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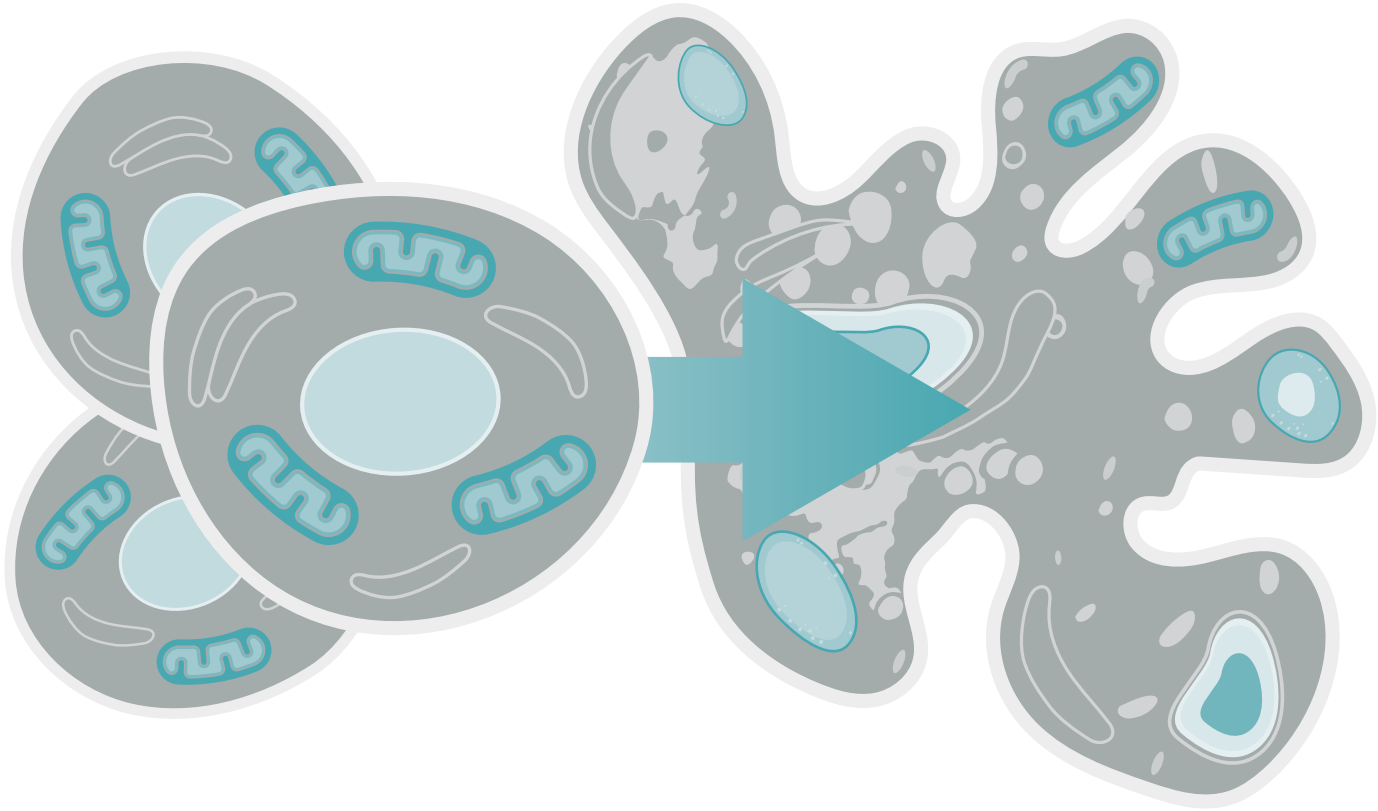
Cell health assay guide

How to identify your best approach to measuring cell viability, cytotoxicity and apoptosis



The analysis of cell viability, cytotoxicity, cell cycle state, cell proliferation, and cell death are critical to most cell-based studies. With many different assays available for assessing cell health, it can be difficult to know where to start. Use this guide to understand your options when you need to assay cell health.

Contents

Why measure cell health?

Understanding the general health of your cells is a fundamental aspect in most cell culture experiments and some tissue analysis experiments.

In addition to identifying levels or localization of a particular protein of interest and examining activation of pathways, measuring aspects of general cell functions, such as metabolic rates, cell death, cell proliferation and cell movement, can offer critical insights into the question you're trying to answer.

There are three main reasons why you would want to assess aspects of general cellular behavior:

1. You are investigating the mechanisms involved

Understanding normal, healthy function is critical to understanding the effects of disease. You may want to measure the mechanisms behind continuing cellular activities, such as metabolism or cell movement.

2. You are researching the effect of a drug or disease

This is the most common need for assessing cell health function. Monitoring the parameters of cell function in a model of disease allows researchers to assess if treatment with a compound offers protection or supports recovery. Assessing parameters of cell function can also be used to assess the progression of disease, such as cancer.

The assays described in this guide are often used by researchers looking to understand the effect of a drug or disease, as well as the mechanisms behind that effect. Cell health assays are also used to quantify the response to treatment with a drug or other stimuli.

3. You need to assess your sample for future experiments

Understanding the health of your cellular population is important as it can have a large impact on your results. If you have 1000 cells, but over 50% of them are dead, your experiment is going to have a different outcome that if all 1000 cells were viable. Additionally, cell death mechanisms, such as apoptosis, can present specific markers or proteins, which could alter your results. Measuring cell health is therefore key to understanding your starting point so that you can assess the effect of your experiment on your cells. If cell viability is low, further experiments on the culture would not be recommended.

You may have a need to assess the health of your cells for one of the reasons above, or potentially all three. Whatever your sample type and the question you are asking, this guide will take you on a journey to find the right assay or combination of assays to measure the health of your cells.

Selecting the right methods for your needs

It is almost always most effective to combine several different methods of assessing cell health, as no one method gives a perfect view of cell viability, proliferation, or cell death. Each method has its own advantages and disadvantages. Selecting the most appropriate method for your needs will depend on factors such as:

What you want to measure

Understand what it is you want to measure and define what your endpoint will be. The most appropriate assay will depend on whether you're looking to determine the number of living versus dead cells, quantify cell damage or understand the underlying mechanisms. Using multiple endpoints may be the best approach.

Many viability assays, such as MTT, don't measure cell number but overall metabolic activity. Therefore, your result may indicate a change in cell number due to either cell proliferation or cell death, or a combination of the two, as well as a change in metabolism. Depending on what you're looking to measure, you may need to combine MTT with another assay to be sure.

Knowing the markers of cell viability, cytotoxicity and apoptosis will enable you to select an assay that will help you answer your question.

The model system that you're using

It is important to identify and consider any aspects of the model system that you're using that will affect the outcome of your assay. For example, cell lines have a higher metabolic rate than primary cells.

In addition, suspension cells will benefit from an assay that has minimal or no steps that require removal of the media as this will reduce potential cell loss during these wash steps.

Number and type of cells

If you have a lot of samples, this is likely going to affect which assay you choose. Some assays require manual counting, such as the viability assay trypan blue, which is more hands-on and time consuming than MTT, for example, and so is not practical for large numbers of cells.

On the other hand, if you have a low number of cells, you will need an assay with a higher sensitivity.

Whether you're using cell culture or tissue samples

You sample type will also influence your choice of assays due to factors such as the inability of some reagents to penetrate through multiple layers of cells.

For example, TUNEL assays and proliferation assays such as BrdU are commonly used for tissue analysis, whereas MTT assays are more commonly used in cell culture.

Many of the cell functions being measured with the assays described in this guide, such as metabolism, proliferation and apoptosis are interlinked[1] by a complex web of processes, therefore the best approach to selecting assays is to thoroughly understand how they work.

How frequently should I assess cell health?

At a minimum, it is recommended that you assay cell health prior to your experiment and at any critical timepoints, such as cell passaging.

Depending on the experiment you're doing and the reason why you're measuring the health of your cells, you may want to assay cell health more often, to provide insight into ongoing changes or identify any issues with your cell culture process. Consider, however, that most assays do not allow for continued culture. Frequent assays could therefore risk using up all your cells, especially slow growing or more precious cultures.

Instrumentation

Available instrumentation will be another consideration when reviewing your assay options. Analysis commonly requires a fluorescence microscope, flow cytometer or microplate reader.

Most assays described in this guide utilize fluorescence, luminescence or absorbance as endpoints, therefore a well-optimized plate reader capable of detecting the right endpoint is required. It's also worth considering what kind of plates to use – generally clear plates are recommended for absorbance assays, white for luminescence assays and black for fluorescence assays.

Cell viability assays

Overview of cell viability assays

Briefly, cell viability is the number of live, healthy cells in a sample[2]. Calculated as a percentage of control, 80-95% cell viability indicates a healthy culture. This number may be slightly lower in suspension cultures as dead cells do not get washed away during trypsinizing.

The following assays can be used to measure cell viability:

- Metabolic assays
- Cytotoxicity assays
- Cell proliferation and cell cycle assays

These assays rely on metabolic activity, ATP content or cell proliferation as indicators of cell health. Loss of membrane integrity and other markers of cell damage or death can be used to measure cell viability indirectly.

