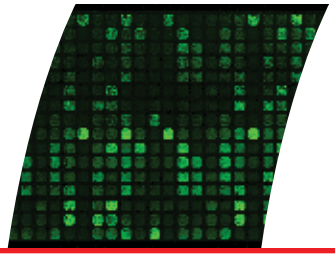

In-Cell ELISA (ICE)

Platform Guide

Validated antibodies, dyes, and kits for the analysis of fixed adherent and suspension cells

MitoSciences®

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Introduction

The In-Cell ELISA (ICE) assay platform is a rapid, high-throughput technology to quantify target protein levels within fixed cells grown in 96- or 384-well microplates. ICE is a quantitative immunocytochemistry technique that combines the specificity of Western blotting and the throughput and reproducibility of traditional sandwich ELISAs. ICE is also referred to as In-Cell Western™ or fixed-cell ELISA.

This ICE Platform Guide includes a discussion of the advantages and validation of the ICE assay platform as well as MitoSciences' proprietary application of suspension cells to ICE. In addition, we provide example datasets from both focused and broad experiments to demonstrate the reproducibility and flexibility of ICE.

MitoSciences offers complete ICE assay kits for defined applications, dozens of ICE-validated mitochondrial and metabolic antibodies (available in bulk or in sample ICE Packs) as well as support packs and detection antibodies to get you started with ICE experiments of your own design.

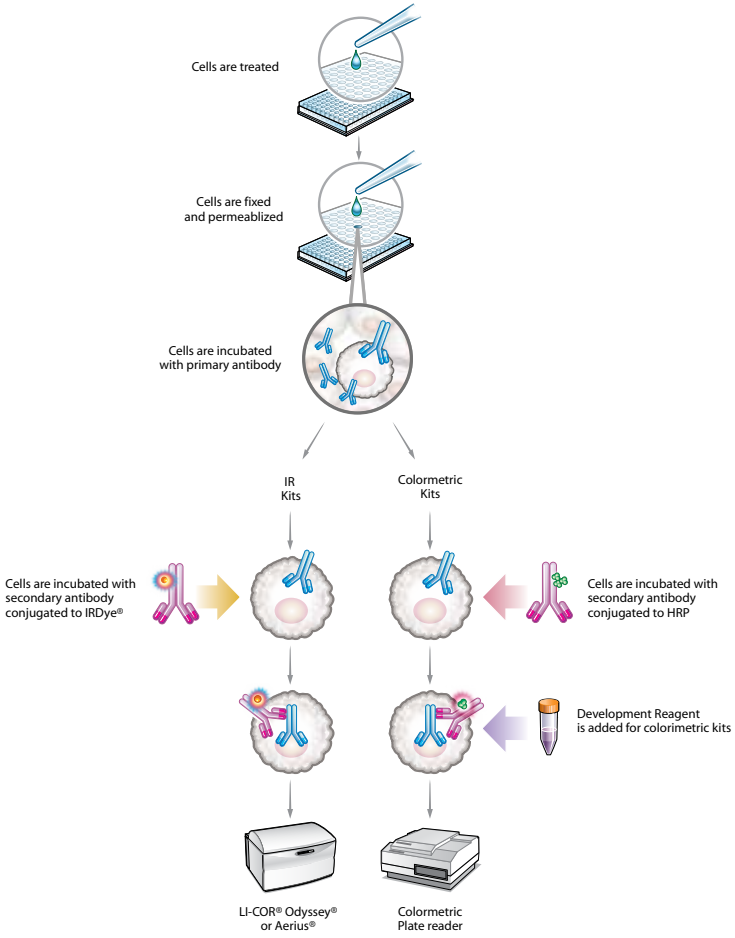


Figure 1. A schematic of infra-red and colorimetric ICE methods. *Treatments and duration of treatment before cell fixing are at the user's discretion. IR kits and detection method require LiCor® Odyssey® or similar instrument. Colorimetric kits and detection require a standard microplate reader.*

Key Features

- Cells are cultured and fixed directly in a microplate. Following fixation, cells are briefly permeabilized, blocked, and exposed to primary antibody (typically for an overnight incubation). Secondary (detection) antibodies are then applied and then the plates are scanned. A standard absorbance plate reader is used to detect HRP signal (Figure 1, right) and a LiCor® Odyssey® or Aerius® is used to detect infrared IR-dye signal (Figure 1, left). Note that duplexing is possible when using IR-dye detection.
- Fixation to data acquisition requires ~30 minutes of hands-on time. Alternatively, fixed cell plates are stable for weeks to months at 4°C in the presence of sodium azide.
- Antibody signal can be normalized to Janus Green whole cell stain to account for any differences in seeding density between wells.

Benefits of In-Cell ELISA (ICE) Technology

1. **FLEXIBILE**
    a. Measure specific protein level or post-translation modifications (e.g. phosphorylation or cleavage events).
    b. Suitable for adherent and non-adherent cell types.
    c. Colorimetric or IR-dye detection methods (IR-dyes afford duplexing within wells).
    d. Microplate format makes it easy to (1) test a few analytes across a range of culture conditions, drug treatments, or cell lines in parallel or (2) test many analytes across a few conditions.

2. FAST AND INEXPENSIVE
    a. Cells are fixed and assayed directly in microplates. No lysate preparation or lysate transfer required.
    b. 96- and 384-well formats are high-throughput and primed for automation.
    c. No capture antibody means less reagent cost than sandwich ELISAs.

3. **SOUND SCIENCE: SPECIFIC, REPRODUCIBLE, QUANTITATIVE**
    a. Direct fixation in microplates freezes and preserves cells in their biologically relevant state.
    b. Microplate format ideally suited for duplicate or triplicate measurements (CVs <10% are expected).
    c. Signals can be normalized to a total protein readout or to cell number.

4. **MITOSCIENCES SUPPORT**
    a. MitoSciences' antibodies are rigorously validated for ICE platform.
    b. Optimized assay kits available.
    c. Support packs and a spectrum of detection antibodies simplify experimental design.

Detection Methods

ICE data can be collected with a LiCor® Odyssey® or Aerius® scanner using IRdye®-labeled secondary antibodies or a standard absorbance microplate reader using HRP-labeled secondary antibodies. When a LiCor® infrared reader is available, duplexing readouts within a single well is possible using IR-800 and IR-680 labeled secondary antibodies. IRdye® secondary antibodies and IRDye® kits offer much greater dynamic range and sensitivity. Protocols for IR and HRP detection methods are identical up to the addition of secondary antibody (see Figure 1).

MitoSciences provides anti-mouse and anti-rabbit HRP-conjugated secondary antibodies, also anti-mouse and anti-rabbit IRdye® conjugated secondary antibodies. In addition, MitoSciences offers isotype-specific anti-mouse IRdye® conjugated secondary antibodies to allow duplexing with two mouse monoclonal antibodies of differing isotype. All secondary antibodies are included in ICE reagent kits or can be purchased separately.

Suitable for Adherent and Non-Adherent Cells

Adherent cells are inherently suited for ICE analysis, as they are readily fixed onto the surface of the microplate on which they are cultured. MitoSciences has developed a novel method to firmly attach suspension cells to a microplate surface yielding high quality data previously seen only with adherent cell lines. This approach has also been particularly useful in the analysis of cells that are loosely adherent, for example apoptotic cells that would otherwise be lost during plate wash steps (and thus not quantified). For more details on analyzing detaching/ apoptotic cells, see page 18.

References

A sampling of literature reports describing use of the ICE technique with diverse analytes and cell types: (1) kinetic studies of β-Catenin stabilization in mouse fibroblasts, (2) myosin phosphorylation in primary myocytes and (3) multiple phospo-protein analysis following kinase inhibitor treatment in a variety of immortalized cell lines.

1. Hannoush RN. 2008. Kinetics of Wnt-driven beta-catenin stabilization by quantitative and temporal imaging. *PLoS One.* 3(10): e3498.

2. Aguilar HN. 2010. Quantification of rapid Myosin regulatory light chain phosphorylation using high-throughput In-Cell Western assays: comparison to Western immunoblots. *PLoS One.* 5(4):e9965.

3. Chen H. 2005. A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. *Anal Biochem.* 338(1):136-42.

Antibody Validation

ICE results provide accurate, quantitative measurements of cellular antigen concentrations. However, ICE does not provide internal confirmation of antibody binding specificity with each experiment, unlike traditional Western blots or immunocytochemistry, which allow confirmation by molecular weight or subcellular localization, respectively. Therefore, confidence in antibody specificity is critical to ICE data interpretation and reliability. All of MitoSciences' ICE-validated antibodies have been screened rigorously for specificity by immunoprecipitation / mass spectrometry, Western blotting and by fluorescence immunocytochemistry under the conditions used for ICE. Documentation for each antibody and kit contain these data, plus recommended working concentrations, host species, antibody isotype and species cross-reactivity. Examples of validation by immunocytochemistry, mass spectrometry, and Western blot are shown in Figures 2, 3, and 4, respectively.

