abcam

Mitochondrial toxicity application guide

Contents

Introduction

A key role of mitochondria is the conversion of substrates into usable energy in the form of ATP. However, mitochondria also have roles outside of energy regulation: they are involved in calcium signaling and intermediary metabolism, and they regulate the extrinsic apoptotic pathway following cell insult. Recent studies show that mitochondria are implicated in many diseases including neurological disorders such as Parkinson's[1] and Alzheimer's disease[2], as well as in cancer[3].

Mitochondria can be affected by drug treatment, resulting into cardio- and hepatotoxic side effects that can lead to drug withdrawal from the market. Therefore, testing for mitochondrial function has become a key aspect of drug discovery. In particular, there is increasing emphasis on testing the impact on mitochondria early on in the drug development process to reduce failure rates during preclinical and clinical phases[4]. This guide outlines how to analyze various aspects of mitochondrial toxicity, and the points you should consider when planning your experiment.

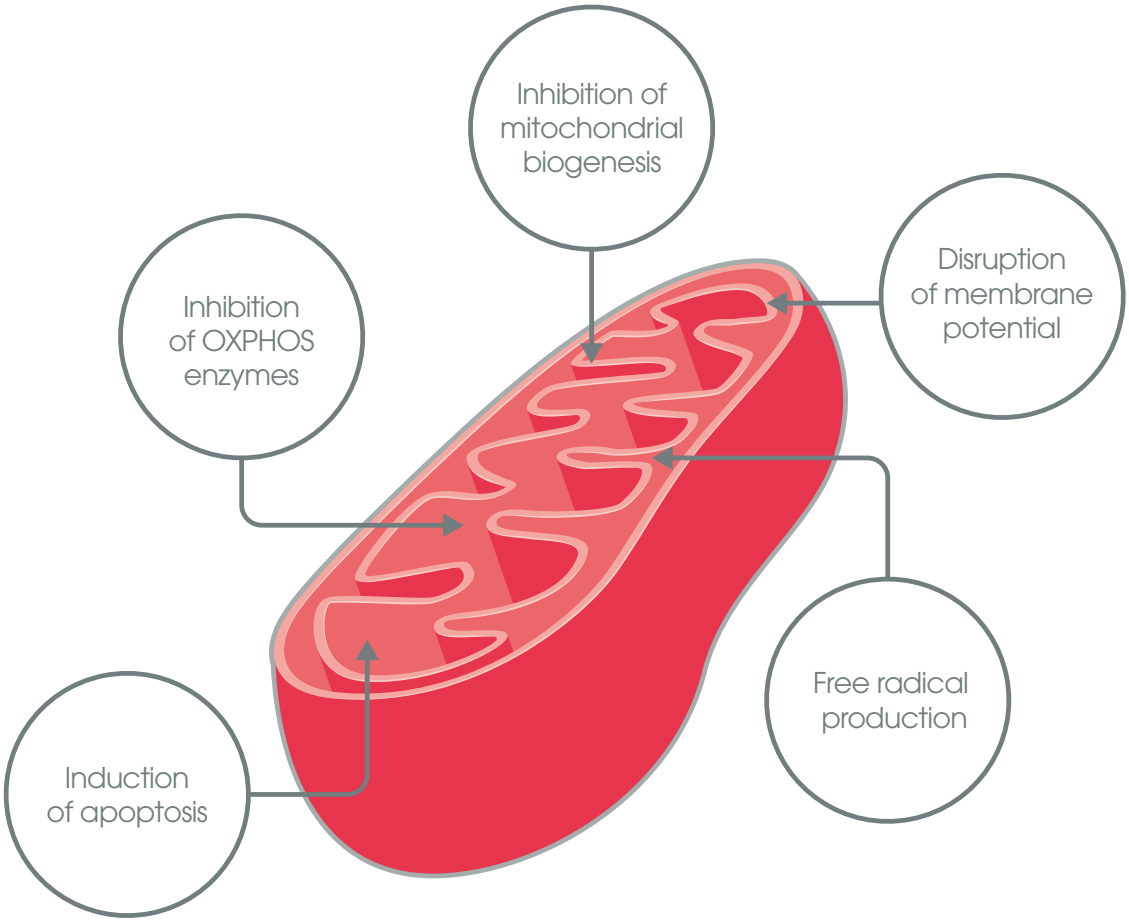


Figure 1. Processes involved in mitochondrial dysfunction.