See below to learn more about these assay methods, or review our most popular assay kits here:

- Cell viability assays: **MTS** , **resazurin**, **TMRE**, **calcein violet**, and **ATP luminescence**.
- Cytotoxicity assays: **LDH assay**, **DRAQ7®**, and our **combined dye live:dead cell assay**.
- Cell proliferation and cell cycle assays: **EdU**, **propidium iodide**, and **CFSE**.

What's the difference between a viability assay and a cytotoxicity assay?

Although metabolic assays, cytotoxicity assays and cell proliferation and cell cycle assays can all be used to assess viability, they each provide different perspectives.

Cell viability assays identify markers of healthy cell function, such as metabolism, DNA synthesis and cell division, and whilst these are used to measure the number of living cells, they can also be used as an indication of cytotoxicity because they can tell you if the level of metabolic activity and healthy function has decreased. Viability assays only tell you the number of remaining viable cells in a sample, and not whether this number is the result of cytotoxic or antiproliferative effects.

In contrast, cytotoxicity assays measure the toxicity of a chemical on cells by detecting markers of severe cell damage, such as loss of membrane integrity. It's possible to use cytotoxicity assays to measure cell viability indirectly.

Combining a viability assay with a cytotoxic assay may be a good solution if you're looking for a fuller picture of what's happening in your cells.

Metabolic assays

Metabolism is a complex process that lies at the core of biology. Changes to metabolism are involved in a huge range of outcomes, from cancer to neurodegeneration, and more.

Within the cell, metabolism encompasses multiple enzymatic reactions that produce energy and maintain life. Measuring these processes can be a critical part of research, whether it's to inform future experiments or to assess disease progression.

Metabolic assays indicate cell viability by quantifying the presence of enzymes and proteins involved in metabolism. Cells undergoing proliferation increase their metabolic rate[3], therefore metabolic assays can also be used to assess proliferation.

Factors to consider when selecting a metabolic assay include:

- Assay sensitivity
- Signal to noise ratio
- Ease of use
- Reagent stability[4]

Generally, luminescence-based assays are more sensitive than fluorescence or absorbance-based assays.

Metabolism assays are also used for purposes other than to assess viability, such as to measure metabolites and metabolic enzymes. See our **guide to measuring metabolism**.

1. Dye reduction assays

Dye reduction assays are broadly split into two types: tetrazolium assays and resazurin assays. To discover the proportion of viable cells in a population, these assays incubate a reagent with the cell sample. The compounds in the reagent form a dye in a metabolically active environment, and the resulting color change can be quantified to indicate the extent of cell viability (Figure 1).

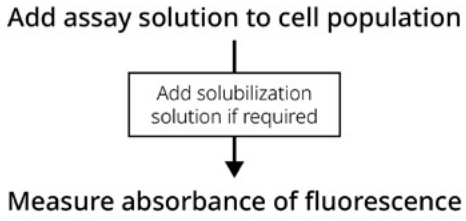


Figure 1. Dye reduction assay overview.

Tetrazolium assays

Tetrazolium cell viability assays rely on cellular dehydrogenases to form a colored formazan product, which is measured by absorbance.

The most commonly used tetrazolium assays are split into two groups:

- MTT assay – a positively charged tetrazolium salt that readily penetrates cells.
- MTS, XTT and WST-1 assays – negatively charged tetrazolium salts that do not penetrate cells easily[5].

MTT assay

The MTT assay was developed to provide a non-radioactive alternative to the tritiated thymine incorporation assay. However, the MTT assay is a measure of cell viability and not cell proliferation.

MTT is converted into formazan via an electron transfer reaction with substrates in the cell, such as NADH and NADPH (Figure 2). However, the resulting formazan crystals are insoluble, and form as precipitate inside the cells and culture medium. For the color change to be detected via a spectrophotometer, the crystals must be solubilized. Solutions such as DMSO and SDS can be used to dissolve the formazan crystals however this, along with the toxic nature of MTT, means that the MTT assay must be used as an endpoint assay. MTT is also light-sensitive, therefore must be kept and used in the dark.

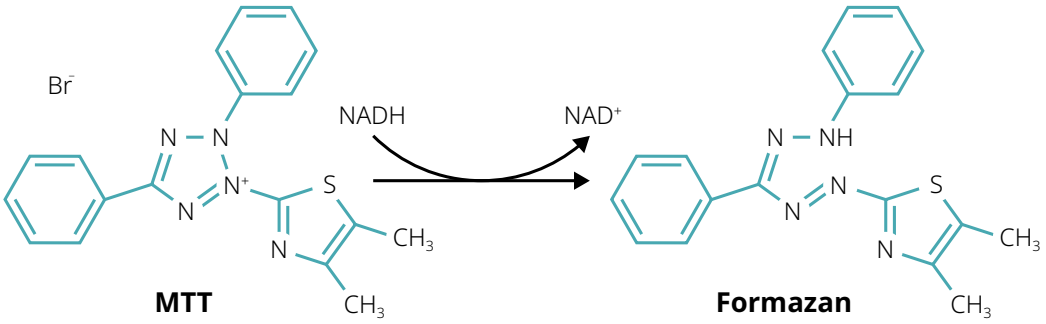


Figure 2. Reduction of MTT in metabolically active cells to form insoluble formazan.

Advantages:

- Simple
- Easy to use
- Widely used

Disadvantages:

- Toxicity
- Susceptible to chemical interference
- Less sensitive than fluorescent and luminescent cell viability assays

MTS, XTT and WST-1 assays

The MTS, XTT and WST-1 assays produce products that are soluble in culture medium. Although this removes the solubilization step, these tetrazolium salts do not easily penetrate cell membranes. Therefore, an intermediate electron acceptor reagent is needed. This intermediate reagent enters the cell, is itself reduced, and then exits the cell, where it can then transfer electrons to the tetrazolium salt. The salt is then converted to a soluble formazan product (Figure 3). However, the intermediate reagent may be toxic to the cells, so it is important to optimize the reaction depending on your sample and assay conditions.

As the product is soluble, multiple readings can be taken from the same plate across a time-course, although incubations longer than 4 h should be avoided. Additionally, background readings for MTS, XTT and WST-1 assays are generally higher than those for MTT assays, with absorbance readings of 0.3 and 0.05 respectively. However, these readings are dependent on culture medium and pH.

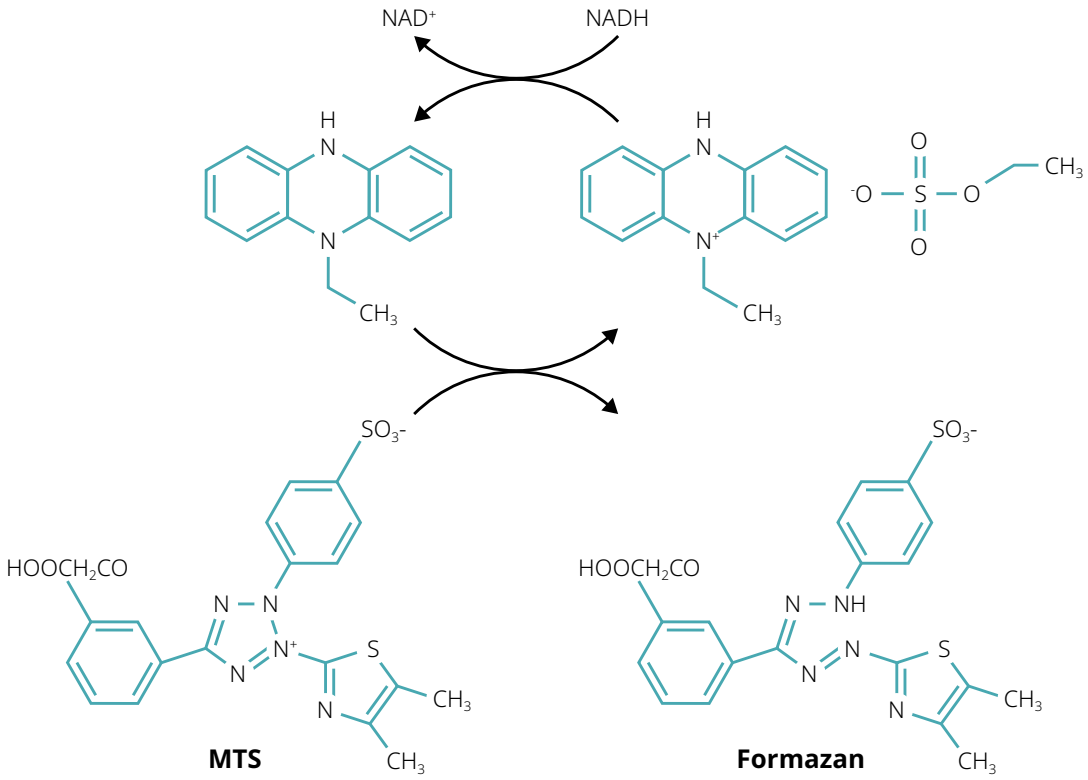


Figure 3. Reduction of MTS to form soluble formazan via the intermediate electron acceptor pheazine ethyl sulfate (PES).