Fluorescence Immunocytochemistry

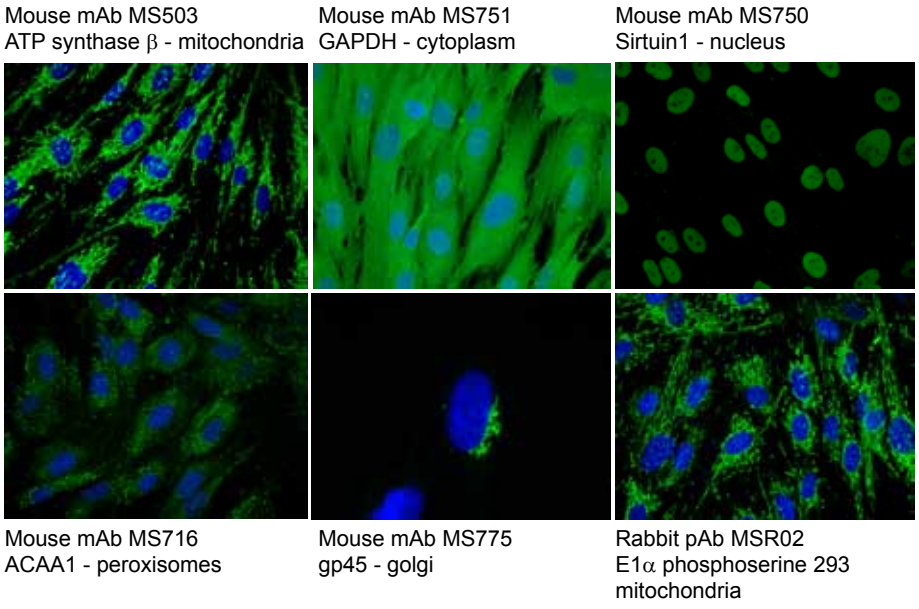


Figure 2. Fluorescence immunocytochemistry confirms the correct cellular locations of target proteins. *ICE was performed on adherent fibroblast cells using a selection of mouse and rabbit antibodies according to MitoSciences' ICE adherent cell protocol (MS901 ICE Support Pack). For fluorescence microscopy, wells were incubated with (green) Alexa Fluor® 488 labeled secondary antibody used at a 1/1000 dilution for 1 hour. DAPI was used to stain the cell nuclei (blue).*

Immunoprecipitation and Mass Spectrometry

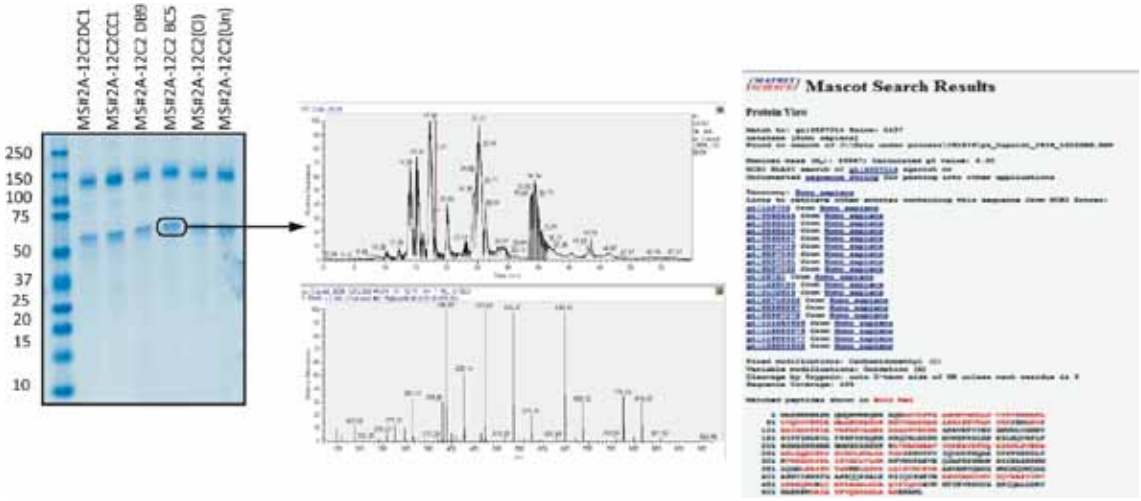


Figure 3. Mass spectrometry is used to confirm the identity of targets after immunoprecipitation. *As an example, a group of hybridomas that secrete mouse IgG antibodies suspected against catalase were used to immunoprecipitate their targets from liver tissue extract. For each clone, a single target band of appropriate size (60 kDa) was identified (the upper 150 kDa band is IgG antibody). The band of 60 kDa band was excised from the gel, trypsinized, and analyzed by tandem mass spectrometry. The resulting data are analyzed by Matrix Sciences' Mascot informatics system, which generates a MOWSE score based on the expected molecular weight of the trypsinized peptides. These data confirmed the target of this ICE validate antibody MS721. While a MOWSE score of 65 is considered sufficient to provide confidence in the accuracy of the identification of the target, MitoSciences' antibodies typically score many times that number (typical MOWSE scores for MitoSciences' antibodies are between 1000 and 3000).*

Western Blotting

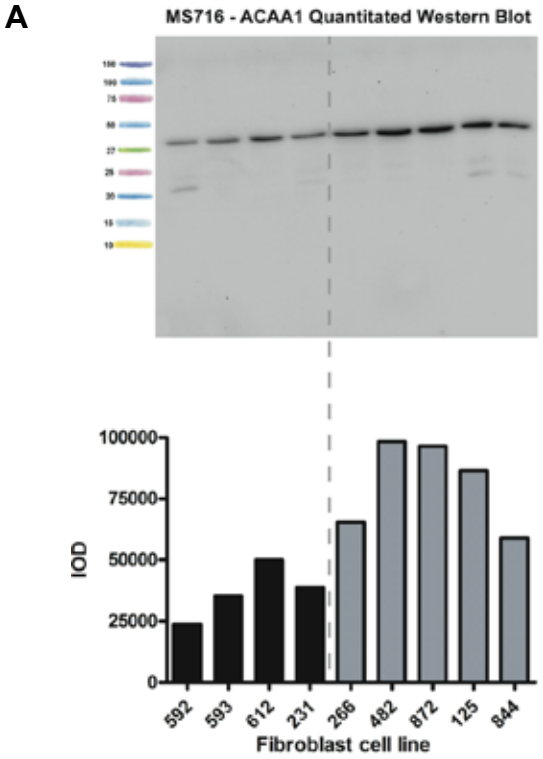
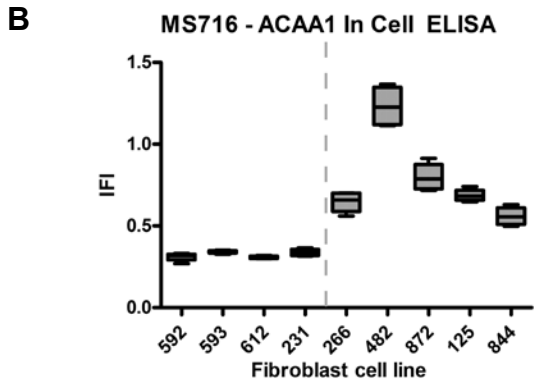


Figure 4. Many ICE validated antibodies also function in Western blot so targets can be confirmed by mass dependant migration. *Additionally Western blot confirms relative quantitation. In this example human cell lines deficient in various enzymes of mitochondrial fatty acid beta-oxidation (gray) are compared to a series of normal fibroblast cell lines (black). By ICE analysis (A) and Western blot (B) the patients had significantly higher levels of peroxisomal 3-ketoacyl-CoA thiolase (ACAA1) - indicative of a compensatory increase in peroxisomal fatty beta-acid oxidation. Notice that patient 482 has the highest signal in both methods but is relatively lower by Western blotting. This is likely due to saturation of enzyme catalyzed signal in Western blot, while IR fluorescent ICE has a broader dynamic range.*



Reproducibility

ICE is a highly reproducible technique. To demonstrate this within the wells of a single 96-well plate, tissue culture cells were treated with Chloramphenicol (CAM) or dideoxycitidine (ddC) continuously for 7 days. CAM is an antibiotic that inhibits mitochondrial protein synthesis, ddC is nucleoside analog and reverse transcriptase inhibitor which also inhibits mitochondrial DNA replication. By different mechanisms both reduce the expression of mitochondrial DNA encoded polypeptides, including OXPHOS Complex IV subunit COX-I. As shown below (Figure 5), CAM treatment caused an 80% decrease in the expression of COX-I, relative to DMSO treated cells.

Coefficients of variation (CVs) are typically <10% for ICE experiments. In this example, the Z factors for DMSO vs. background and DMSO vs. CAM are 0.68 and 0.64, respectively, indicating that this is a robust assay.

Similar reductions were seen in HepG2 cells when treated with either CAM or ddC then analyzed on three separate days (Figure 6). Three 96 well plates containing treated tissue culture cells were analyzed on subsequent days for inter-assay variation.