Mitochondrial biogenesis

Mitochondrial biogenesis – the addition of new mitochondrial material within a cell – is essential to provide enough ATP to meet the energy demands of a tissue. It is affected by factors such as exercise, low temperature and cell division.

Mitochondrial biogenesis requires the coordinated synthesis of 13 proteins encoded in the organelle's own DNA and thousands of nuclear DNA-encoded subunits, all of which are shipped to and transported into the growing organelle by way of cell defined transport processes.

Mitochondria possess their own DNA and protein synthesis apparatus, which are evolutionary remnants of the early symbiotic fusion of bacterial cells, making these components quite similar to their bacterial counterparts. Because of this similarity, mitochondrial biogenesis can be easily impacted by anti-viral[5] and anti-bacterial drugs, and any evaluation of drug designed to target bacterial polymerases or their protein synthesis must be evaluated for their effect on mitochondria.

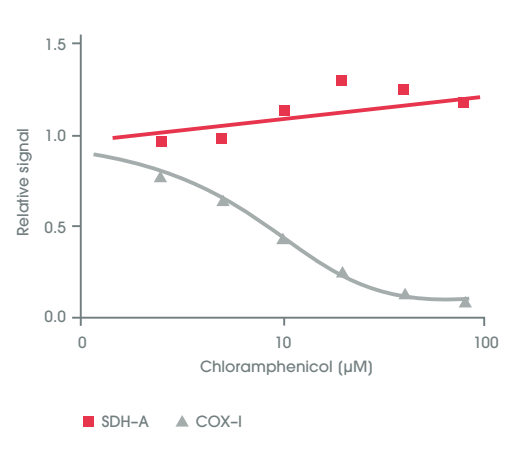
Measuring mitochondrial biogenesis

Options for assessing mitochondrial biogenesis are shown below. Multiple approaches should be used to confirm your results.

Technique/ Method	How it works	Advantages	Disadvantages
ELISA or western blot quantification of mitochondrial-encoded proteins	Ratio of mitochondrial-encoded protein with total cell protein is calculated to indicate change in mitochondria number	- Does not require specialized equipment - ELISA is high throughput	- Does not measure mitochondrial turnover
ELISA or western blot quantification of mitochondrial biogenesis markers	Proteins including mtTFA, NRF-1/2 and PGC-1 are involved in mitochondrial gene transcription and biogenesis coordination: upregulation of these proteins indicates that the biogenesis program is turned on	- Does not require specialized equipment - ELISA is high throughput	- Proteins are only transiently upregulated - Does not measure mitochondrial turnover
Real-time PCR quantification of mitochondrial DNA	Amount of mitochondrial DNA is proportional to the number of mitochondria	- Does not require specialized equipment	- Does not measure mitochondrial turnover
Fluorescence microscopy	Mitochondria are visualized using fluorescent dyes such as CytoPainter mitochondrial dyes to give the relative area occupied by mitochondria	- Fluorescent microscope is the only equipment required	- You must ensure that dyes are not membrane potential dependent
Transmission electron microscopy	Fixed tissue sections are visualized to measure the relative or absolute area occupied by mitochondria	- Provides direct visible results - Provides absolute values for mitochondrial quantification	- Laborious to prepare and stain tissue - Requires access to a transmission electron microscope

Technique/ Method	How it works	Advantages	Disadvantages
Radiolabeling	Rate of incorporation of a radioactive amino acid is measured to give the translation rate of mitochondrial DNA-encoded protein	- Takes mitochondrial turnover into account	- A large dosage of radioactivity is required
Oxygen Consumption	Measuring oxygen consumption in the presence of FCCP (maximal respiratory capacity) identifies increased mitochondrial capacity caused by mitogenesis	- Measures functional / metabolic consequences of mitogenesis facilitating further mechanistic delineation	- Is an indirect measure and is impacted by upstream metabolic regulation (e.g. substrate restriction)

Product highlight



Chloramphenicol inhibition of mitochondrial biogenesis

Inhibition of mitochondrial biogenesis by chloramphenicol, assessed using MitobiogenesisTM In-Cell ELISA kit (Colorimetric) (ab110217) by monitoring the relative amounts of COX-I (mitochondrial DNA encoded) and SDH-A (nuclear DNA encoded)

"I am using this kit for high throughput screening of more than 500 compounds. The kit is highly reproducible and I did not observe any lot to lot variation. I would highly recommend this kit."