Advantages:

- Easy to use
- No DMSO dissolution required
- More sensitive and accurate than MTT (WST-1 being the most sensitive)

Disadvantages:

- Intermediate reagent required

Assay	Instrument	Notes
MTT	Plate reader	Original tetrazolium assay; still very popular. Only tetrazolium assay that needs a wash / solubilization step.
MTS		Most popular assay. More heavily used than WST-1.
WST-1		More sensitive than MTT, XTT or MTS.
Cell Counting Kit-8 / CCK-8 / WST-8		–
XTT assay		–

Resazurin assays

Resazurin assays are based on the same principle as tetrazolium assays and use electron transfer to convert one compound into another (Figure 4). In this case, resazurin forms a dark-blue solution when dissolved in physiological buffers, which is converted to resorufin.

Resorufin is a pink, fluorescent product, and, as fluorescence is generally more sensitive than absorbance measurements, this is an advantage over tetrazolium assays. Additionally, resazurin can penetrate cells, meaning that an intermediate electron acceptor is not required, although inclusion can speed up signal generation.

Resazurin assays are relatively inexpensive and can be used in combination with other methods to achieve a greater understanding of the cytotoxicity mechanisms. Despite these advantages, care must be taken to avoid fluorescent interference from other compounds.

As with the other dye reduction assays, extended incubation times are not recommended and should be optimized to balance sensitivity and toxicity.

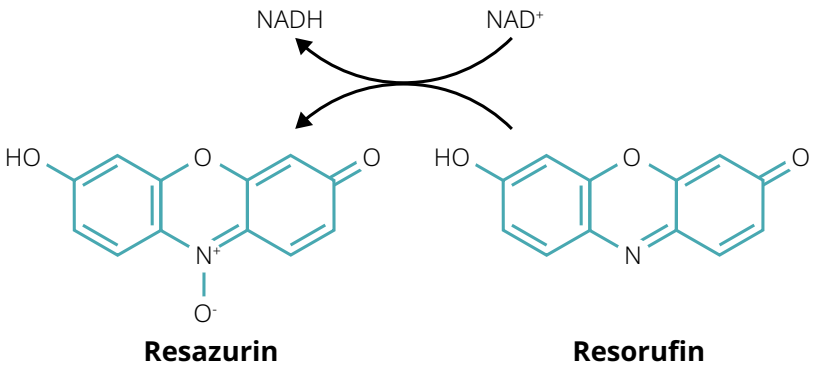


Figure 4. Reduction of resazurin to resorufin in viable cells.

Advantages:

- Relatively inexpensive
- Increased sensitivity over tetrazolium assays

Disadvantages:

- Risk of fluorescence interference from other compounds

Assay	Instrument	Notes
Resazurin	Plate reader, microscope, flow cytometer	Fluorometric (Ex/Em 535–560/560–615) or colorimetric. No-wash assay. Fluorescent readout enables multiplexing with other assays.

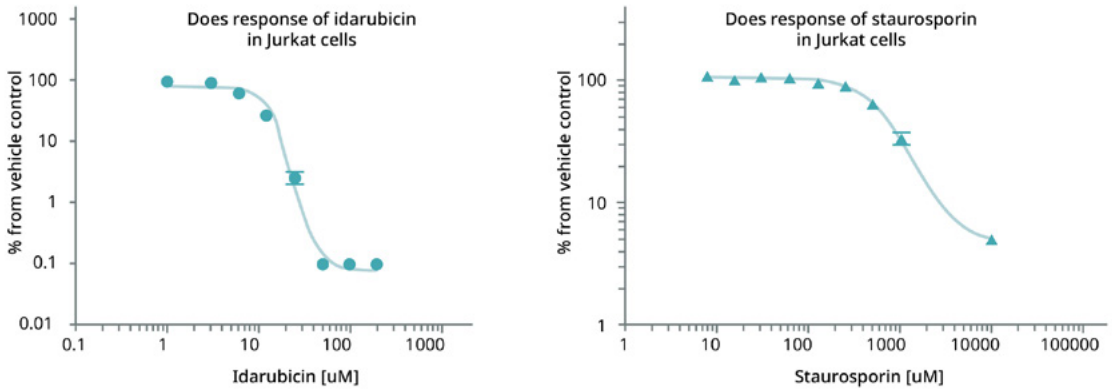


Figure 5. Jurkat cells treated with idarubicin (left) or staurosporin (right) were analyzed with Resazurin assay **(ab129732)**.

2. Mitochondrial membrane potential-dependent dyes

Membrane potential is closely linked to a cell's ability to generate ATP and therefore can be used as an indicator of cell health. There are several dyes available that accumulate in mitochondria due to the mitochondrial membrane potential and you can use these to identify viable cells.

A loss of membrane potential and loss of staining is used to assay for apoptosis.

Advantages:

- TMRE is reversible and suitable for live cell analysis
- JC-1 and JC-10 are ideal for comparative measurements

Disadvantages:

- Some assays not suitable for fixed samples
- Controls and technical details need careful attention to ensure performance[6]

Assay	Instrument	Notes
TMRE/TMRM	Plate reader, microscope, flow cytometer	Most popular Abcam mitochondrial membrane dye assay. Ex/Em 549/575 nm. Washed out of mitochondria after fixation.
JC-1/JC-10		JC-1 (Ex/Em 530/530–570) and JC-10 (Ex/Em 590/520–570) form red aggregates at high concentrations (unaggregated dye is green). Loss of membrane potential causes loss of dye and increased green fluorescence. JC-10 is more soluble than JC-1. Best suited for endpoint analysis. Washed out after fixation.
Mitotracker Red		Ex/Em 579 /599.Not washed out after fixation.
Rhodamine 123		Ex/Em 507/529.Washed out after fixation.
MitoNIR		Ex/Em 635/660.
MitoOrange	Plate reader, flow cytometer	Ex/Em 540/590.

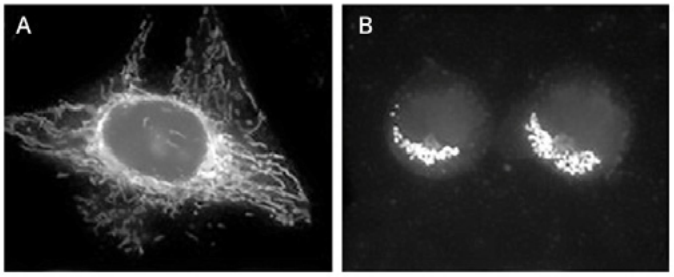


Figure 6. Cell staining with TMRE kit (**ab113852**). A: Healthy HeLa cells. B: Healthy Jurkat cells.

3. Esterase cleavage

Calcein and similar hydrophobic dyes diffuse into cells and are cleaved by intracellular esterases in live cells, providing a measurement of enzymatic activity which is a proxy for metabolic health and membrane integrity. The hydrophilic fluorescent product is retained within the cell.

Advantages:

- Non-toxic

Disadvantages:

- Potential fluorescent interference from other compounds

Assay	Instrument	Notes
Calcein AM	Plate reader, microscope, flow cytometer	Ex/Em 495 / 515 nm
Calcein violet AM	Plate reader, microscope, flow cytometer	Ex/Em 405/460 nm
Esterase-cleaved blue	Plate reader	Ex/Em 405/460360/450 nm
Esterase-cleaved green	Plate reader, microscope	Ex/Em 490/520 nm

4. ATP assays

Most assays use a cell membrane permeabilization agent to release ATP; light is produced using ATP-dependent luciferase. Other ATP assays use the ATP-dependent phosphorylation of glycerol (or other substrates).

Advantages:

- Sensitive
- Fast

Disadvantages:

- Requires cell lysis

Assay	Instrument	Notes
Luminescence ATP assay	Luminometric plate reader	No-wash assay.
Luminescence ADP/ATP assay		No-wash assay. After ATP analysis, ADP is converted to ATP for detection.
ATP phosphorylation assay	Plate reader	No-wash assay used with cell lysates. Not as sensitive as luminescence assays. Fluorometric (Ex/Em 535/587 nm) is more sensitive than colorimetric.

5. Oxygen consumption and glycolysis assays

The rate of oxygen consumption indicates the level of cellular metabolic activity. Analysis of intracellular oxygen levels and glycolysis activity allow deeper investigation of cell health.

Advantages:

- Non-destructive and fully reversible, enabling assay time courses and drug treatments.
- Assays do not require specialized equipment or probes; signal is measured in a standard fluorescence or TR-F plate reader.
- Can be used with isolated mitochondria, cell cultures, tissues, enzyme preparations, and small organisms.