Cells were fixed and stored at 4°C. ICE analysis was performed on each plate on consecutive days - 1,2,3. Measurements were made in duplicate using IRdye® detection. The treatment induced reduction in COX-I signal over three days was 89%±3 (CAM) and 72%±2 (ddC). The inter assay variability of the untreated sample was <7% across the three days (not shown). The ICE technique has excellent inter-plate reproducibility; it is determined that the degree of experimental reproducibility is principally dependant on consistent tissue culture and cell seeding.

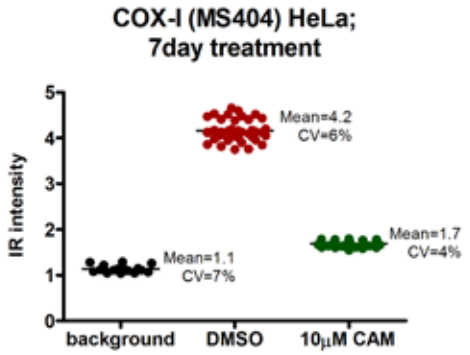


Figure 5. ICE intra-plate variability. *HeLa cells were treated with 10μM Chloramphenicol (CAM) or Vehicle (DMSO) for 7 days and analyzed by ICE for levels of OXPHOS Complex IV (COX-I, MS404). Statistics are shown for background (no primary antibody, 16 data points) ,DMSO and CAM treatments (40 data points each, CVs=6% and 4%, respectively) using IRdye® detection.*

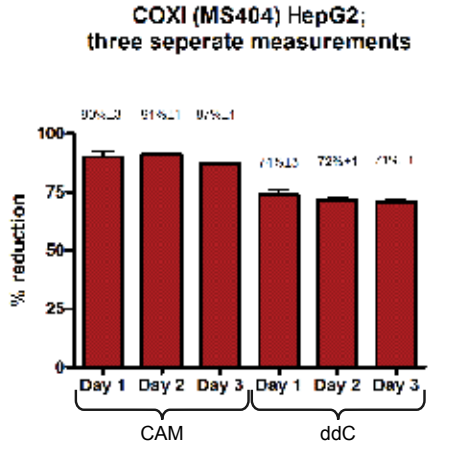


Figure 6. ICE inter-plate variability. *HepG2 cells were treated with 10μM Chloramphenicol (CAM), 10μM dideoxycytidine (ddC) or Vehicle (DMSO) for 7 days and analyzed by ICE for levels of OXPHOS Complex IV (COX-I, MS404).*

ICE Kits

Overview

Screening new drug candidates early in the safety screening process requires an assay that is adequately quantitative, specific, and reproducible, and which is flexible enough to be suitable for a variety of cell types. It must also have a minimum of sample steps and, ideally, be adaptable to automation. MitoSciences' ICE kits provide researchers and toxicologists with a solution that meets all of these requirements.

Kit 1 - MitoBiogenesis In-Cell ELISA (MS642, MS643)

Background

MitoBiogenesis ICE Kits are high-throughput assays for measuring the ratio of steady-state levels of a mitochondrial DNA- and a nuclear DNA-encoded protein in cultured or primary cells.

The addition of "new" mitochondrial material within a cell, i.e. mitochondrial biogenesis, requires the coordinated synthesis of 13 proteins encoded on the organelle's own DNA and made on mitochondrial ribosomes, along with thousands of nuclear DNA-encoded subunits, all of which are imported into the organelle by way of now well-defined transport processes. Mitochondrial DNA and ribosomes differ from the chromosomal DNA and cytosolic ribosomes in several aspects, reflecting their prokaryotic origin in evolution. The similarity between mitochondrial biogenesis and bacterial/viral replication represents a significant challenge for drug developers designing novel antibacterials and antivirals, as many of these drugs can cause serious mitochondrial toxicity. Measuring RNA levels using reverse transcription qPCR is occasionally discussed as a useful high-throughput technique for identifying effects on mitochondrial biogenesis, but this approach has a fundamental limitation: its inability to identify inhibition of protein translation. If such an effect is occuring then RNA levels might in fact increase in the cell, since mitochondrial translation is unable to process transcription products. The only way to easily and simultaneously identify inhibition of both mitochondrial transcription and translation is to ratio the levels of an mtDNA encoded protein with total cell protein and/or an nDNA-encoded protein. This can be done by Western blotting or by ICC microscopy, but neither technique is suitably quantitative, nor are they amenable to high-throughput nor clinical applications. MitoSciences offers the only solution for measuring drug-induced effects on mitochondrial biogenesis early in the safety screening process. Our MitoBiogenesis ICE Assay is a true duplexing 96/384-well assay that ratios both an mtDNA- and an nDNA-encoded protein in cultured or primary cells, and which requires very little sample prep and few overall steps.

Assay Principle

Cells (human, rat or mouse) are seeded in 96- or 384-well microplates, and after exposure to experimental compounds for several cell doublings, the levels of two mitochondrial proteins are measured simultaneously by specific antibodies in each well by ICE. The two proteins are each subunits of a different oxidative phosphorylation enzyme complex, Complex IV (COX-I), which is mtDNA-encoded, and the 70kDa subunit of Complex II (SDH-A), which is nDNA-encoded. After IR or colorimetric analysis, cells can be stained with the whole cell stain Janus Green for the purposes of normalizing signals. Normalizing levels of both proteins to whole cell staining can provide additional information regarding the effects of drugs or conditions on mitochondrial mass and biogenesis per cell. Drugs that adversely affect mitochondrial biogenesis such as antibiotics or anti-retroviral nucleoside analogs are identified easily with this assay (Figure 7), and dose response series are easily run to quickly establish IC50 data (Figure 8).

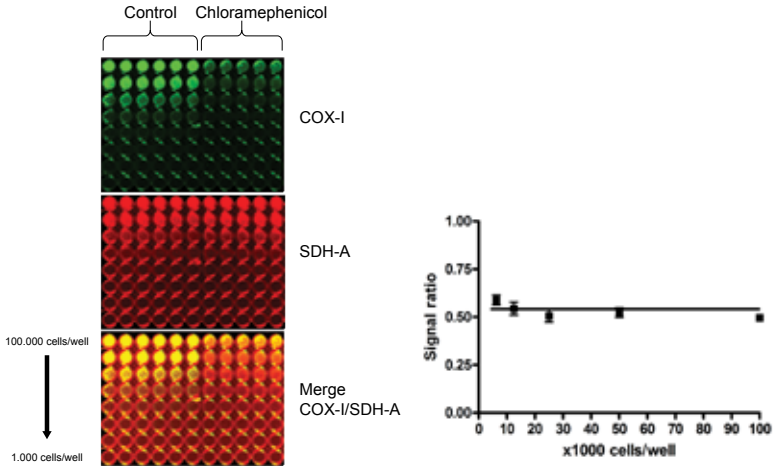


Figure 7. mtDNA-encoded protein expression is reduced specifically in rat H9C2 cardiomyocytes treated in vitro with the antibiotic chloramphenicol. *A dilution series of rat cardiomyocytes is shown treated with the antibiotic chloramphenicol at 10 µM. Using MS642 the levels of mtDNA-encoded COX-I (green 800 channel), nuclear DNA-encoded SDH-A (red 700 channel) and relative ratios COX-I/SDH-A (merged) are shown in each microwell. At all cell densities a constant ratio of mtDNA encoded protein expression (COX-I) to nuclear DNA-encoded mitochondrial protein expression (SDH-A) is observed. Therefore, normalizing COX-I to SDH-A simplifies data analysis and eliminates the need to perform all tests at the same cell concentration.*

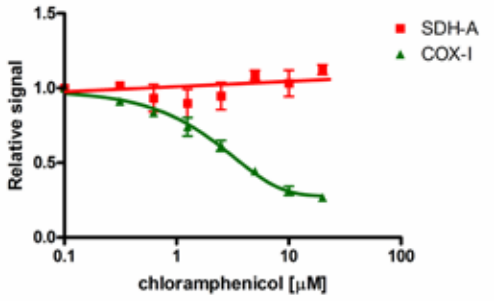


Figure 8. Inhibition of mitochondrial biogenesis by chloramphenicol. *The IC50 of a drug's effect on mitochondrial protein translation can be determined quickly using the MitoBiogenesis ICE (IR). In this example, cells were seeded at 3000 cells/well, allowed to grow for 3 cell doublings in a drug dilution series and then the relative amounts of COX-I, and SDH-A were measured in each well. Chloramphenicol inhibits mtDNA-encoded COX-I protein synthesis relative to nuclear DNA-encoded SDH-A protein synthesis by 50% at 3.5 µM.*

Availability

The kit includes all necessary reagents and a detailed protocol for conducting the assay, and versions are available for both IR (MS642) and colorimetric detection (MS643).

Product Information

Cat. No.	Product Name	Amount	Reactivity
MS642	MitoBiogenesis™ In-Cell ELISA Kit (IR)	2 x 96 tests	human, rat, mouse, bovine
MS643	MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric)	2 x 96 tests	human, rat, mouse, bovine

Kit 2 – PhosphoPDH In-Cell ELISA (MSP47, MSP48)

Background

MitoSciences' PhosphoPDH ICE Kits are high-throughput assays for measuring phosphorylation at all three E1α regulatory serines: Ser232, Ser293 and Ser300 and up- or down-regulation of total pyruvate dehydrogenase (PDH) subunit E1α. The assay is designed for use with cultured adherent cells in a 96/384 well microplate format.