– Dr Andaleeb Sajid

Mitochondrial viability and activity

Drug treatments, disease, and environmental conditions can impact mitochondrial activity. The following assays can be used to get an overall picture of whether mitochondrial function is being impacted.

Oxygen consumption

Oxygen consumption is one of the most informative and direct measures of mitochondrial function. Diminished oxygen consumption and ATP synthesis together may signal mitochondrial dysfunction. Reduced ATP production rate without a corresponding decrease in oxygen consumption rate signals mitochondrial uncoupling.

Measuring oxygen consumption

Oxygen consumption is typically measured either electrochemically or fluorometrically, with the most appropriate method being dependent on the requirements of your assay design. Electrochemical analysis (Clark electrode) is typically used for low number of samples, while fluorescence-based assays are used in instances where higher throughput, microplate compatibility and/or multiparametric analysis is necessary.

Clark oxygen electrode

In the Clark oxygen electrode, an anode and cathode are in contact with an electrolyte solution covered by a semi-permeable membrane. Oxygen diffuses through the membrane to the cathode, where it is reduced. The current produced by the electrode is proportional to the oxygen tension in the solution.

When to use	Advantages	Disadvantages
- For measuring immediate impact of drug or substrate addition on oxygen consumption - When sample number are low (one sample at a time) - When detailed mechanistic information is required	- Provides high resolution - Provides good sensitivity - Compounds can be added during measurement	- Very low sample throughput (one sample at a time) and large assay volumes - Cell need to be in suspension and continuous stirring may damage cells/ mitochondria - Measurement consumes oxygen

Fluorescence-based oxygen consumption assays

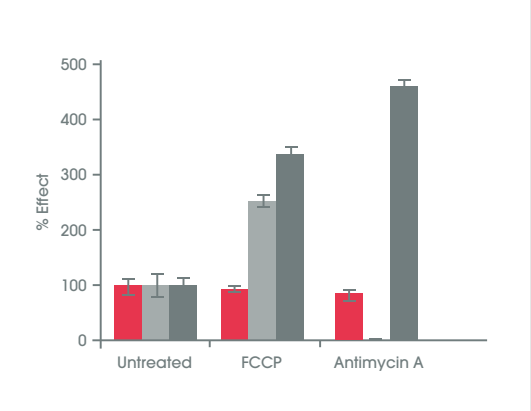
These assays use oxygen-sensitive fluorescent probes and are based on the ability of oxygen (O_2) to quench the excited state of the probe. As oxygen concentration is reduced by cellular respiration, probe signal increases and the rate of signal increase is therefore indicative of oxygen consumption rate (OCR). Measurement is performed on standard microtiter plates using conventional fluorescence plate readers.

When to use	Advantages	Disadvantages
- For measuring the impact of drug treatment, or other treatment of oxygen consumption - When microplate compatibility or medium-high throughout is required (96/384 well plate) - When multi-parametric analysis is required - When measurement of 3D cultures is required	- Simple and easy to use - No dedicated specialist equipment required - Potential to multiplex with other relevant metabolic parameters (e.g. JC-1, glycolysis, ROS etc.) - Compatible with adherent cells, suspension cells, isolated mitochondria and 3D culture microplates - Compounds are added prior to measurement and tested in parallel making assay compatible with conventional microplate screening workflow	- Standard protocol alteration required to facilitate compound addition during measurement

Glycolytic rate

Mitochondrial dysfunction can force cells to increase glycolytic flux in an attempt to maintain ATP supply. The resultant increase in conversion of pyruvate to lactate can be detected as an increase in extracellular acidification (ECA). ECA-based glycolytic flux measurements can therefore be used as a means to monitor altered glucose metabolism and, in conjunction with OCR measurements (described above), as an additional confirmation of mitochondrial dysfunction. An example of this type of analysis id presented below and illustrates how multi-parametric analysis (O_2 consumption, glycolytic flux, cellular ATP) can be used to generate a comprehensive overview of fluctuations in cellular bioenergetics.

Product highlight



Cellular and energy flux

HepG2 cells (seeded at 6.5 x 10^4 cells/well) were treated with 1 µM antimycin A and 2.5 µM FCCP. Oxygen consumption (light grey column, ab197243), extracellular acidification rate (dark grey column, ab197244) and ATP concentration (red column, ab113849) data are shown as percentage of untreated control.

Membrane potential

Mitochondrial membrane potential is at the heart of mitochondrial function: it controls ATP synthesis, ROS generation, mitochondrial calcium sequestration, protein import and mitochondrial membrane dynamics.