Disadvantages:

- May require alteration of atmospheric conditions in the incubator

Assay	Instrument	Notes
Extracellular oxygen consumption	Plate reader	No-wash assay. Dye signal (Ex/Em 380/650 nm) increases as respiration lowers O_2 levels. No need for specialized instrument.
Intracellular oxygen levels		Dye fluorescence (Ex/Em 340/642) is quenched by intracellular oxygen. No-wash assay.
Glycolysis activity		No-wash assay. Lactate production causes extracellular acidification and increased dye fluorescence (Ex/Em 340-380/615 nm).

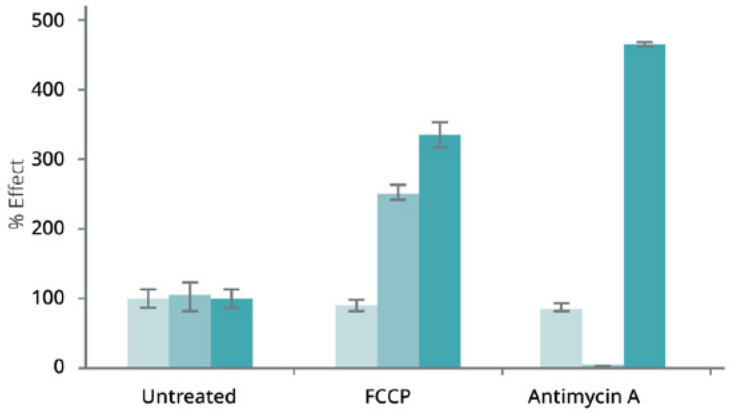


Figure 7. HepG2 cells treated with antimycin A and FCCP and tested with assays for ATP (light green, **ab113849**), oxygen consumption (green, **ab197243**) and glycolysis (dark green **ab197244**).

Cytotoxicity assays

Cytotoxicity is a measure of how toxic a substance is to a cell. In response to a toxic substance, a cell may either cease to proliferate, or die as a result of apoptosis or necrosis.

Cytotoxicity assays are commonly used to screen for any cytotoxic effects of a compound in drug screening. To determine the toxicity of a chemical agent, the assays detect markers of severe cellular damage or cell death, most commonly, damage to the cell membrane.

Methods that assess damage to the cell membrane include:

- Measurement of the activity of enzymes that leak into the extracellular medium
- Amine-reactive dyes bind weakly to the surface of live cells and create a brighter staining in dead cells
- Membrane impermeable dyes that enter and stain cells upon membrane damage. Membrane impermeable dyes. Often used with dyes that stain live cells, in combined live:dead cell assays.
- Membrane impermeable dyes that enter and stain cells upon membrane damage. Often used with dyes that stain live cells in combined live:dead cell assays.

In addition to causing cell membrane damage, cytotoxic agents can also affect cells by stopping protein synthesis, irreversibly binding to receptors, or causing other losses of structure or function[7].

Alternatives to methods that assess damage to the cell membrane include the **SRB assay**, which uses the level of binding of the fluorescent Sulforhodamine B dye as a proxy for the number of live cells. The **Crystal violet assay** works similarly. Both methods should be used with adherent cell cultures as they rely on the detachment of adherent cells from cell culture plates during cell death.

See below to learn more about these assay methods, or review our most popular cytotoxicity assay kits including the **LDH assay**, **DRAQ7®**, and our **combined dye live: dead cell assay**.

1. Enzyme leakage

These assays measure the activity of enzymes that leak into the extracellular medium on cell membrane damage. The most popular assay is for lactate dehydrogenase (LDH).

Advantages:

- Fast
- Reliable
- Sensitive
- Non-toxic to healthy cells
- Can be automated for high throughput applications

Disadvantages:

- Susceptible to interference from other compounds including serum (LDH only)

Assay	Instrument	Notes
LDH/Lactate dehydrogenase	Plate reader	LDH oxidizes lactate and a colored, or fluorescent (Ex/Em 535/587 nm), product is formed.
AK/Adenylate kinase		AK converts ADP to ATP with detection via luciferase light-generation. AK activity is not as enduring as LDH.

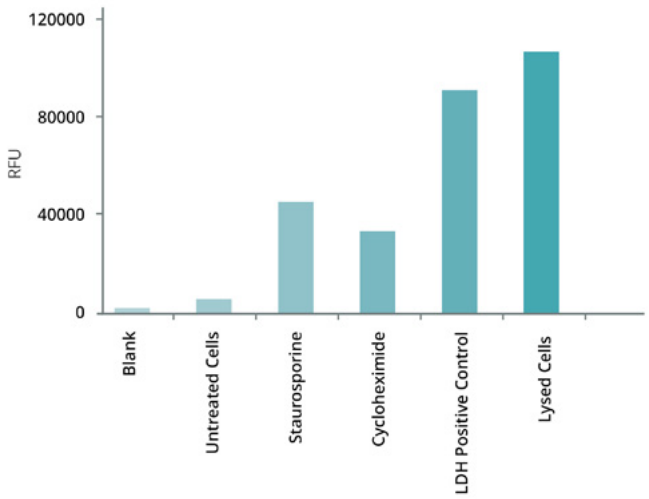


Figure 8. LDH assay used with staurosporine or cycloheximide HeLa cells, untreated cells, LDH positive control, and lysed cells.

2. Membrane impermeable dyes (dye exclusion assays)

These assays use membrane-impermeable fluorescent dyes (mostly DNA stains) that stain cells with damaged cell membranes. They offer a simple and widely used method for determining the membrane integrity of cells in suspension[7].

The once commonly used propidium iodide has largely been replaced by DRAQ7™ and 7-AAD for cell viability assays due to its broad emission spectra and tendency to bind to live cells.

Advantages:

- Simple
- Rapid
- Can be used for low numbers of cells

Disadvantages:

- Trypan blue requires cell counting and is therefore susceptible to error

Assay	Instrument	Notes
DRAQ7™	Flow cytometer, microscope	Ex/Em 633 & 647/665–800 nm. DNA stain.
7-AAD		Ex/Em 488/647 nm. DNA stain.
Propidium Iodide		Ex/Em 536/617 nm. DNA stain. Leaches from cells over time.
Ethidium homodimer-1		Ex/Em 528/617. DNA stain.
Trypan blue	Microscope	Non-fluorescent cell stain. Classic cell viability assay that requires cell counting. Tedious and prone to manual error. Best for small sample numbers.

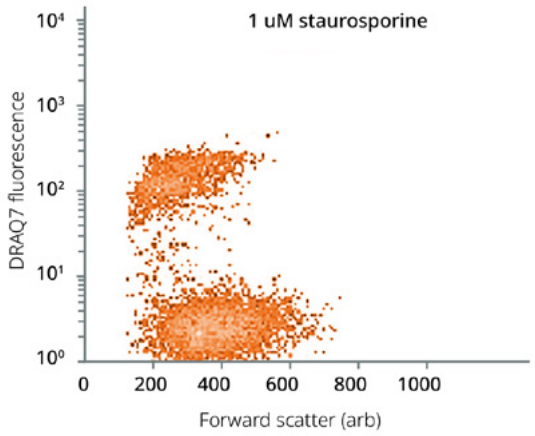


Figure 9. Jurkat cells treated with staurosporine to induce cell death show DRAQ7™ staining (top half of the plot).

3. Amine-reactive dyes for live:dead cell assays

Amine-reactive dyes weakly stain viable cells by binding to cell surface amines and strongly stain membrane-compromised cells by reacting with intracellular amines. Dead and live cells can be differentiated by fluorescence level.

Advantages:

- Accurate
- Clear distinction between dead and live cells
- Fixation compatible – allowing for post-fixation analysis as required for immunophenotyping experiments[8]

Disadvantages:

- Potential background fluorescence
- Requires a flow cytometer

Assay	Instrument	Notes
Amine-reactive dyes	Flow cytometer	Ex/Em 410/450 nm. Fixation compatible (applies to all dyes in this table).
		Ex/Em 408/512 nm
		Ex/Em 398/550 nm
		Ex/Em 353/442 nm
		Ex/Em 498/521 nm
		Ex/Em 547/573 nm
		Ex/Em 583/603 nm
		Ex/Em 649/660 nm

4. Combined dye live:dead cell assays

Multiple dyes can be combined in a single live:dead cell assay to differentiate between dead and live cells. Kits allow for rapid quantitation of cell viability and are often suitable for proliferating and non-proliferating cells.

Advantages:

- Rapid
- More robust and accurate than other viability/cytotoxicity assays

Disadvantages:

- Potential background fluorescence

Assay	Instrument	Notes
Combined dye live:dead cell assays	Flow cytometry or fluorescent microscopy	Popular live and dead cell assay with ethidium homodimer to label dead cells and an esterase-cleaved dye for live cells.
	Readily adapted for a variety of fluorescence platforms such as microplate assays, fluorescence microscope	Fluorometric dual green/red assay, includes a red DNA staining dye for dead cells and a green esterase-cleaved dye for live cells.