The pyruvate dehydrogenase complex is the key regulatory site in cellular metabolism, in that it links the citric acid cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis, as well as with both lipid and amino acid metabolism. Not surprisingly, given its central role in metabolism, PDH is under tight and complex regulation, which includes regulation by reversible phosphorylation in response to the availability of glucose. In humans, PDH activity is inhibited by site-specific phosphorylation at three sites on the PDH E1α subunit (Ser232, Ser293 and Ser300), which is catalyzed by four different pyruvate dehydrogenase kinases (PDK1-4). Each of the four kinases has a different reactivity for these three sites. Interestingly, phosphorylation at any one site leads to the inhibition of the complex and a shift to glycolytic metabolism of pyruvate. Two pyruvate dehydrogenase phosphatases (PDP1 and PDP2) dephosphorylate the E1α and activate the enzyme. Both the kinases and phosphatases are differentially expressed in tissues. Each of the PDKs and PDPs is under transcriptional control in response to different cellular stress events. In addition, the kinases are activated by acetyl Coenzyme A, NADH and ATP, meanwhile the availability of pyruvate and ADP leads to their inhibition.

The regulation of glucose metabolism by PDH phosphorylation is of key interest in hypoxia and tumor cell metabolism and is therefore, a target for cancer therapy as well as other metabolic diseases such as diabetes and obesity.

Assay Principle

Cells (human, rat or mouse) are seeded in 96- or 384-well microplates, and after exposure to treatment, the levels of phosphorylated PDH E1α at each of three regulatory serines is measured while simultaneously measuring total E1α subunit. Optionally, a relative cell density stain is included for normalization. An example of a treatment affecting the phosphorylation state of PDH E1α is exposure to dichloroaceteate (DCA) an inhibitor of PDH kinases. Figure 9 shows images from a plate where a dilution series of DCA was added to growing cells. DCA decreased the signal from each of the three phosphoserine sites, while total E1α is unaffected. This result is confirmed by Western blotting in Figure 10 using the same antibodies. For Western blotting sample preparation, the cells must be harvested and washed in DCA to maintain the dephosphoryalted state. In ICE such limitations do not exist, cells are fixed instantaneously in a dephosphoryalted state. Finally using this kit, a drug dilution series can easily be performed in multiple cell types, as shown in Figure 11, where 3 different cell types have IC50 response to DCA but the magnitude of the response differs by cell type and by phosphorylation site.

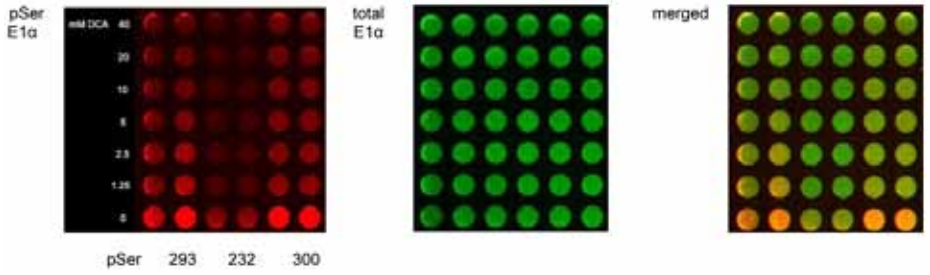


Figure 9. DCA inhibits kinases and reduces phosphorylation at all three sites while total E1α is unaffected. *Cells (HepG2 shown) were seeded at 50,000 cells per well and treated with millimolar concentrations of dichloroacetate (DCA) in vehicle (1% DMSO) for 2 hours. Cells were then fixed and processed according to the protocol. To account for any cell density variability between wells the ratio of PDH E1α pSer : total E1α signal should be determined.*

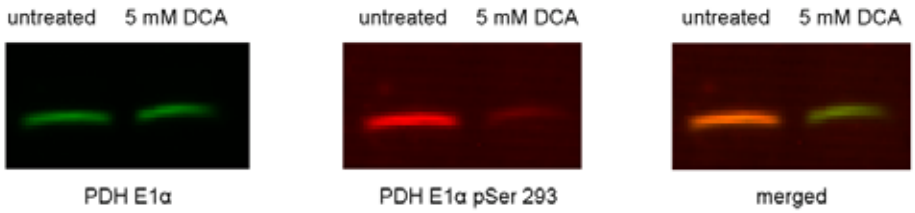


Figure 10. Antibody specificity demonstrated by Western Blot. *HepG2 cells were treated with 5 mM dichloroacetate (DCA) to inhibit PDH kinase activity. To maintain the PDH in the maximal dephosphorylated state the cells were harvested quickly and washed in DCA containing buffers before Western blot analysis. Shown the PDH E1α and pSer293 analysis with antibodies used in this kit.*

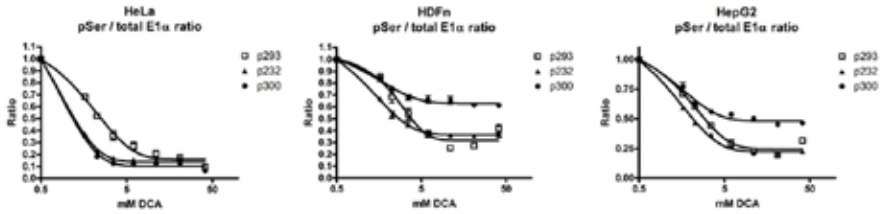


Figure 11. Kinase inhibiton by dichloracetate in HepG2, HeLa and fibroblasts (HDFn) cells results in decreased phosphoserine signal. *The ratio of PDH E1α pSer232, 293 and 300 to the total E1α signal was normalized to the untreated (DMSO) sample for each concentration of DCA from 0-40 mM, showing that DCA is effective at inhibiting the PDH kinases and reducing phosphorylation at all three regulatory serine residues.*

Availability

The kit includes all the necessary reagents and a detailed protocol for conducting the assay. Versions are available for both IR (MSP47) and colorimetric detection (MSP48).

Product Information

Cat. No.	Product Name	Amount	Reactivity
MSP47	PhosphoPDH In-Cell ELISA Kit (IR)	2 x 96 tests	human, rat, mouse, bovine
MSP48	PhosphoPDH In-Cell ELISA Kit (Colorimetric)	2 x 96 tests	human, rat, mouse, bovine

Kit 3 - PARP-1 (cleaved) In-Cell ELISA (MSA43)

Background

The PARP-1 (cleaved) ICE Kit is a highly specific and high-throughput assay for measuring cleaved PARP1 (89kDa fragment) in human adherent and suspension cells.

PARP is a 113 kDa nuclear DNA-repair enzyme that transfers ADP-ribose units from NAD^+ to variety of nuclear proteins including topoisomerases, histones and PARP itself. Via poly ADP ribosylation, PARP1 is responsible for regulation of cellular homeostasis including cellular repair, transcription and replication of DNA, cytoskeletal organization and protein degradation. In response to DNA damage, PARP1 activity is increased upon binding to DNA strand nicks and breaks. Excessive DNA damage leads to generation of large branched ADP-ribose polymers and activation of a unique cell death program. During apoptosis, PARP1 is cleaved by activated caspase-3 between Asp214 and Gly215, resulting in the formation of an N-terminal 24 kDa fragment containing most of the DNA binding domain and a C-terminal 89 kDa fragment containing the catalytic domain. The proteolysis of PARP through this cleavage renders the enzyme inactive and this further facilitates apoptotic cell death. Thus the presence of 89 kDa PARP fragment is considered to be an important and general biomarker of apoptosis.

The assay is designed for use with cultured adherent and suspension cells in a 96-well microplate format. The adherent cells undergoing apoptosis readily detach from a culture plate. The cell detachment often leads to their loss and thus underestimation of the proportion of apoptotic cells. This assay was developed to eliminate the loss of apoptotic cells. In addition, this assay is also applicable for suspension cells.

Assay Principle

This assay uses a combination of a plate coating and simple centrifugation steps to achieve efficient fixation of cells to the plate. Adherent cells are seeded directly into the provided assay plate and exposed to experimental conditions of interest. Suspension cells, after exposure to experimental conditions, are transferred to the assay plate. Then the protein levels of cleaved PARP are measured. The primary antibody used in this assay reacts with the N-terminal end of the 89 kDa PARP fragment formed by the caspase cleavage adjacent to Asp214; it thus recognizes only the apoptosis-specific 89 kDa catalytic domain fragment, but it does not recognize the full-length PARP or the 24 kDa DNA binding domain fragment. Optionally, a cell density stain is included for normalization. Examples using both adherent and suspension cells are shown in Figures 12 and 13. Note the cell type-specific response of cleaved PARP to Staurosporine and high efficiency of crosslinking of apoptotic cells to the assay plate (Figure 13).