Mitochondrial membrane potential is generated by electron flow through the mitochondrial electron transport chain, which powers complexes I, III and IV to pump protons from the matrix into the intermembrane space. Mitochondrial depolarization occurs when protons flow back into the matrix, bypassing ATP synthase (Complex V). Depolarization may occur as a result of damage to the inner mitochondrial membrane or opening of the mitochondrial permeability transition pore.

Detecting membrane potential with fluorescent dyes

Membrane potential is often measured using fluorescent dyes that enter polarized mitochondria. These dyes are lipophilic cationic compounds that accumulate according to the charge across the membrane: a mitochondrion with a more polarized membrane will accumulate more dye.

Numerous dyes are available for this purpose. Use the guide below to help you select the most appropriate for your experiment:

1. Quenching or non-quenching mode?

Mode	How it works	When to use
Quenching	A relatively high concentration of fluorescent dye is used (50–100 nM). Dye molecules aggregate as they enter the mitochondria, causing fluorescence quenching. Depolarization releases the dye, reducing the quenching and giving a transient increase in signal.	- To detect short-term effect of treatments applied after the dye has been loaded.
Non-quenching	Lower dye concentrations are used (0.5–30 nM) to avoid aggregation, so a higher signal equates to a higher membrane potential.	- To follow changes in membrane potential over a longer time frame - To determine differences in membrane potential between two populations

2. Selecting a probe

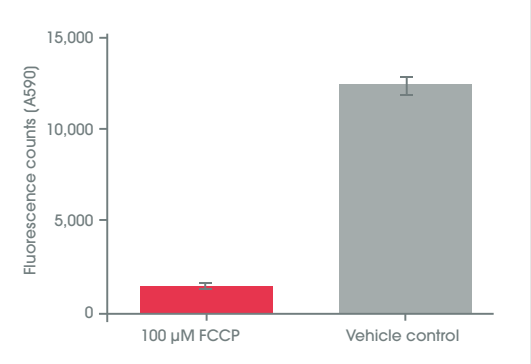
When selecting a probe, you should consider the following: whether you want to use the dye in quenching or non-quenching mode, equilibration rates, toxicity and how much the dye accumulates into mitochondria[6,7].

Probe	How it works	Advantages	Disadvantages
JC-1/JC-10	Emission spectra shifts from red to green with increasing concentration and aggregation	- Suitable for relatively crude assessment of whether a cell population is largely polarized or largely depolarized - JC-10 is more soluble than JC-1	- Not suitable for quenching studies, as aggregation does not result in quenching. - Highly sensitive to probe loading concentrations and loading times: consistency is crucial - Aggregates do not equilibrate as quickly as monomers, which can falsely report differences in membrane potential between populations - Not suitable for detecting small changes in membrane potential
TMRE/TMRM	Accumulates across charged membranes	- Least toxic to mitochondria of all commonly used probes. - Equilibrates quickly: good for non-quenching studies	- Results can be hard to interpret when used in quenching mode
Rhod123	Accumulates across charged membranes	- Suitable for short-term quenching studies	- Higher mitochondrial binding and toxicity, and equilibrates more slowly than TMRE

3. Selecting a read-out method

Fluorescent dyes can be detected by microscopy, flow cytometry or fluorescent plate reader. Short-term quenching studies are analyzed by fluorescent or confocal microscopy, so that changes can be monitored at individual cell resolution. Longer term population studies are best analyzed by flow cytometer or plate reader.

Product highlight



Tracking mitochondrial membrane potential.

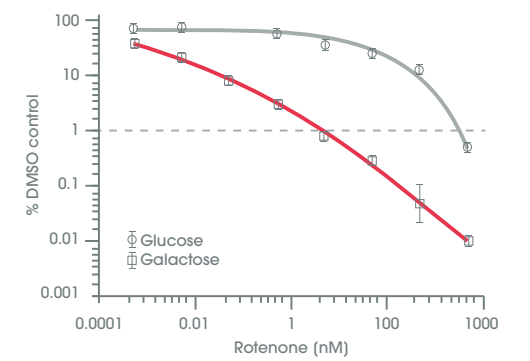
Mitochondrial changes were monitored with JC-1 Mitochondrial Membrane Potential Assay Kit (ab113850). HL60 cells were labelled and treated for 4 h with 100 µM FCCP or vehicle/diluent control (DMSO). JC-1 dye (ab141387) can also be purchased separately.