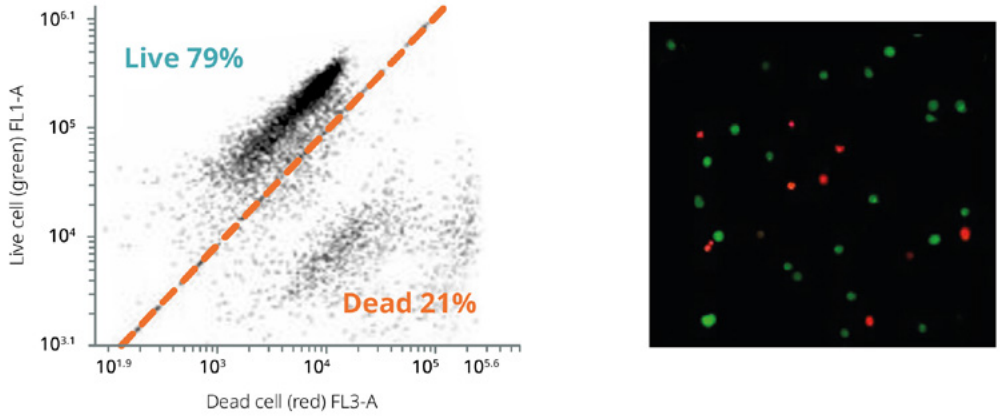


Figure 10. Left: Live:dead cell assay (**ab115347**) used with live (upper left) and dead (lower right) cells. Right: Etoposide treated cells stained with **ab115347**. Live cells stain green and dead cells are red.

Cell proliferation and cell cycle assays

Proliferation is the process by which cells increase in number, usually through the phases of the cell cycle (mitosis). In addition to being key to development and size attainment in organisms, dysfunction of proliferative mechanisms can lead to diseases such as cancer[9].

Cell proliferation assays measure the number of cells in a population that are actively dividing[4], whereas viability assays measure all live cells, which can also be quiescent or senescent. Proliferation assays can additionally provide an indication of cell viability, as only viable cells can proliferate. The number of dividing cells can be measured through analysing DNA levels, DNA synthesis, metabolic activity, or proliferation-specific proteins.

Proliferation can be used as an indication of prognosis in cancer, with a high rate of proliferation associated with aggressive cancers. For this reason, proliferation assays are commonly used in tissue analysis, using well-known markers such as Ki67, proliferating cell nuclear antigen (PCNA), and minichromosome maintenance (MCM)[10] – see protein markers of proliferation below.

Methods include:

- DNA staining dyes for cell cycle analysis
- Dye dilution assays
- Incorporation of nucleoside analogs during DNA synthesis

Our most popular assay kits include: **EdU**, **propidium iodide,** and **CFSE**.

Cell proliferation can also be measured using metabolic assays – see the section in this guide on metabolic assays above.

1. Using DNA content to measure proliferation (cell cycle assays)

DNA-staining dyes are commonly used in flow cytometry to measure the DNA content in cell populations and assay for cell cycle state. These dyes emit fluorescence once bound to DNA. They bind in proportion to the amount of DNA present in the cell, therefore cells that are undergoing DNA replication and preparing to divide (phases S and G2) will take up proportionally more dye and will fluoresce more brightly. Propidium iodide is the mostly commonly used dye.

Advantages:

- Widely used
- Straight forward

Disadvantages:

- Some dyes require cells to be fixed or permeabilized (propidium iodide, DAPI), which is often incompatible with fluorescent proteins and some surface markers.

Dye	Instrument	Notes
Propidium iodide	Flow cytometer	Ex/Em 536/617 nm
Nuclear Green CCS1		Ex/Em 490/525 nm
Nuclear Red CCS1		Ex/Em 490/620 nm
DRAQ5TM		Ex/Em 633&647/665–800 nm
DAPI		Ex/Em 358/461
Hoechst 33342		Ex/Em 350/461
Hoechst 33258		Ex/Em 350/461
7-AAD		Ex/Em 488/647 nm

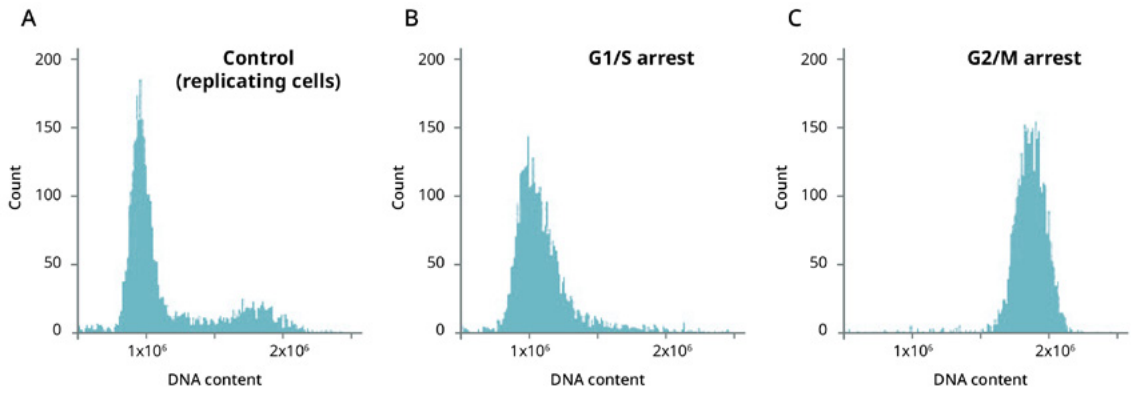


Figure 11. Propidium iodide flow cytometry kit (**ab139418**) used with thymidine (B) and nocodazole (C) treated HeLa cells. Peaks show 2N and 4N DNA content.

2. Incorporation of nucleoside analogs during DNA synthesis

The most reliable and accurate method of assessing cell proliferation is a measurement of DNA-synthesis. This relies on incubating live cells with compounds capable of being incorporated into newly synthesized DNA. These compounds can then be detected with a reporter.

Thymidine analogs are the compound of choice for incorporation into DNA, substituting thymidine during DNA replication. This labels proliferating and daughter cells. However, it is important to be aware that these thymidine analogs can lead to mutations and DNA damage in some instances and thereby affect the cycle cycle[11,12].

Bromodeoxyuridine (BrdU) and ethynyldeoxyuridine (EdU) assays measure the incorporation of BrdU or EdU into newly synthesized DNA during DNA replication. Unlike BrdU, which is detected using antibodies, EdU can be easily directly labeled, either with a fluorescent dye or biotin for colorimetric or fluorometric detection via streptavidin-HRP. Edu staining is consistent with further antibody staining, unlike the harsher BrdU protocol.

This method is suitable for immunohistochemistry (IHC), immunocytochemistry (ICC), ELISAs, flow cytometry, and some multiplex assays. Bromo-deoxyuridine (BrdU) can be detected with immuno-detection, whilst chemical detection is used for ethinyl-deoxyuridine (EdU)[13].

For more information on these assays, review our **cell proliferation guide**.

Advantages:

- Accurate and reliable
- High- and low-throughput options

Disadvantages:

- Protocol can be lengthy and complex
- DNA denaturation prohibits subsequent co-staining experiments (BrdU Only)

Assay	Instrument
EdU	Microscope, flow cytometry, plate reader
BrdU	Plate reader, microscope

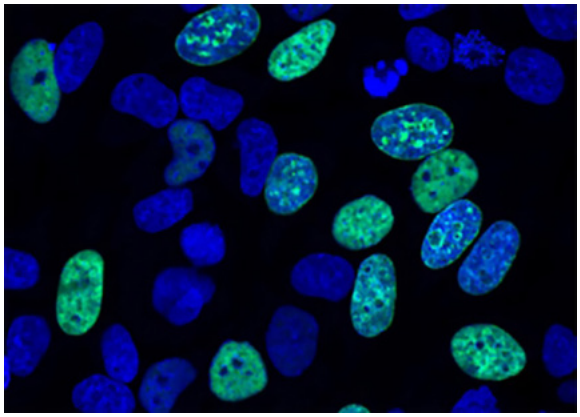


Figure 12. EdU staining of proliferating HeLa cells. DNA (blue) was stained with Hoechst 33342 (**ab145597**). Green cells are EdU + Hoechst-positive.

3. Dye Dilution Assays

The dyes in dye dilution assays (otherwise known as fluorescent dye proliferation assays) are retained within cells over multiple generations. Daughter cells receive half of the dye of parent cells and assays are analyzed on a flow cytometer. Carboxyfluorescein succinimidyl ester (CFSE) is the longest established dye.

Advantages:

- Fluorescence is retained after formaldehyde and alcohol fixation
- Enables multicolor analysis

Disadvantages:

- CFSE is cytotoxic at higher concentrations (non-toxic to cells at appropriate concentrations)

Dye	Instrument	Notes
CFSE	flow cytometer	Ex/Em 492/517 nm. Cytotoxic at higher concentrations.
CytoLabel Blue	flow cytometer, microscope	Ex/Em 403/454 nm
CytoLabel Green		Ex/Em 511/525 nm
CytoLabel Red		Ex/Em 628/643 nm
CytoLabel Orange		

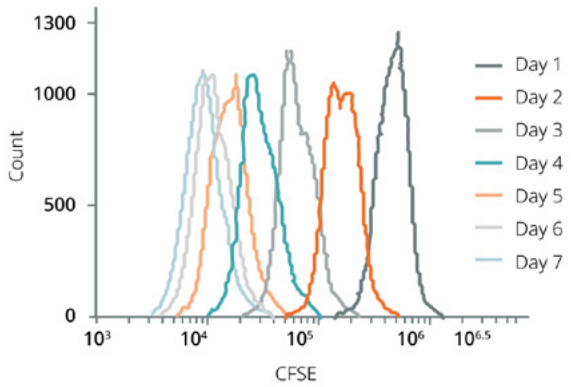


Figure 13. Flow cytometry analysis of CFSE (ab113853) dilution assay.

