal
   PNAS, Aug 2007; 104: 13182 - 13186.
6. Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation
   Erin Lepic, Dylan Burger, Xiangru Lu, Wei Song, and Qingping Feng
   Am J Physiol Cell Physiol, Dec 2006; 291: C1240 - C1246.
7. Injection of IL-12- and IL-18-encoding plasmids ameliorates the autoimmune pathology of MRL/Mp-Tnfrsf6lpr mice: synergistic effect on autoimmune symptoms
   Detlef Neumann, Thomas Tschernig, Daniela Popa, Andreas Schmiedl, Guillermo Pérez de Lema, Klaus Resch, and Michael Uwe Martin
   Int. Immunol., Dec 2006; 18: 1779 - 1787.
8. Activation of Mammalian Target of Rapamycin Signaling Promotes Cell Cycle Progression and Protects Cells from Apoptosis in Mantle Cell Lymphoma
   Evangelia Peponi, Elias Drakos, Guadalupe Reyes, Vasiliki Leventaki, George Z. Rassidakis, and L. Jeffrey Medeiros
   Am. J. Pathol., Dec 2006; 169: 2171 - 2180.
9. Rac1 Is Crucial for Hair Follicle Integrity but Is Not Essential for Maintenance of the Epidermis
   Anna Chrostek, Xunwei Wu, Fabio Quondamatteo, Rong Hu, Anna Sanecka, Catherin Niemann, Lutz Langbein, Ingo Haase, and Cord Brakebusch
   Mol. Cell. Biol., Sep 2006; 26: 6957 - 6970.
10. Activation of Mammalian Target of Rapamycin Signaling Pathway Contributes to Tumor Cell Survival in Anaplastic Lymphoma Kinase–Positive Anaplastic Large Cell Lymphoma
    Francisco Vega, L. Jeffrey Medeiros, Vasiliki Leventaki, Coralyn Atwell, Jeong Hee Cho-Vega, Ling Tian, Francois-Xavier Claret, and George Z. Rassidakis
    Cancer Res., Jul 2006; 66: 6589 - 6597.

Appendix

12

Apoptosis-related Parameters – Abbreviations and References

Parameter	Full length name	Reference	Roche Applied Science 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.	
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. 11 086 090 001 Calpain inhibitor II, Cat. No. 11 086 103 001
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. 11 835 238 001

Parameter	Full length name	Reference	Roche Applied Science product
crm A	Cytokine response modifier A	■ Zhou Q. et al. (1997) *J. Biol. Chem.* **272**, 7797. ■ Ogasawara J. et al. (1993) *Nature* **364**, 806.	
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. 11 835 246 001 Cell Death Detection ELISA^{PLUS}, Cat. No. 11 744 425 001 Cell Death Detection ELISA, Cat. No. 11 544 675 001 Cellular DNA Fragmentation ELISA, Cat. No. 11 585 045 001 *In Situ* Cell Death Detection Kit, Fluorescein, Cat. No. 11 684 795 910 In Situ Cell Death Detection Kit, TMR, Cat. No. 12 156 792 910 *In Situ* Cell Death Detection Kit, AP, Cat. No. 11 684 809 910 *In Situ* Cell Death Detection Kit, POD, Cat. No. 11 684 817 910
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. 11 835 238 001
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. 10 107 921 001 DNase I, recombinant RNase free, Cat. No. 04 716 728 001 DNase I, grade II, Cat. No. 10 104 159 001
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.	

Parameter	Full length name	Reference	Roche Applied Science product
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.	
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.	
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. 11 457 756 001
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.	

Parameter	Full length name	Reference	Roche Applied Science product
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.	
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.	
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. 03 703 126 001 Annexin-V-FLUOS, Cat. No. 11 828 681 001 Annexin-V-Biotin, Cat. No. 11 828 690 001
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. 11 371 843 001, 11 088 939 001
TNF-α receptor		■ Nagata S. (1997) *Cell*, **88**, 355. ■ Tartaglia L. A. et al. (1993) *Cell* **74**, 845.	TNF-α, mouse, Cat. No. 11 271 156 001 TNF-a ELISA, human, Cat. No. 11 425 943 001

Parameter	Full length name	Reference	Roche Applied Science product
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.	
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 10: Published sources that contain more information about the components of the apoptosis pathways.