Mitochondrial viability assay

Many cell lines used in drug discovery screening prefer to use glycolysis rather than oxidative phosphorylation to produce ATP when grown in glucose, a phenomenon known as Crabtree effect[7]. This can be potentially problematic when looking for adverse effects of a compound on energy metabolism, particularly when assessing effects on oxidative phosphorylation. Substituting glucose for galactose plus glutamine in the growth media forces cells to rely on mitochondrial oxidative phosphorylation rather than glycolysis, which in term leads to an increased susceptibility to compounds that are toxic for mitochondria[7].

Growing cell lines on galactose-containing and glucose-containing media in parallel for toxicity screens is becoming a common assay in drug screening activities. This comparison allows to discriminate between compounds that affect overall cellular metabolism and viability from those that specifically affect mitochondria[8].

Product highlight



Mitochondrial viability stain in long term toxicity.

HepG2 cells were cultured in glucose or galactose substrate media. Cells were then treated for 72 hours in presence of rotenone (0.5 pM – 5 µM) prior to addition of Mitochondrial Viability Stain (ab129732). Plates were incubated for 4 h before fluorescent plate readout.

Investigational assays

Once it has been established that a treatment impacts mitochondrial function, activity assays are necessary to pinpoint the cause of mitochondrial dysfunction.

Oxidative phosphorylation complexes

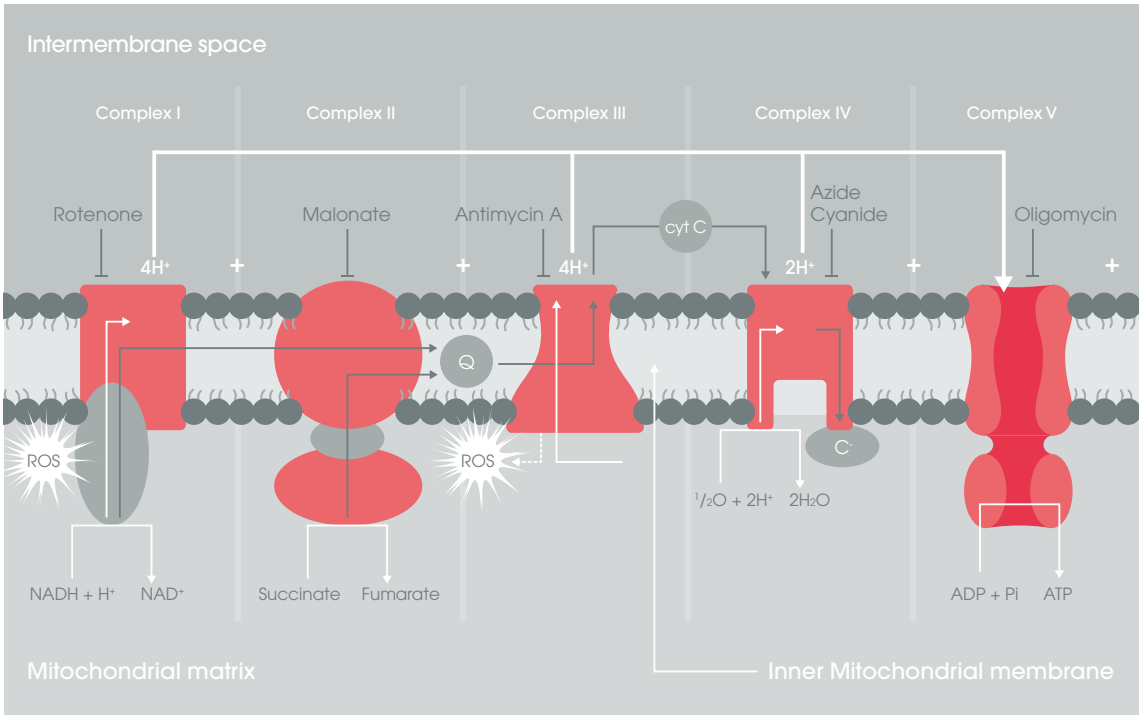


Figure 2. The mitochondrial electron transport chain and its inhibitors and substrates.