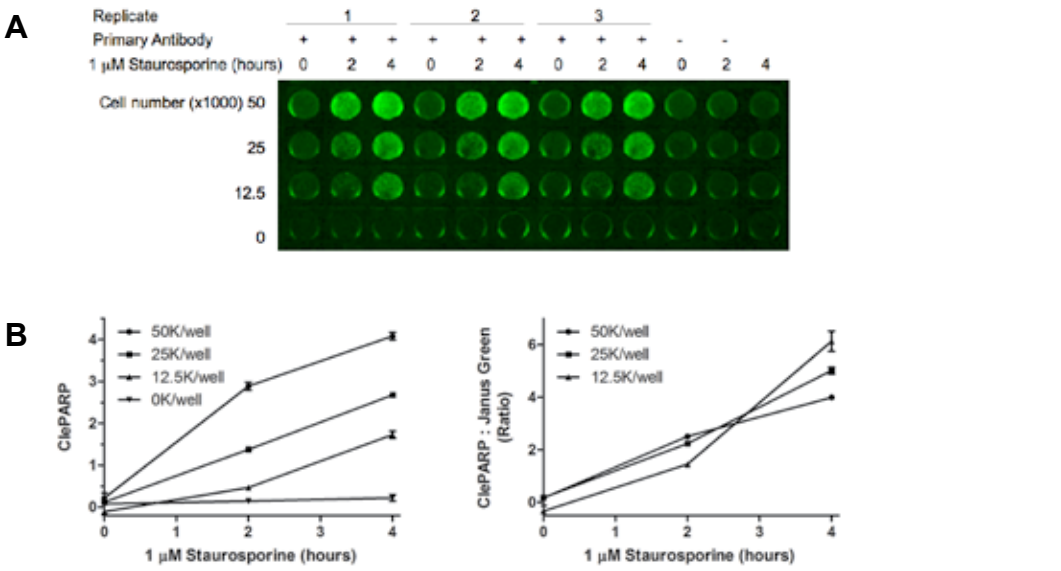


Figure 12. Time-dependence of PARP cleavage in adherent cells treated to undergo apoptosis. *HeLa cells were seeded as indicated in the assay plate, allowed to attach overnight, treated with Staurosporine (STS) and fixed. The plate was analyzed by ICE to measure cleaved PARP using MSA43 kit. All steps were performed as described in MSA43 Protocol. (A) Image of the assay plate analyzed by the LI-COR® Odyssey® imaging system. (B) Quantification of cleaved PARP signal after subtraction of background signal (left panel) and normalization of cleaved PARP to cell amount measured by Janus Green (right panel). Mean and standard error of the mean (n=3) is shown. Note that the normalized cleaved PARP signal is independent of cell amount used (B, right panel).*

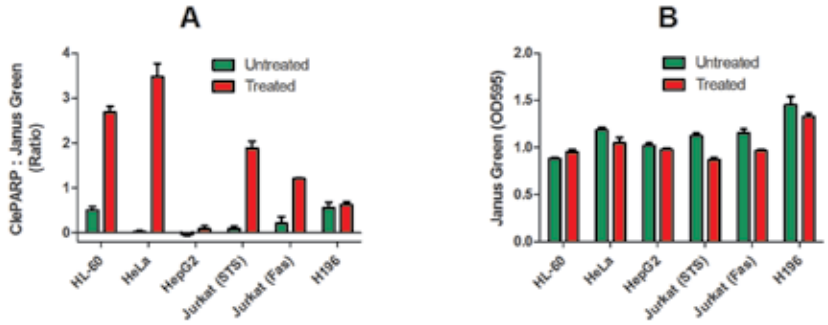


Figure 13. Cell-line dependent induction of PARP cleavage. *Adherent cell lines were seeded (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) directly in assay plate, allowed to attach overnight and treated with 1 µM Staurosporine (STS) as indicated. Suspension cells were treated with 1 µM STS or 50 ng/mL Fas antibody as indicated and transferred (HL-60 at 300,000 per well, Jurkat at 200,000 per well) in media containing 10% serum to the assay plate. Cells were fixed and the plate was analyzed by ICE to measure the cleaved PARP using MSA43. All steps were performed as described in MSA43 Protocol. Mean and standard error of the mean (n=3) is shown. (A) Cleaved PARP normalized to cell amount measured by Janus Green cell stain. Note HepG2 cells are resistant to undergoing apoptosis under these conditions, consistent with all of our previous observations. (B) Cell amount measured by Janus Green. Note no or very small differences between Janus Green staining of treated and untreated cells indicating that the treated cells undergoing apoptosis are efficiently crosslinked to the assay plate.*

Availability

This kit (MSA43) is available in IR detection version. It includes all necessary materials, reagents and a detailed protocol for conducting the assay.

Product Information

Cat. No.	Product Name	Amount	Reactivity
MSA43	PARP-1 (cleaved) In-Cell ELISA Kit (IR)	2 x 96 tests	human

Custom ICE Kits

Take advantage of the flexibility of ICE to design your own experiments around your pathway(s) of interest. MitoSciences provides all of the reagents required to build your own customized ICE experiment:

ICE-Validated Antibodies:

A collection of MitoSciences' ICE-validated mouse monoclonal and rabbit primary antibodies. Available in 50 µg or smaller aliquots in 3, 4, or 10 antibody packs.

Labeled Secondary Antibodies:

Select from IRDye® and HRP labeled secondary detection antibodies.

ICE Support Packs:

Reagents and protocols for performing ICE assays with adherent or suspension cells. ICE support packs provide sufficient reagents for 5x96 well-plate assays.

ICE-Validated Antibodies

MitoSciences offers a wide selection of mouse and rabbit antibodies pre-validated for performance in ICE. Antibodies are thoroughly tested for the qualities described in the validation section above. With the appropriate choice of IRDye® labeled secondary antibodies, mouse and rabbit antibodies or two mouse antibodies of different isotype can be used in the same well, simultaneously generating two independant measurements. Alternatively, antibodies can be detected using the available HRP conjugated secondary antibodies with one antibody per well.

The list of currently available antibodies, at the time of writing, is shown on the next page. Most antibodies are available on MitoSciences' website in 100 µL (100 µg) amounts. Additionally, all antibodies are available in ICE Packs. ICE Packs are completely customizable selections of 3, 5, or 10 antibodies packaged in smaller amounts - enough for a minimum of 50 wells for each antibody. In this way, a panel of antibodies against similar targets can be easily obtained for pathway analysis. The MitoSciences ICE webpage contains a filter tool for selecting individual antibodies as part of an ICE Pack. Included in the filter criteria are protein, species reactivity, isotype, and metabolic pathway.

Check www.mitosciences.com frequently as we are continually adding to our collection of ICE-validated antibodies.