Synonyms

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	Synonyms
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)

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	–

Ordering Information

Product	Cat. No.	Pack Size
Products for Measuring Apoptosis—Caspase Activity		
Anti-Poly (ADP-Ribose) Polymerase (Anti-PARP)	11 835 238 001	100 µl
Caspase 3 Activity Assay	12 012 952 001	1 kit (96 tests)
Homogeneous Caspases Assay, fluorimetric HTS	03 005 372 001 12 236 869 001	100 to 400 tests 1,000 to 4,000 tests
M30 CytoDEATH*	12 140 322 001 12 140 349 001	50 tests 250 tests
M30 CytoDEATH, Fluorescein*	12 156 857 001	250 tests
Products for Measuring Apoptosis—Membrane Alterations		
Annexin-V-FLUOS	11 828 681 001	250 tests
Annexin-V-FLUOS Staining Kit	11 858 777 001 11 988 549 001	1 kit (50 tests) 1 kit (250 tests)
Annexin-V-Alexa 568	03 703 126 001	250 tests
Annexin-V-Biotin	11 828 690 001	250 tests
Products for Measuring Apoptosis—DNA Fragmentation		
Apoptotic DNA Ladder Kit	11 835 246 001	1 kit (20 tests)
Cell Death Detection ELISA^{PLUS}	11 774 425 001	1 kit (96 tests)
Cell Death Detection ELISA^{PLUS}, 10 x HTS	11 920 685 001	1 kit (10 x 96 tests)
Cell Death Detection ELISA	11 544 675 001	1 kit (96 tests)
In Situ Cell Death Detection Kit, AP	11 684 809 910	1 kit (50 tests)
In Situ Cell Death Detection Kit, Fluorescein	11 684 795 910	1 kit (50 tests)
In Situ Cell Death Detection Kit, POD	11 684 817 910	1 kit (50 tests)
In Situ Cell Death Detection Kit, TMR red	12 156 792 910	1 kit (50 tests)
TUNEL Enzyme	11 767 305 001	2 x 50 µl (20 tests)
TUNEL Label Mix	11 767 291 910	3 x 550 µl (30 tests)
TUNEL AP	11 772 457 001	3.5 ml (70 tests)
TUNEL POD	11 772 465 001	3.5 ml (70 tests)
TUNEL Dilution Buffer	11 966 006 001	2 x 10 ml
Products for Measuring Cytotoxicity		
Cytotoxicity Detection Kit^{PLUS} (LDH) HTS	04 744 926 001 04 744 934 001	1 kit (400 tests) 1 kit (2,000 tests)
Cytotoxicity Detection Kit (LDH)	11 644 793 001	1 kit (2,000 tests)
Cellular DNA Fragmentation ELISA	11 585 045 001	1 kit (500 tests)
Products for Measuring Cell Proliferation		
5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I	11 296 736 001	1 kit (100 tests)
5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II	11 299 964 001	1 kit (100 tests)
5-Bromo-2'-deoxy-uridine Labeling and Detection Kit III	11 444 611 001	1 kit (1,000 tests)
Cell Proliferation ELISA, BrdU (colorimetric)	11 647 229 001	1 kit (1,000 tests)
Cell Proliferation ELISA, BrdU (chemiluminescent)	11 669 915 001	1 kit (1,000 tests)
In Situ Cell Proliferation Kit, FLUOS	11 810 740 001	1 kit (100 tests)
Cell Proliferation Kit I (MTT)	11 465 007 001	1 kit (2,500 tests)
Cell Proliferation Kit II (XTT)	11 465 015 001	1 kit (2,500 tests)
Cell Proliferation Reagent WST-1 HTS	05 015 944 001 11 644 807 001	8 ml (800 tests) 25 ml (2,500 tests)
ATP Bioluminescence Assay Kit HS II	11 699 709 001	1, 000 assays (microplate) 500 assays (tube)
ATP Bioluminescence Assay Kit CLS II	11 699 695 001	1, 600 assays (microplate) 800 assays (tube)
Anti-Bromodeoxyuridine, formalin grade	11 170 376 001	50 µg
Anti-Bromodeoxyuridine-Fluorescein	11 202 693 001	50 µg
Anti-Bromodeoxyuridine-Peroxidase, Fab fragment	11 585 860 001	15 units

HTS = Suitable for high-throughput screening.

*The M30 antibody is made under a license agreement from Peviva AB, Sweden. US Patent No. 6,296,850; 6,706,488 and 6,716,968.