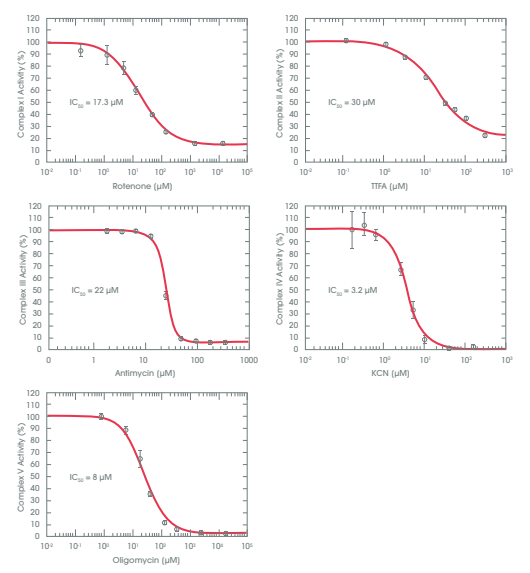
Measuring oxidative phosphorylation (OXPHOS) complex activity

Many drugs are direct inhibitors of one or more of the OXPHOS complexes. When these compounds enter the mitochondria, their interaction with the respiration complexes can be a primary cause of cell toxicity.

Spectrophotometric plate-based assays can be used to measure the activity of oxidative phosphorylation complexes in response to potential inhibitors in vitro, using isolated mitochondria, or tissue or cell lysates[9]. Substrates for the complex in question are added, and conversion of substrate to product is measured spectrophotometrically, often by detecting NADH or NADPH.

When to use	Advantages	Disadvantages
- To get catalytic information about maximum activities from frozen or fresh tissue	- Easy to reproduce - Can be used with a variety of sample types including tissue or homogenates, or isolated mitochondria. - Can be used with tissue that has been frozen	- *In vitro assays*: conditions may not be physiological - Does not allow for the evaluation of respiratory coupling

Product highlight



Dose response curves of mitochondrial complexes

Dose response curves of mitochondrial complexes I – V after treatment with specific inhibitors[10]. Activity was monitored with the MitoTox™ Complete OXPHOS Activity Assay panel (ab110419), using isolated bovine heart mitochondria provided in the kit.

Other metabolic enzyme activity assays

Drugs can also impact the activity or expression of key enzymes in mitochondrial metabolic pathways other than oxidative phosphorylation. Maximum activity of these enzymes can be measured in isolated mitochondria, or cell or tissue lysates using spectrophotometric assays.

Product highlight

Enzyme	Pathway	Product code
Aldehyde dehydrogenase (ALDH2)	Alcohol detoxification	ab115348
Carboxylesterase 1 (CES1)	Xenobiotic detoxification	ab109717
Citrate synthase	TCA cycle	ab119692
Enolase 1 (ENO1)	Glycolysis	ab117994
Acyl-CoA dehydrogenase medium-chain (ACADM)	Fatty acid oxidation	ab118182
Fumarase	TCA cycle	ab110043
Lactase dehydrogenase, subunit B (LHB)	Glycolysis	ab140361
Malate dehydrogenase (MDH2)	TCA cycle	ab119693
Monoamine oxidase B (MAOB)	Xenobiotic deamination	ab109912
Pyruvate dehydrogenase (PDH)	Link between glycolysis and TCA cycle	ab110671
Transketolase	Pentose phosphate pathway	ab187398

Oxidative stress

Increased free radical production is a feature of mitochondrial dysfunction. The mitochondrial respiratory chain is the predominant source of reactive species, and inhibition of the respiratory chain by pharmacological compounds often leads to oxidative stress.

Highly reactive oxygen species (ROS) or nitrogen species (RNS), such as superoxide or nitric oxide respectively, can damage phospholipids, proteins and DNA. Antioxidant systems are present to counteract reactive species production under normal physiological conditions, but when these are overwhelmed, damage occurs in the cell.

Analyzing oxidative stress

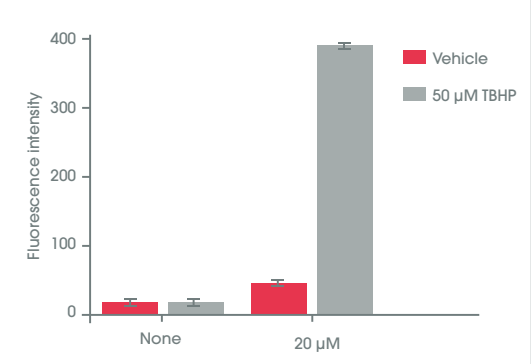
Multiple approaches exist to measure oxidative stress. These broadly fall into two categories: direct quantification of reactive species, or indirect detection by measuring the impact of oxidative stress on macromolecules and antioxidant systems.