ICE Validated MOUSE Monoclonal Antibodies

Cat. #	Antibody Name	Pathway(s)	Reactivity*	Isotype	Availability
MS711	2,4-dienoyl-CoA reductase (DECR1)	Fatty Acid Oxidation (Mito)	h	IgG1	ICE pack / 100 µg
MS716	Acetyl-CoA acyltransferase, peroxisomal (ACAA1)	Fatty Acid Oxidation (Perox)	h, m, r	IgG1	ICE pack / 100 µg
MS718	Acetyl-CoA acetyltransferase (ACAT1)	Ketogenesis	h	IgG2a	ICE pack / 100 µg
MS786	Aconitase (ACO2)	Krebs Cycle	h, m, r	IgG2a	ICE pack / 100 µg
MS767	Aldehyde dehydrogenase, mitochondrial (ALDH2)	Alcohol Metabolsim	h, m, r, b	IgG2a	ICE pack / 100 µg
MS753	Alpha-ketoglutarate dehydrogenase (OGDH) subunit E2	Krebs Cycle	h	IgG1	ICE pack / 100 µg
MSA09	Apoptosis-inducing factor (AIF)	Apoptosis	h	IgG1	ICE pack / 100 µg
MS503	ATP synthase subunit beta	OXPHOS	h, m, r, b, mk	IgG1	ICE pack / 100 µg
MS776	Bcl-2	Apoptosis	h	IgG1	ICE pack
MS778	BNIP3	Apoptosis	h, m	IgG2b	ICE pack
MS722	Carnitine palmitoyltransferase 2 (CPT2)	Fatty Acid Oxidation (Mito)	h, m, r, b	IgG1	ICE pack / 100 µg
MS721	Catalase	Free Radical Scavenging, Fatty Acid Oxidation (Perox)	h, r, b	IgG1	ICE pack / 100 µg
MS101c	Complex I immunocapture	OXPHOS, Apoptosis	h, m, r, b	IgG2b	ICE pack / 100 µg
MS103	Complex I subunit GRIM-19	OXPHOS, Apoptosis	h, m, r, b	IgG2b	ICE pack / 100 µg
MS204	Complex II subunit 70 kDa Fp	OXPHOS	h, m, r, b	IgG1	ICE pack / 100 µg
MS304	Complex III subunit Core 2	OXPHOS	h, m, r, b	IgG1	ICE pack / 100 µg
MS404	Complex IV subunit 1	OXPHOS	h, m, r, b	IgG2a	ICE pack / 100 µg
MSA04	Cyclophilin D (PPIF)	Permeability Transition Pore	h, m, r, b	IgG1	ICE pack / 100 µg
MSA06	Cytochrome c	OXPHOS, Apoptosis	h, m, r, b	IgG2a	ICE pack / 100 µg
MS723	Delta(3,5)-delta(2,4)-dienoyl-CoA isomerase (ECH1)	Fatty Acid Oxidation (Perox)	h, r	IgG2b	ICE pack / 100 µg
MS704	Dicarbonyl/L-xylulose reductase (DCXR)	Xenobiotic Metabolism	h	IgG1	ICE pack / 100 µg
MS782	Electron transfer flavoprotein (ETF) subunit alpha (ETFA)	Fatty Acid Oxidation (Mito)	h, m, r	IgG2b	ICE pack / 100 µg
MS758	Epoxide hydrolase 1 (EPHX1)	Xenobiotic Metabolism	h	IgG1	ICE pack / 100 µg
MS709	Fumarase (FH)	Krebs Cycle	h, r, b	IgG2a	ICE pack / 100 µg
MS751	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Glycolysis	h	IgG2b	ICE pack / 100 µg
MS725	Hydroxymethylglutaryl-CoA lyase (HMGCL)	Ketogenesis	h	IgG2b	ICE pack / 100 µg
MS728	Hydroxysteroid dehydrogenase-like protein 2 (HSDL2)	Oxioreductase	h	IgG1	ICE pack / 100 µg
MS779	Hypoxia induction factor 1α (Hif1 α)	Transcription	h	IgG1	ICE pack
MS783	Malate dehydrogenase, mitochondrial (MDH2)	Krebs Cycle, Gluconeogenesis	h, m, r, p	IgG1	ICE pack / 100 µg
MS726	Medium-chain acyl-CoA dehydrogenase (MCAD)	Fatty Acid Oxidation (Mito)	h, m, r, b	IgG1	ICE pack / 100 µg
MS765	Microsomal glutathione S-transferase 3 (MGST3)	Peroxidase	h, m, r	IgG2a	ICE pack / 100 µg
MS732	Mitochondrial superoxide dismutase 2 (SOD2)	Free Radical Scavenging	h, m, r, b	IgG1	ICE pack / 100 µg
MS702	Mitochondrial trifunctional protein (TFP) subunit alpha (HADHA)	Fatty Acid Oxidation (Mito)	h	IgG2b	ICE pack / 100 µg
MS733	Mitochondrial trifunctional protein (TFP) subunit beta (HADHB)	Fatty Acid Oxidation (Mito)	h	IgG1	ICE pack / 100 µg
MSM02	Mitofilin (MF)	Mitochondrial Morphology	h	IgG1	ICE pack / 100 µg
MS750	NAD-dependent deacetylase sirtuin 1 (SIRT1)	NAD+ NADH Cycling	h, m, r	IgG1	ICE pack / 100 µg
MS701	Nicotinamide nucleotide transhydrogenase (NNT)	NAD+ NADH Cycling, OXPHOS	h, m, r, b	IgG1	ICE pack / 100 µg
MS703	Nitrotyrosine	Oxidative stress	h, m, r, b	IgG2b	ICE pack / 100 µg

**Reactivity: h = human, m = mouse, r = rat, b = bovine, mk = monkey, hr=hamster, p = pig*

ICE Validated MOUSE Monoclonal Antibodies (cont.)

Cat. #	Antibody Name	Pathway(s)	Reactivity*	Isotype	Availability
MS777	Poly [ADP-ribose] polymerase 1 (PARP-1) (cleaved)	Apoptosis	h	IgG1	ICE pack / 100 µg
MS774	Pyruvate carboxylase, mitochondrial (PC)	Gluconeogenesis	h, m, r, b	IgG1	ICE pack / 100 µg
MSP03	Pyruvate dehydrogenase (PDH) subunit E1α	Glycolysis, Krebs Cycle	h, m, r, b	IgG1	ICE pack / 100 µg
MSP05	Pyruvate dehydrogenase (PDH) subunit E2	Glycolysis, Krebs Cycle	h, b	IgG1	ICE pack / 100 µg
MSP49	Pyruvate dehydrogenase kinase isoform 1 (PDK1)	Glycolysis, Krebs Cycle	h, m, r, b	IgG1	ICE pack / 100 µg
MS766	Quinone oxidoreductase (CRYZ)	Xenobiotic Metabolism	h	IgG2a	ICE pack / 100 µg
MS773	Serine-pyruvate aminotransferase (AGXT)	Xenobiotic Metabolism	h, r, b	IgG1	ICE pack / 100 µg
MS706	Short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD)	Fatty Acid Oxidation (Mito)	h	IgG1	ICE pack / 100 µg
MS764	Transitional endoplasmic reticulum ATPase (VCP)	Transport	h, m, r	IgG1	ICE pack / 100 µg

ICE Validated RABBIT Antibodies

Cat. #	Name	Pathway	Reactivity*	Availability
MSR14	α-Tubulin	Microtubules	h, m, r, b, mk	ICE pack
MSR08	Acetyl-CoA carboxylase1 (ACC1)	Fatty Acid Synthesis	h, m, r	ICE pack
MSR12	Acetyl-CoA carboxylase1 (ACC1)	Fatty Acid Synthesis	h, m, r, hr	ICE pack
MSR13	Acetyl-CoA carboxylase 2 (ACC2) Phospho (pSer221)	Fatty Acid Synthesis	h, m, r	ICE pack
MSR24	Bak	Apoptosis	h	ICE pack
MSR25	Bax	Apoptosis	h, mk	ICE pack
MSR26	Bcl-X	Apoptosis	h, m, r	ICE pack
MSR27	Caspase 3 (cleaved)	Apoptosis	h, m, r, mk	ICE pack
MSR19	Complex I assembly factor NDUFAF1	OXPHOS	h, m, r	ICE pack
MSR09	Fatty acid synthase (FAS)	Fatty Acid Synthesis	h, m, r, b	ICE pack
MSR11	Glucose 6 phosphate dehydrogenase (G6PD)	Glycolysis, Pentose Phosphate	h, m, r	ICE pack
MSR05	Glucose transporter 1 (GLUT1)	Glycolysis	h, m, r	ICE pack
MSR06	Glucose transporter 4 (GLUT4)	Glycolysis	h, m, r	ICE pack
MSR10	Hexokinase II (HK2)	Glycolysis	h, m, r, mk	ICE pack
MSR04	Hypoxia induction factor 1α (Hif1 α)	Transcription	h, r	ICE pack
MSR23	Lactate dehydrogenase (LDH)	Glycolysis	h, m, r	ICE pack
MSR28	Mcl-1	Apoptosis	h	ICE pack
MSR18	Parkin	Protein Degradation	h, m, r	ICE pack
MSR07	Phosphoglycerate kinase (PGK1)	Glycolysis	h	ICE pack
MSR17	Prohibitin	Chaperone / Mitochondrial Morphology	h, m, r	ICE pack
MSR29	Puma	Apoptosis	h	ICE pack
MSR01	Pyruvate dehydrogenase E1α PSer232	Glycolysis, Krebs Cycle	h, m, r	ICE pack
MSR02	Pyruvate dehydrogenase E1α PSer293	Glycolysis, Krebs Cycle	h, m, r	ICE pack
MSR03	Pyruvate dehydrogenase E1α PSer300	Glycolysis, Krebs Cycle	h, m, r	ICE pack
MSR15	Pyruvate kinase isoforms M1/M2 (PKM1/PKM2)	Glycolysis	h, m, r, mk	ICE pack
MSR16	Pyruvate kinase isoform M2 (PKM2)	Glycolysis	h, m, r, mk	ICE pack
MSR21	Sirtuin 2 (SIRT2)	NAD+ NADH Cycling	h	ICE pack
MSR20	Superoxide dismutase (SOD1)	Free Radical Scavenging	h, r	ICE pack

**Reactivity: h = human, m = mouse, r = rat, b = bovine, mk = monkey, hr=hamster, p = pig*

Labeled Secondary Antibodies for ICE

Mitosciences offers a wide selection of IRDye® labeled secondary antibodies. IR measurement offers superior sensitivity and greater dynamic range than colorimetric methods. IRDyes® require a LiCor® Odyssey® or Aerius®, are read in the 700 or 800 channel, and can therefore be duplexed. For the purposes of ICE this allows the simultaneous in-well analysis of two analytes detected by two antibodies which are differentiated by host species, or isotype and can therefore be distinguished by a carefully chosen pair of secondary detection reagents. For users without an IR imaging system, colorimetric detection using HRP conjugated antibodies in a standard absorbance microplate reader is recommended using species specific HRP conjugated secondary antibodies. After IR or colorimetric analysis, cells can be stained with the whole cell stain Janus Green for the purposes of normalizing signals.

A selection of IRDye® labeled secondary antibodies allow duplex detection of mouse and rabbit antibodies or mouse antibodies of different isotypes in an IR Imager.