4. Protein markers of proliferation

Another method to study cell proliferation is by looking at specific proteins that are expressed in proliferating cells but absent from non-proliferating cells. This requires the use of specific primary antibodies against the antigens expressed during proliferation.

These antigens are typically expressed in the perinuclear or nuclear interior regions across all cell cycle phases except G0, making them excellent cellular markers for proliferation. Ki67 is a very popular proliferation marker and is routinely used in pathology labs due to its diagnostic and prognostic power in cancer. PCNA is another common marker, yet multiple studies have shown that Ki67 is more sensitive and specific when evaluating cell proliferation in tumors from various origins[14–17]. A marker growing in prominence is MCM-2, and recent work suggests this may be a better choice for informing cancer prognoses than Ki67 and PCNA[18,19].

However, much of the data is inconclusive regarding a 'best' marker of proliferation, especially in a clinical context.

These immunoassays are excellent for fixed tissue samples and analysis by IHC.

Advantages:

- Accurate and reliable
- Large body of supporting data
- Clinical diagnostic and prognostic value in some cases

Disadvantages:

- Limited high-throughput options
- Scoring of results can be subjective
- Conflicting data around the 'best' marker of cell proliferation in a clinical setting

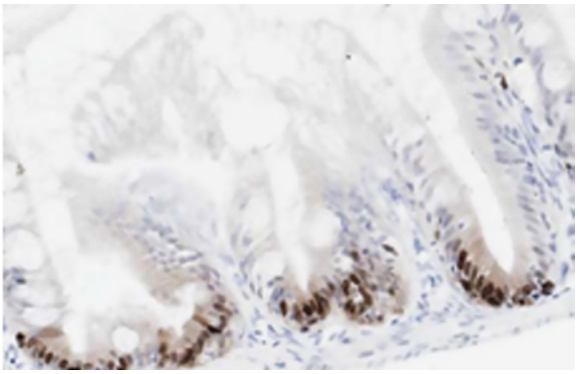


Figure 14. Immunohistochemical analysis of frozen sections from adult zebrafish intestine, labeled with an anti-PCNA antibody [PC10] (**ab29**).

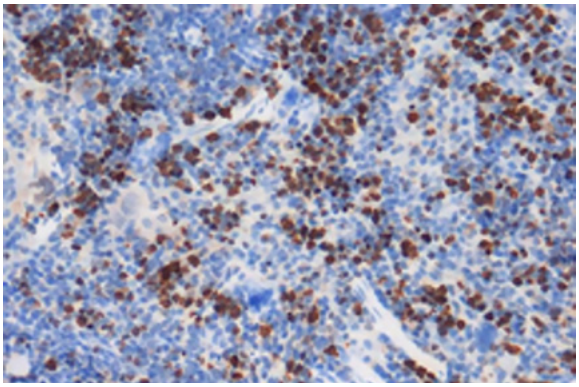


Figure 15. Immunohistochemical analysis of formalin/PFA-fixed paraffin-embedded sections from mouse spleen, labeled using an anti-Ki67 antibody (**ab15580**).

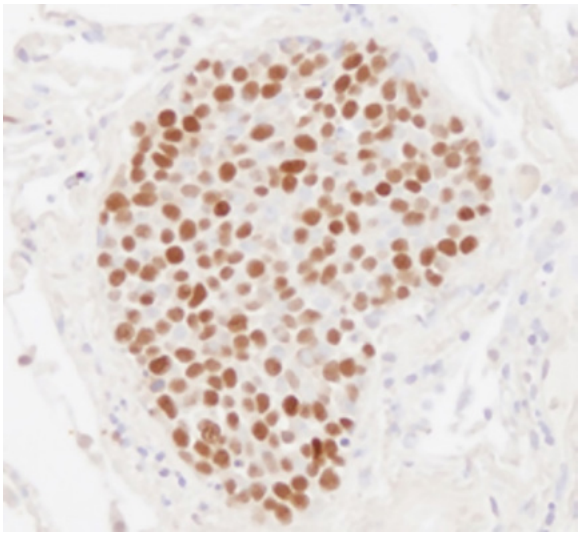


Figure 16. Immunohistochemical analysis of formalin/PFA-fixed paraffin-embedded sections from human small cell lung cancer tissue, labeled with an anti-MCM2 antibody (**ab4461**).

Find more information on staining and scoring cell proliferation in our **cell proliferation guide**.

5. Clonogenicity assays

Although little used for high throughput, the classical method of assaying cell proliferation is to use a clonogenic/clonogenicity assay. In this assay, cells are plated out at a low density and then the number of colonies formed is counted.

Advantages:

- Low risk of interference[20]

Disadvantages:

- Susceptible to error due to manual counting
- Time-consuming
- Limited to adherent cells

6. Senescence assays

Senescence is thought to be a tumor suppressive mechanism and an underlying cause of aging. Senescence represents an arrested metabolic state in which the cells remain viable, but not actively dividing.

The most common marker of senescent cells is the overexpression and accumulation of the endogenous lysosomal beta-galactosidase (SA-beta-gal). Beta-gal activity is detected using a colorimetric or fluorometric substrate.

Advantages:

- Suitable for cells or tissues
- Simple
- Reliable

Disadvantages:

- Requires fixation of cells (colorimetric assay only)
- Certain cells (osteoclasts and macrophages) have higher levels of β-gal activity, potentially leading to false positives[21]

Assay	Instrument
Beta-gal	Microscope, plate reader
	Flow cytometer

Scoring proliferating cells

Scoring the extent of proliferation is especially important in a clinical setting. The percentage of Ki67-positive cells, for example, can be used to score the severity and course of cancer. There are several scoring techniques available for use with the proliferation proteins methods, each with their own strengths and limitations.

See our complete **proliferation guide** for more information on identifying and scoring proliferating cells.

Apoptosis

What is apoptosis and when does it occur?

Apoptosis is a form of cell death characterized by several features including cell shrinkage, membrane blebbing, chromosome condensation, nuclear fragmentation, DNA laddering, and the eventual engulfment of the cell by phagosomes. It maintains a balance between cell death and proliferation in a cell population, regulating the total number of cells[22]. Apoptosis also plays vital roles in the immune system[23] and some developmental processes.

Dysfunctions in apoptosis can lead to disease by causing excessive cell death, such as in abnormal development and degenerative diseases, or by allowing too many cells to survive, an imbalance in the opposite direction which is known to cause cancer and allow viral infections to persist. To further understand these disease areas and work towards effective therapies in these fields, identifying and exploring the role of apoptosis is a fundamental first step[24].

Using apoptosis assays as part of your analysis can help to identify early indicators of cell health changes. You can assay apoptosis using a number of different approaches. As with cell viability assays, the most appropriate apoptosis assay will depend on factors such as your sample type and number of cells. It is often best to combine more than one assay.

Apoptosis markers

Apoptosis occurs via a complex signaling cascade. Figure 17 shows the main stages of apoptosis and the approximate relative time when markers for those events are likely to be detected.

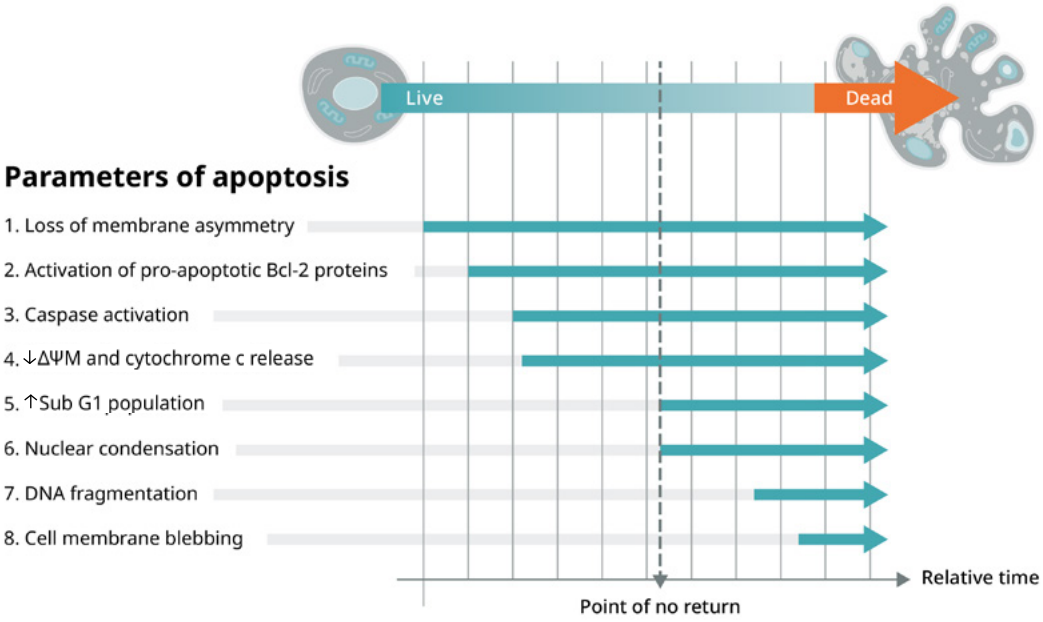


Figure 17. The parameters of apoptosis and the approximate relative time when markers for those events are likely to be detected.