Probes to quantify reactive species

The presence of reactive species can be directly measured using fluorogenic dyes, such as 2,7-dichlorofluorescein (DCFDA). Once inside the cell, DCFDA is cleaved by esterases and can be oxidized to the fluorescent product DCF by a variety of reactive species.

When to use	Advantages	Disadvantages
- To detect localization of ROS production - To obtain quantitative data on ROS levels	- Effective for detecting intracellular ROS - Provides quantitative data - Can be used with flow cytometry and fluorescent microscopy - Enables localization of ROS production within tissues	- Prone to artifacts and requires careful interpretation[11] - The reaction leading to fluorescence is very indirect: conditions that affect steps in the pathway will lead to erroneous results - The intermediate DCF/ radical reacts with oxygen to form superoxide, leading to false signal amplification. - DCFH oxidation is catalyzed by cytochrome c and redox-active metals

Product highlight



ROS quantification

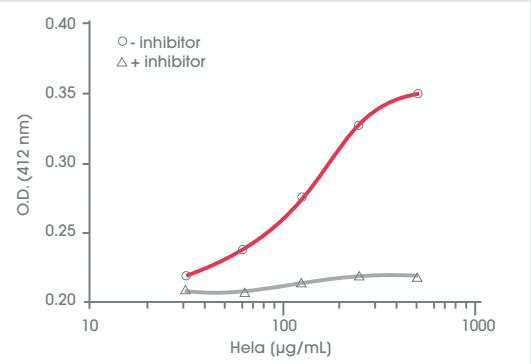
Detection of reaction oxygen species with DCFDA Cellular Reactive Oxygen Species Assay Kit (ab113851). Jurkat cells were labeled with 20 µM DCFDA or unlabeled, and then cultured for 3 h in presence or absence of 50 µM tert-butyl hydrogen peroxide (TBHP).

Antioxidant activity levels

An indirect approach to quantify the impact of oxidative stress is to measure the levels and activities of key antioxidant enzymes. After prolonged exposure to oxidative stress, cells increase the expression of antioxidant enzymes to overcome the oxidative stress[12].

These antioxidant systems comprise enzymes (superoxide dismutase, catalase, the glutathione system, and thioredoxin system), macromolecules such as albumin and ferritin, and an array of small molecules, including ascorbic acid, α-tocopherol, reduced glutathione, bilirubin and uric acid.

Product highlight



TXNRD1 activity quantification

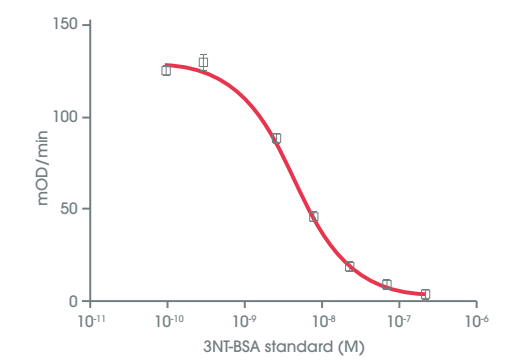
HeLa cells were left untreated (circles) or treated with 20 µM aurothiomalate (triangles), an TXNRD1 inhibitor. Cells were lysates and TXNRD1 activity was measured in endpoint mode using Thioredoxin Reductase 1 (TXNRD1) Activity Assay Kit (ab190804).

Measuring Reactive species-induced damage to macromolecules

Reactive species directly oxidize or nitrate cellular macromolecules such as proteins, lipids and DNA. Detecting these modifications can provide a useful indication of whether elevated reactive species are present.

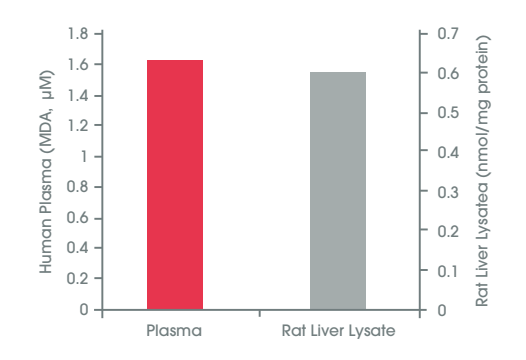
Modification	How to detect
Lipid modification	- Malondialdehyde (MDA) is a common byproduct of lipid peroxidation that can be detected using a TBARS assay, in which thiobarbituric acid reacts with malondialdehyde to yield a fluorescent product - 4-hydroxynonenal (4-HNE) is another common byproduct of lipid peroxidation that can be detected using specific antibodies
Protein modification	- Use specific antibodies to detect 3-nitrotyrosine (3NT), a ROS-mediated tyrosine nitration - Detect protein carbonyl groups
DNA modification	- Detect 8-hydroxy-deoxyguanosine

Product highlight



Quantification of nitrotyrosine

3-Nitrotyrosine-BSA (3NT-BSA) standard was quantified using the competitive Nitrotyrosine ELISA Kit (ab113848).