Cat. No.	Antibody Name	Reactive Against	Amount	# ICE Plates
MS923	Goat anti-mouse IRDye®800CW	all subclasses	50 µL	12
MS924	Goat anti-rabbit IRDye®680LT	all subclasses	50 µL	12
MS925	Goat anti-mouse IRDye®680LT	anti-IgG1	50 µL	12
MS926	Goat anti-mouse IRDye®800CW	anti-IgG2a	50 µL	12
MS927	Goat anti-mouse IRDye®800CW	anti-IgG2b	50 µL	12

HRP labeled secondary antibodies for detection of mouse or rabbit primary antibodies.

Cat. No.	Antibody Name	Reactive Against	Amount	# ICE Plates
MS928	Goat anti-mouse HRP, IgG (H+L)	all subclasses	50 µL	12
MS929	Goat anti-rabbit HRP, IgG (H+L)	all subclasses	50 µL	12

Janus Green whole cell stain is included in ICE kits and support packs but is also available separately.

Cat. No.	Antibody Name	Amount	# ICE Plates
MS930	Janus Green cell normalization stain	11 mL	2

ICE Support Pack

The In-Cell ELISA (ICE) Support Pack is for use with suspension, apoptotic/detaching cells and adherent cell lines. As outlined in the introduction, ICE requires that the assayed cells are fixed onto the surface of a microplate in which the assay is performed. The pack contains five 96-well microplates, buffers and protocols to perform ICE with MitoSciences ICE-validated antibodies. Two protocols are available when using this kit. Protocol 1 is for use with suspension cells and cells likely to detach under experimental conditions (for example, adherent cells undergoing apoptosis readily detach from a culture plate). Protocol 2 is available for use with normal adherent cells. These protocols can be used with any of MitoSciences' ICE-validated antibodies.

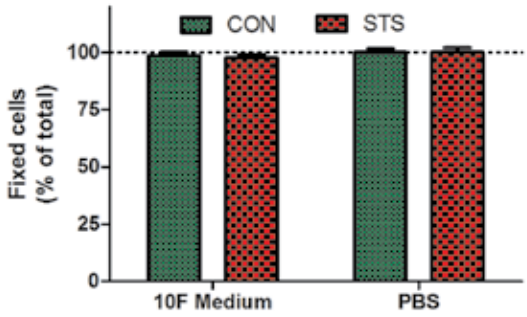


Figure 14. Suspension cells are efficiently crosslinked to the assay plate. *Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 µM) Jurkat cells were seeded at 200,000 cells per well into the assay plate in media containing 10% bovine fetal serum (10F Medium) or in PBS, and fixed as described in the MS922 protocol. After the fixation the number of cells attached (fixed) to the plate was determined and expressed as percentage of total seeded cells. Mean and standard error of the mean (n=2) is shown. Note that virtually all cells (untreated or STS-treated) attached to the plate whether the fixative was added to cells in 10F media or PBS.*

Adherent cells are typically fixed in the same plate in which the cells are grown under desired experimental conditions. Thus, depending on the type of adherent cells, an appropriate microplate surface, that promotes optimal cell growth and attachment, should be chosen. The ability of adherent cells to attach to a microplate makes these cells naturally suitable for the ICE analysis.

The suspension cell protocol relies on combination of a coating of the assay plate and simple centrifugation steps. The suspension cells, treated as desired, are transferred into the provided assay plate, sedimented by centrifugation and, after the addition of fixative, sedimented again. When this protocol is used, nearly all suspension cells are crosslinked to the assay plate (see Figure 14). Crosslinked cells remain attached on the plate within the course of ICE assay (see Figure 15). In addition, it is advantageous that no serum-washing step is required; the suspension cells can be fixed onto the assay plate directly in culture medium containing serum (see Figures 14 and 15). Several other products on the market use filter-based microplates to capture the suspension cells. However, these products have some limitations, mainly: (1) very low- or no serum-containing medium should be used to avoid clogging the filter plate and (2) the ICE detection is limited to endpoint colorimetric measurements.

For ICE assays using either suspension cells or adherent cells, MitoSciences offers an ICE Support Pack (MS922) that includes assay plates, reagents and protocol to perform the assay with MitoSciences ICE-validated antibodies (available for purchase separately).

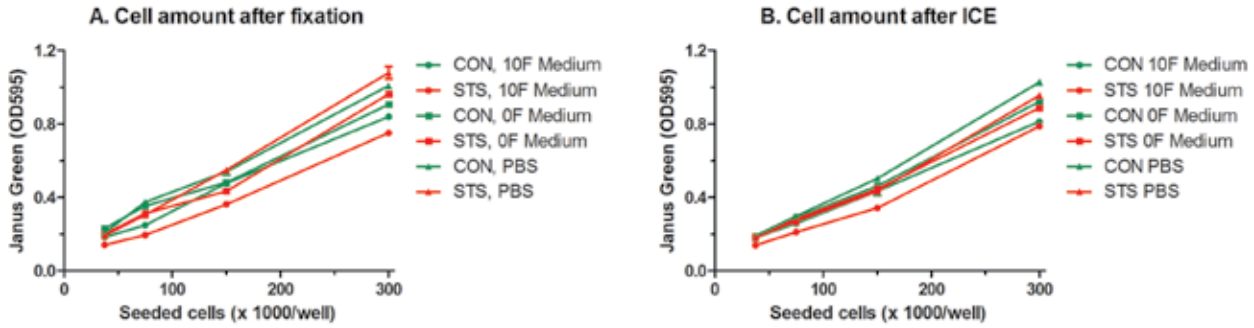


Figure 15. Suspension cells remain firmly attached to the assay plate within the ICE assay. *Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 µM) HL-60 cells were seeded in the indicated amounts into the assay plate in media containing 10% bovine fetal serum (10F Medium), media without serum (0F Medium) or PBS, and fixed as described in the MS922 protocol. The cell amount attached to the assay plate was determined by Janus green staining either just after the fixation (Panel A) or at the end of ICE assay (Panel B). Mean and standard error of the mean (n=2) is shown. Note: (1) that virtually all fixed cells remained attached to the plate within the duration of the ICE assay (compare the cell amounts in Panel A to the cell amounts in Panel B), (2) that the STS-treated cells attached nearly as efficiently as the untreated cells and (3) that the cells attach efficiently even in media containing 10% serum.*

Cells that may become detached – e.g. Apoptosis

Many experimental conditions cause detachment of adherent cells. These detached cells are often lost (and thus unaccounted for in the analysis) during fixation and washing steps of a regular ICE protocol. For example, adherent cells undergoing apoptosis readily detach from a culture plate. The detachment of apoptotic cells often leads to their loss and thus underestimation the proportion of apoptotic cells. MitoSciences has developed a protocol that eliminates the loss of the apoptotic/detaching cells and thus this protocol is recommended for ICE on adherent cells undergoing apoptosis/detachment (see Figure 13, PARP-1 (cleaved) ICE Kit MSA43). As for the suspension cells, this protocol relies on the combination of a coated assay plate and simple centrifugation steps. The adherent cells are seeded directly into the assay plate, allowed to attach and treated as desired. The treated cells are sedimented by centrifugation and, after the addition of fixative, sedimented again. Therefore, when performing and ICE assay on adherent cells and cell detachment is of concern, we recommend to use the ICE Support Pack for suspension cells (MS922) which includes assay plates, reagents and a protocol which contains specific details for efficient crosslinking of detaching cells to the assay plate.

Availability

The ICE Support Pack is available for adherent cells and suspension (or detaching) cells (MS922).

Product Information

Cat. No.	Product Name	Amount
MS921	In-Cell ELISA (ICE) Support Pack *(does not include culture treated imaging plates.)*	5 x 96 tests
MS922	In-Cell ELISA (ICE) Support Pack *(Includes 5x tissue-culture treated 96-well black/clear imaging plates.)*	5 x 96 tests

MetAbArray

MitoSciences has applied the flexibility, reproducibility and throughput of ICE to create the MetAbArray, a versatile tool to monitor an increasingly comprehensive panel of metabolic analytes in a single experiment. The MetAbArray leverages MitoSciences' collection of ICE-validated antibodies to enable researchers to track modulation of multiple analytes simultaneously (making intra- and inter-pathway analysis possible). By monitoring a broader array of analytes (including antibodies for protein level, phosphorylation and cleavage events, etc), the ICE MetAbArray enables new discoveries and hypotheses without the need for expensive and specialized technologies (e.g. mass spectroscopy).

Sample Applications of the MetAbArray:

1. Monitor analyte changes following alterations in culture conditions, e.g.
    a. Altering energy generation: glucose vs. galactose cultures
    b. Metabolic reprogramming in response to hypoxia
2. Assess differences between defined samples, e.g.
    a. Clinical patient fibroblasts
    b. Wile-type vs. knock-out cells for protein of interest
    c. Intrinsic differences between cell lines
3. Discover effects of pathway pertubations or chemical inhibition, e.g.
    a. siRNA or overexpression of pathway components
    b. Effect(s) of specific metabolic inhibitor(s) or apoptotic inducer(s)
    c. Off-target or downstream metabolic effects of your compound of interest

The MetAbArray is inherently flexible since analyte selection is at the discretion of the researcher. Project-specific MetAbArray panels can be assembled from MitoSciences' list of ICE-validated antibodies (see pages 15-17). Sample pathway-specific panels are listed below. Visit www.mitosciences.com to assemble panel ICE packs of your own design.