These stages do not happen in a sequential order, and many of them will overlap and occur at the same time.

As cell death can occur by several different pathways, including apoptosis, necrosis, autophagy, and necroptosis, some of which share characteristics, you may need to examine multiple apoptosis markers to confirm that this is the mechanism of cell death in your experimental system.

The table below shows the main apoptosis markers and the most common methods used to study them.

Apoptosis marker	Detection methods
Loss of membrane asymmetry/ PS exposure	Flow cytometry analysis of annexin V binding
Cleavage of anti-apoptotic BCL-2 family proteins	Western blot assessment of protein cleavage
Caspase activation	Colorimetric / fluorometric substrate-based assays in microtiter plates
	Detection of cleavage of the fluorometric substrate in flow cytometry/ microscopy or by microtiter plates analysis
	Western blot analysis of pro- and active caspase
	Flow cytometry/microscopy analysis with antibodies specifically recognizing the active form of caspases
	Microplate spectrophotometry analysis with antibodies specifically recognizing the active form of caspases
Caspase substrate (PARP) cleavage	Microplate spectrophotometry analysis with antibodies specific for cleaved PARP
	Western blot analysis of cleaved PARP
Non-caspase proteases (cathepsins and calpain) activation	Colorimetric/fluorometric substrate-based assays in microtiter plates
Mitochondrial transmembrane potential (δ ψm) decrease	Flow cytometry/ microscopy/microplate spectrophotometry analysis with Δ ψm sensitive probes
	Oxygen consumption studies
Cytochrome C release	Western blot analysis of the presence of cytochrome C in the cytosol
	Antibody-based microscopy analysis of the presence of cytochrome C in the cytosol
Increase of sub G1 population	Flow cytometry analysis of sub G1 peak
Nuclear condensation	Flow cytometry analysis of chromatin condensation
	Microscopy analysis of chromatin condensation
DNA fragmentation	Analysis of DNA ladder in agarose gel
	Analysis of DNA fragmentation by TUNEL
Membrane blebbing	Light microscopy analysis of membrane blebbing
	Western blot analysis of cleaved substrate (gelsolin, ROCK1)

Apoptosis assays

You can determine how cells are dying by measuring markers that are activated in different types, and at different stages, of cell death.

There are a number of methods for running an apoptosis assay to measure these markers of apoptosis.

1. Annexin V binding of cell surface phosphatidylserine

Loss of membrane asymmetry in apoptosis can be detected using Annexin V. Annexin V binds to phosphatidylserine, which migrates to the outer plasma membrane in apoptosis. Analysis is typically by flow cytometry. Pair Annexin V with a membrane impermeable dye like 7-AAD to distinguish between intact, apoptotic, and necrotic cells.

Advantages:

- Suitable for *in-vivo*, tissue and cell culture studies
- Allows for earlier detection of apoptosis than TUNEL[25]

Disadvantages:

- Not suitable for use in fixed cells

Annexin V conjugate	Instrument	Ex/Em
FITC	Flow cytometry or fluorescence microscope	495/519
Cy3		548/561
Cy5		647/665
PE		496/576
PE-Cy5		565/693
EGFP		488/530
Biotin		

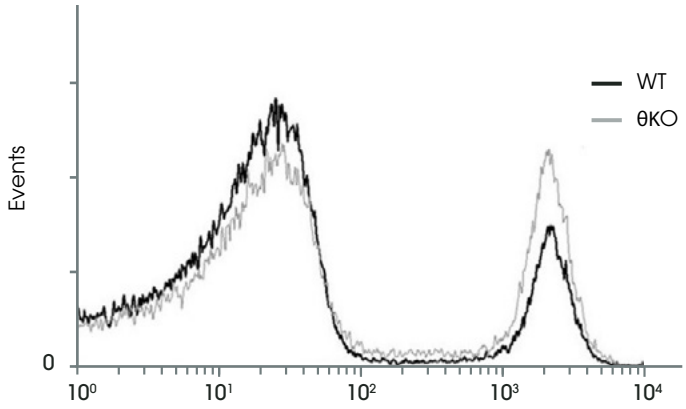


Figure 18. Murine platelets were stimulated with CRP in the presence of annexin V-FITC. The annexin V-positive population is indicated (AnV+ve). Harper MT et al, JBiol Chem. 2012 285:19865-73

2. DNA condensation and fragmentation

Chromatin condensation and genomic DNA fragmentation, together with cell membrane blebbing, are considered morphological hallmarks of the terminal stages of apoptosis.

Chromatin condensation:

During apoptosis, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert, highly condensed form. When stained with DNA-binding nuclear dyes, the compacted chromatin will be brighter than the chromatin from non-apoptotic cells, and the condensed nuclei can be easily identified by fluorescence microscopy (qualitative detection) and/or flow cytometry (quantitative detection).

Assay	Instrument
Beta-gal	Microscope, plate reader

Genomic DNA fragmentation:

Condensed chromatin can be fragmented by a specific nuclease called Caspase-Activated DNase (CAD). Activation of CAD by the caspase cascade leads to specific cleavage of the DNA at the internucleosomal linker sites between the nucleosomes, generating fragments of ~ 200 base pairs known as DNA ladders.

A classical method to detect DNA ladders is to examine fragmented genomic DNA on an agarose gel. This is a simple, semi-quantitative method that provides a robust answer. **Review the protocol** to learn more.

An alternative method for detecting DNA fragmentation involves the identification of nicks (or strand breaks) using a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay.

The TUNEL staining / TUNEL assay method relies on the enzyme terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks. TdT is expressed in certain immune cells and acts during V(D)J recombination – the process that generates antibody diversity.

In TUNEL staining, the nucleotides attached by TdT are tagged either directly with a fluorescent label or with a chemical label that can be indirectly linked to either a fluorescent label or an enzyme.

TUNEL staining is a modern alternative to analyzing the formation of DNA fragments during apoptosis using agarose gel electrophoresis.

TUNEL staining / the TUNEL assay is most commonly analyzed by light microscopy. Fluorescent TUNEL staining / TUNEL assay methods are also suitable for analysis by
flow cytometry.

See our **TUNEL staining / TUNEL assay guide** for more details.

Assay	Instrument
DNA fragmentation	Gel electrophoresis
TUNEL	Flow cytometry, fluorescence microscope, microscope

Advantages:

- TUNEL assay has high sensitivity and widely used
- Detection of DNA ladders by gel electrophoresis is simple and robust

Disadvantages:

- Risk of false positives with TUNEL assay

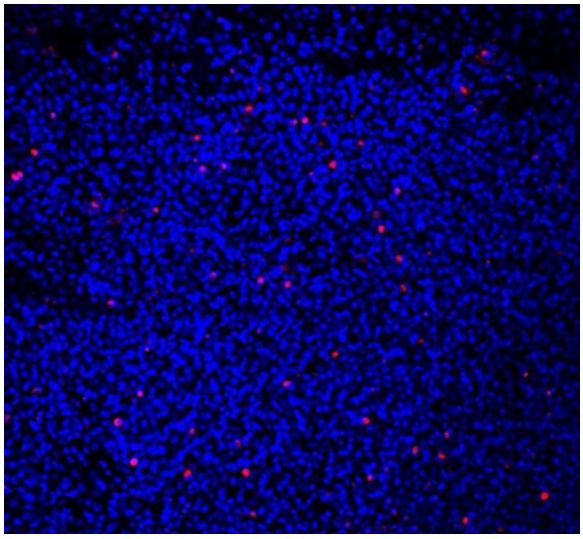


Figure 19. TUNEL staining in whole-mount Hydractinia echinata using red TUNEL assay kit (ab66110) with DAPI counterstain (blue).

3. Increase of G1 population

An increase in the percentage of cells stalled at G1 is another consequence of DNA fragmentation and can be readily detected with a flow cytometer. Once cells are permeabilized with, for example, a 70% ethanol solution, the DNA fragments will leak out and create a population of cells with reduced DNA content. When cells are stained with a DNA staining dye such as propidium iodide, a DNA profile representing cells in the different stages of the cell cycle (G1, S-phase and G2M) can be observed by flow cytometry. The apoptotic cells are easily identified as the subG1 population seen to the left of the G1 peak. The subG1 fraction will include all the dead cells in the population regardless of the type of cell and therefore this parameter on its own is not a good indicator of apoptosis.