Quantification of MDA as lipid peroxidation marker

Malondialdehyde (MDA) was detected in human plasma (20 µL, left) and in rat liver lysate (10 mg, right) using Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric) (ab118970)

Apoptosis

Mitochondria are intricately involved with the intrinsic pathway of apoptosis. A distinctive feature of apoptosis is the disruption of normal mitochondrial function, especially changes that affect the mitochondrial transmembrane potential (ΔΨm); the opening the mitochondrial permeability transition pore (mPTP) leads to the collapse of the ΔΨm and subsequent release of cytochrome c into the cytosol.

The table below shows how to detect various parameters of apoptosis. For more detail, see our apoptosis analysis guide. www.abcam.com/apoptosisebook

Parameters	Detection methods	Sample type	Highlighted Products
Loss of membrane asymmetry/ PS exposure	Flow cytometry analysis of Annexin V binding	Live cells	ab14085
Caspase activation	Colorimetric / fluorometric substrate-based assays in microtiter plates	Cell extracts Tissue extracts	ab39383 ab65607 ab39700
	Detection of cleavage of fluorometric substrate in flow cytometry / microscopy or by microtiter plates analysis	Live cells	ab112130 ab65614 ab65613
	Western blot analysis of pro- and active caspase	Cell extracts Tissue extracts	ab32042 ab138485 ab32539
Caspase substrate (PARP) cleavage	Microplate spectrophotometry analysis with antibodies specific for cleaved PARP	Cells extracts Tissue extracts Live cells (In cell ELISA)	ab174441 ab140362
Mitochondrial transmembrane potential (ΔΨm) decrease	Flow cytometry/microscopy/ microplate spectrophotometry analysis with MMP sensitive probes	Live cells	ab113852 ab113850 ab112134
Increase of sub G1 population	Flow cytometry analysis of subG1 peak	Fixed cells	ab14083 ab139418
Nuclear condensation	Flow cytometry analysis of chromatin condensation	Live cells	ab139479 ab112151 ab115347
DNA fragmentation	Analysis of DNA ladder in agarose gel	DNA	ab66090 ab65627 ab66093
	Analysis of DNA fragmentation by TUNEL	Live cells	ab66110 ab66108

References

1. Perier C, Vila M. Mitochondrial biology and Parkinson's disease. *Cold Spring Harb Perspect Med 2*, a009332 (2012).

2. Moreira PI, Carvalho C, Zhu X, Smith MA, Perry G. Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. Biochim Biophs Acta 1802, 2–10 (2010).

3. Boland ML, Chourasia AH, Macleod KF. Mitochondrial Dysfunction in Cancer. *Front Oncol*, 3; 292 (2013).

4. Nadanaciva S, Will Y. New insights in drug-induced mitochondrial toxicity. *Curr Pharm Des* 17, 2100–2112 (2011).

5. Guidance on Antiviral Product Development – Conducting and Submitting Virology Studies to the Agency. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM387446.pdf

6. Nicholls DG. Fluorescence measurement of mitochondrial membrane potential changes in cultured cells. *Methods Mol Biol* 810, 119–33 (2012).

7. Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques 50, 98–115 (2011).

8. Marroquin LD, Hynes J, Dykens JA, Jamieson JD, Will Y. Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicol Sci, 97 (2), 539-547 (2007).

9. Spinazzi M, Casarin A, Pertegat V, Salviati L, Angelini C. Assessment of mitochondrial chain enzymatic activities on tissues and cultured cells. Nature Protocols 7, 1235–1246 (2012).

10. Nadanaciva S, Bernal A, Aggeler R, Capaldi R, Will Y. Target identification of drug induced mitochondrial toxicity using immunocaptured based OXPHOS activity assays. Toxicol In Vitro 21, 902-11 (2007)

11. Kalyanaraman B, Darley-Usmar V, Davies KJA, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ, Ischiropoulos H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic Biol Med* 52, 1–6 (2012).

12. Franco AA, Odom RS, Rando TA. Regulation of antioxidant gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. *Free Radic Biol Med* 27, 1122–32 (1999).