Example Analyte Panels:

OXPHOS	GLYCOLYSIS	FATTY ACID METABOLISM AND LIPID BIOGENESIS	APOPTOSIS	HYPOXIA
Complex I	GAPDH	DECR	cleaved-Caspase3	HIF1A
Complex II	Hexokinase II	ACAA1	Cleaved-PARP	GLUT1
Complex III	Lactate Dehydrogenase	TFP	AIF	BNIP3
Complex IV	PDHE1alpha	SCHAD	Bax	GAPDH
Complex V	pPDH S293	ECH1	Bak	
Cytochrome C	PKM12	FAS	Bcl-XL	
NNT		ACAT1	Bcl-2	
NDUFAF1		ACC	Mcl-1	
		pACC2 S221	Puma	

Alternatively, if a broader screen is desired, contact the Contract Research division at MitoSciences for information on our custom 64-plex MetAbArray. Grow, treat, and fix your samples and send the plates to us; we run the MetAbArray and send you the results.

Example MetAbArray Studies:

The following are sample datasets generating using the 96-well plate MetAbArray. These studies highlight the flexibility of the ICE platform.

I. Broad analyte comparison of two culture conditions: Glucose vs. Galactose.

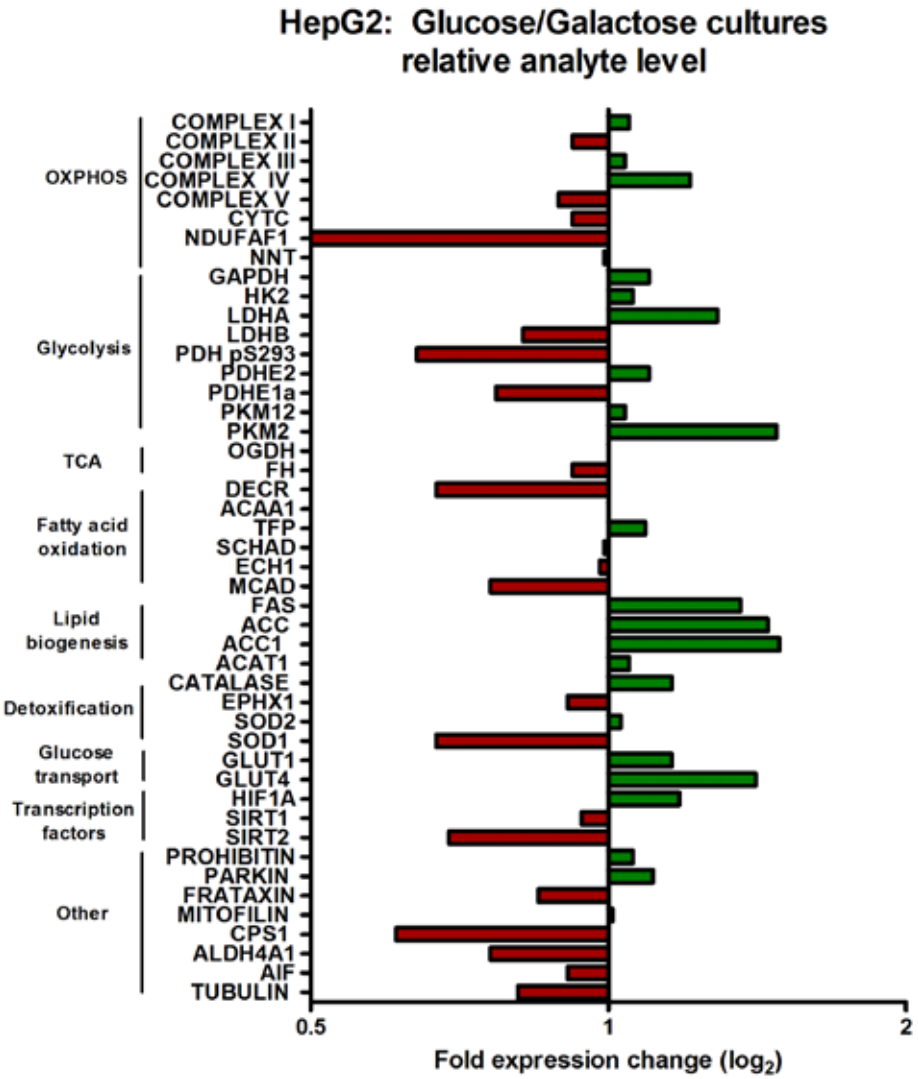


Figure 16. Relative analyte levels between HepG2 cells cultured in Glucose and Galactose. *Long-term cultures of HepG2 cells grown in media containing 5mM glucose or 5mM galactose + glutamine were compared using the ICE MetAbArray. Plotted here on a log2 scale are the mean intensities (triplicate measurements) each analyte from the glucose culture divided by the galactose culture. Thus, all analytes with values >1 (green bars) are present at a greater amount in glucose cultures than in galactose cultures and vice versa for values <1 (red bars). Analytes are grouped by functional pathway.*

II. Comparison of apoptotic readouts across sensitive and insensitive cell lines.

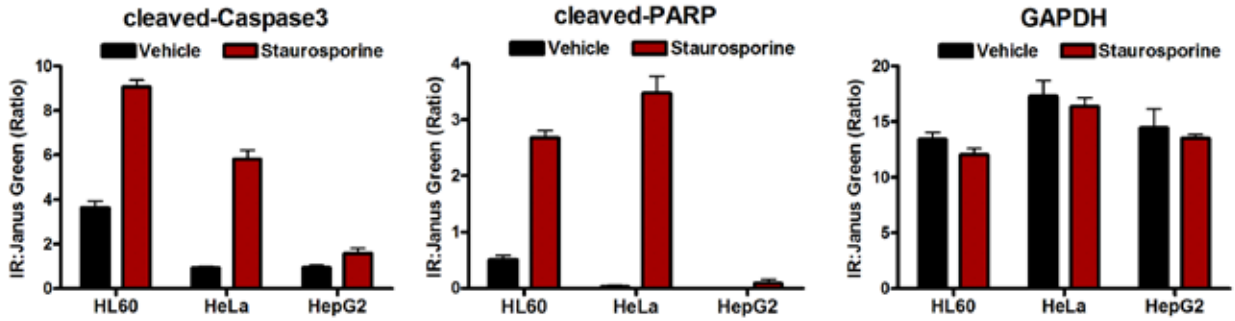


Figure 17. cleaved-PARP, cleaved-Caspase3 and GAPDH were compared across HL60, HeLa and HepG2 cell lines, +/- Staurosporin treatment. *HL60 (suspension) and HeLa and HepG2 (adherent) cell lines were treated +/- 1µM Staurosporine for 4 hours and analyzed by ICE for levels of cleaved-Caspase3 and cleaved-PARP (apoptotic readouts) and GAPDH (a cell normalizing control). All measurements in triplicate. Note that Staurosporine does not induce cleavage of Caspase3 or PARP in HepG2 cells. Stability of GAPDH signal +/- Staurosporin treatment indicates that apoptotic cells are not being lost from the plate. Finally, note that the absolute lack of cleaved-PARP in unstimulated cells can cause the signal to drop below background following background subtraction as in the HepG2 sample.*

III. Comparison of hypoxic response within a cell line.

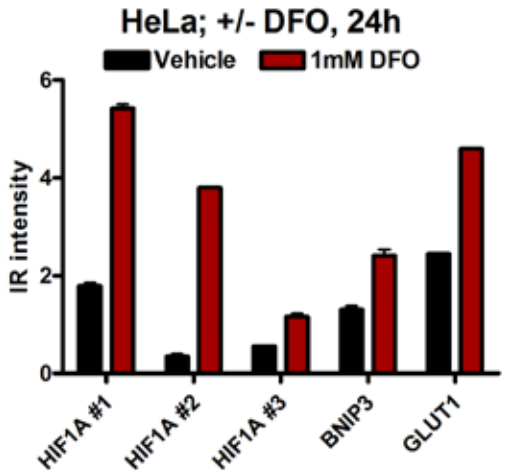


Figure 18. ICE analysis of hypoxic response in HeLa cells following treatment with iron chelator deferoxamine (DFO). *Comparison of three distinct HIF1A (hypoxia inducible factor alpha) antibodies (#1-3) and HIF1A regulated targets BNIP3 (involved in mitochondrial autophagy) and GLUT1 (a glucose transporter). All measurements in duplicate. DFO blocks the degradation of HIF1A protein, a process that requires iron cofactor. Note that the three HIF1A antibodies show increase levels in HIF1A following DFO exposure but the fold increase is dependent on the binding properties of the individual antibodies. Similarly, BNIP3 and GLUT1 show increased levels following DFO treatment.*

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