Trademarks

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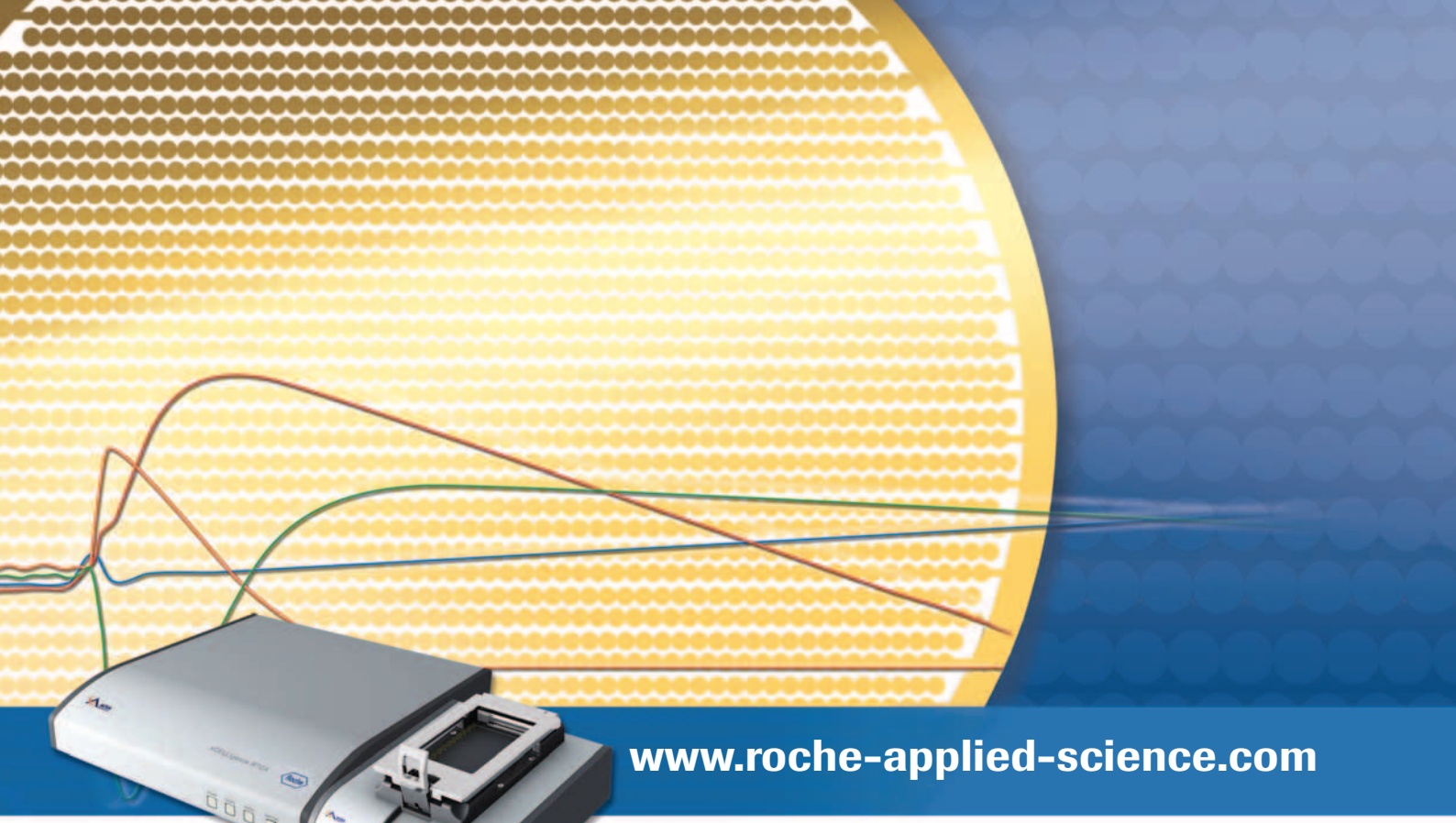

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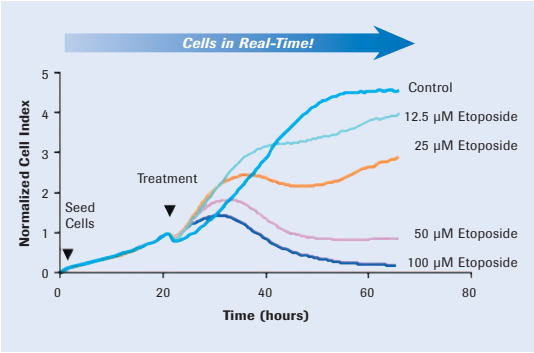

Figure 1: Real-time monitoring of cytotoxicity through DNA damage. Etoposide is a DNA damaging agent which induces apoptosis in high concentrations, while at lower concentrations it leads to S-Phase and/or G2 arrest.

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