Advantages:

- Compatible with cells of any species that can be prepared as a single cell suspension

Disadvantages:

- Not a good indicator of apoptosis on its own
- Requires fixation

Assay	Instrument
Propidium Iodide	Flow cytometry

4. Membrane blebbing

Together with DNA fragmentation, the final execution phase of apoptosis is characterized by dynamic membrane blebbing and cell contraction. During apoptosis, the cell cytoskeleton breaks up, causing some parts of the cell membrane to bulge outwards. The bulges eventually separate from the cell taking a portion of the cytoplasm with them and forming what are known as apoptotic bodies.

Membrane blebbing can be observed in live cells using phase-contrast microscopy. If you are not able to use live cells or would like to use cells that you have prepared for studying other parameters (for example, cells harvested for DNA fragmentation quantification or chromatin condensation), you can detect caspase substrates associated with apoptotic membrane blebbing. Be aware, however, that this is an indirect method and it may give you false positive/negative results.

Caspase substrates associated with apoptotic membrane blebbing include:

- Gelsolin
- ROCK-1 kinase
- P21-activated kinase (PAK)

Advantages:

- Enables real-time analysis of apoptosis within a population[26]

Disadvantages:

- Risk of false positive/negative results with caspase substrates detection method

Assay	Instrument
Membrane blebbing	Phase contrast microscope

5. Active caspase detection

Caspases are a family of conserved cysteine proteases that play an essential role in apoptosis.

Mammalian caspases can be subdivided into three functional groups: initiator caspases (caspase 2, 8, 9 and 10), executioner caspases (caspase 3, 6 and 7), and inflammatory caspases (caspase 1, 4, 5, 11 and 12). Initiator caspases initiate the apoptosis signal while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory cytokine signaling and other types of cell death such as pyroptosis.

Initially synthesized as inactive pro-caspases, caspases become rapidly cleaved and activated in response to granzyme B, death receptors and apoptosome stimuli. Caspases will then cleave a range of substrates, including downstream caspases, nuclear proteins, plasma membrane proteins and mitochondrial proteins, ultimately leading to cell death.

Activated caspases can be detected using antibodies with IHC, western blotting, or flow cytometry. Caspase activity assays either use peptide substrates, which are cleaved by caspases in cell extracts, or similar substrates that bind to activated caspases in live cells. Caspase specificity varies by substrate.

The answer provided by different experimental methods will confirm whether one or more specific caspases are active or inactive. It is always best practice to use more than one method to confirm specific caspase activation.

For information on our assays for caspases 1 through 12, formulated either for cell lysates with analysis by plate reader, or for live cells with analysis by flow cytometer, microscope or plate reader, see **www.abcam.com/caspaseassays**. For more information about caspase substrates, see **www.abcam.com/caspsubguide**.

Advantages:

- Quick and convenient for determining caspase activation

Disadvantages:

- Caspase activation can occur in non-apoptotic events[27]
- Cleavage specificities of caspases overlap, therefore reliance on a single substrate/assay is not recommended for identification of a specific caspase

For more guidance on caspase activity assays, see:

www.abcam.com/kits/factors-to-consider-when-using-caspase-activity-assays

6. Cytochrome C release assays

Usually located in the space between the inner and outer mitochondrial membranes, cytochrome C is released into the cytoplasm following total loss of mitochondrial membrane potential (ΔΨm).

The collapse of the ΔΨm is a catastrophic event. It leads to the opening of the mitochondrial permeability transition pores in the mitochondrial membrane, and the subsequent release of cytochrome C in the cytosol, which in turn triggers other downstream events in the apoptotic cascade.

Once the mitochondrial pores are opened and cytochrome C is released, the apoptotic cascade reaches a "point of no return" from which it is very unlikely that the cell can recover, and death is the most likely outcome.

The most common technique to detect cytochrome C release is through western blot on protein extracted from different subcellular compartments. It is very important in this case to ensure that the different subcellular fractions are not contaminated with other fractions. This can be easily checked with specific and reliable subcellular markers:

- Cytoplasmic markers: **GAPDH**, **actin**
- Mitochondrial markers: **VDAC1**, **PDH-E1**

On the other hand, immunofluorescence staining of fixed cells with a cytochrome C antibody at selected time points can be used to visualize cytochrome C release from the mitochondria into the cytoplasm.

Advantages:

- Enables analysis in living or fixed cells

Disadvantages:

- Accurate quantitative analysis is difficult with western blot[28]

Assay	Instrument
Cytochrome C	Western blot, fluorescence microscope

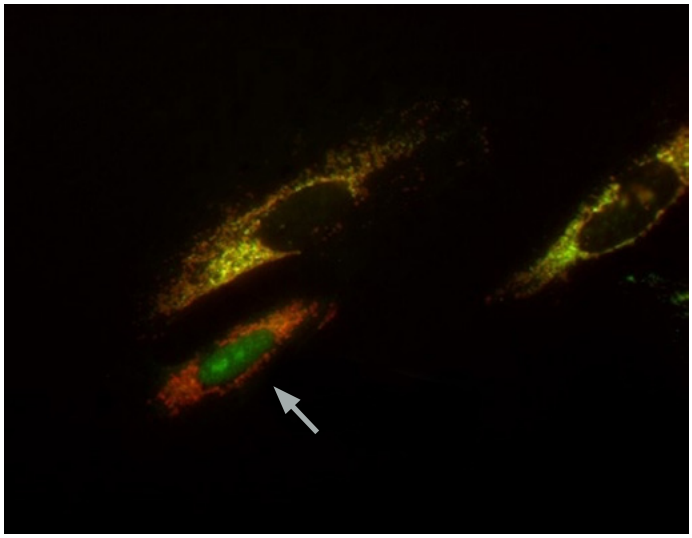


Figure 20: Apotrack™ Cytochrome C Apoptosis ICC Antibody Cocktail (**ab110417**): HeLa cells treated with staurosporine were stained with anti-cytochrome C antibody (green) and anti-ATP synthase subunit alpha antibody (red). The white arrow indicated cytochrome C release from mitochondria.

7. Mitochondrial membrane potential-dependent dyes

Dyes that accumulate in mitochondria due to the mitochondrial membrane potential are also used in the analysis of apoptosis. During apoptosis, several changes happen in the mitochondria, most notably the loss of mitochondrial membrane potential[29].

For more information, see the earlier section on metabolism-based assays. Apoptotic cells stain more weakly with these dyes due to the loss of membrane potential.

8. Glutathione assays

Glutathione (GSH) is a tripeptide which prevents cell damage caused by reactive oxygen species such as free radicals and peroxides. The monitoring of reduced and oxidized GSH in samples is essential for evaluating the redox and detoxification status of the cells and tissues against oxidative and free radicals mediated cell injury. The ratio of oxidised glutathione (GSSG) to reduced glutathione (GSH) is associated with regulation of apoptosis, and as a result, an imbalance towards increased GSSG can lead to cell death[30].

Glutathione assays allow detection of total glutathione changes during cellular response to toxicity, apoptosis and other conditions.

Advantages:

- Simple
- Can be used together with other reagents, such as 7-AAD for multi-parametric study of cell viability and apoptosis

Disadvantages:

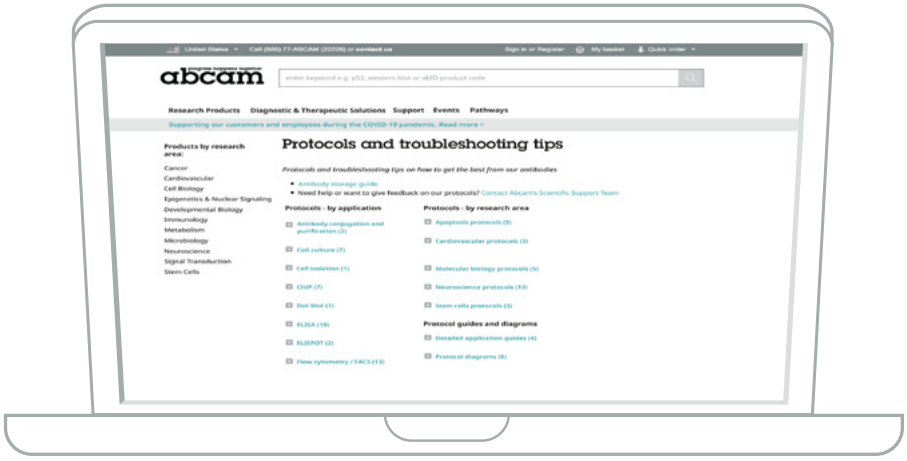
- Assay components have short stability at room temperature, so assay must be performed quickly

Other forms of cell death

Other assays are used to assess **necrosis, anoikis and autophagy.** Learn more about the mechanisms of apoptosis and other forms of cell death in our three comprehensive guides to **apoptosis, necroptosis**, and **autophagy**.

Protocols and troubleshooting

Find **protocols and troubleshooting tips** for cell health assays on our website.



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