Application Note

Cell Analysis

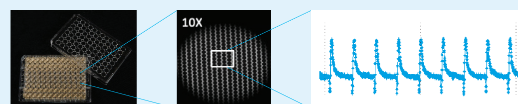
Agilent
Trusted Answers

Assessing the Impact of Drug Treatment on Cardiomyocyte Function

Through combined analysis of contractility, metabolic flux, and cellular oxygenation

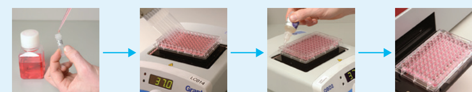
iPS Cardiomyocyte Contractility:

Cells cultured on RTCA E-Plate Cardio 96
Measured on xCELLigence RTCA Cardio
Allows interrogation of Contractility



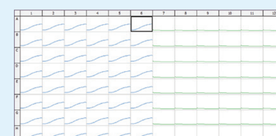
Cell Metabolism:

Cells cultured on E-Plate Cardio 96
Measured on TRF Fluorescence Plate Reader
Agilent assays monitor *mitochondrial function* (MitoXpress Xtra), *glycolytic flux* (pH-Xtra) and *cellular oxygenation* (MitoXpress Intra).



Workflow Integration:

Allows measurement on E-Plates such that metabolism and contractility can be *measured sequentially on the same test plate.*



Authors

Ryan McGarrigle, Conn Carey, and James Hynes
Agilent Technologies, Inc.

Abstract

In this application note, we demonstrate the feasibility of combining microelectrode-based iPS cardiomyocyte contractility measurements with a microplate-based bioenergetics assessment to better characterize cellular responses to drug treatment. Contractility was assessed on 96-well E-Plate Cardio 96 using the Agilent xCELLigence RTCA Cardio system while cell metabolism was measured on the same E-plate using a multiplexed fluorometric measurement of O_2 consumption with Agilent MitoXpress Xtra, glycolytic flux with Agilent pH Xtra, and cellular oxygenation using Agilent MitoXpress Intra.

Introduction

Cardiotoxicity and related cardiac impairment remain one of the main reasons for both drug withdrawal[1] and FDA black box warning[2] and are a significant cause of compound attrition in preclinical development. *In vitro* assays are capable of better characterizing cardiac response to drug treatments and are therefore of significant importance to better predict such adverse effects *in vivo*.

Cardiac tissue requires an uninterrupted supply of respiratory substrates to meet the very high ATP demand imposed by continuous beating. Over 95% of this ATP is generated by oxidative phosphorylation (OXPHOS) with the necessary mitochondrial network taking up approximately one-third of cardiomyocyte cell volume. Energy starvation and mitochondrial dysfunction are therefore significant factors in the progression of cardiotoxicity and so detection of such metabolic dysfunction is an important aspect of cardiotoxicity screening. This detection is best achieved by monitoring the two main ATP generating processes, OXPHOS and glycolysis.

In vivo, the most important respiratory substrates for ATP production are pyruvate and fatty acyl CoA, however, cardiomyocyte metabolism is particularly adaptable and substrates such as amino acids, lactate, and ketone bodies can also be used. Examples of this adaptability include hypoxia inducible factor (HIF) mediated metabolic responses to hypoxia and ischemia and the shift from fatty acid oxidation (FAO) to glucose metabolism that occurs in hypertrophic cardiac tissue. These adaptions highlight the importance of information on substrate preference and oxygenation when designing and interpreting *in vitro* cardiomyocyte analyses.

As cardiac contraction is the main ATP consumer, the coupling of contractility to ATP production, and by extension, mitochondrial activity, is critically important to normal cardiomyocyte function, particularly as the mitochondrial reticulum also regulates intracellular calcium homeostasis and a multitude of critical signally pathways. The ability to relate cardiomyocyte beating to alter metabolic activity would therefore be of significant utility.

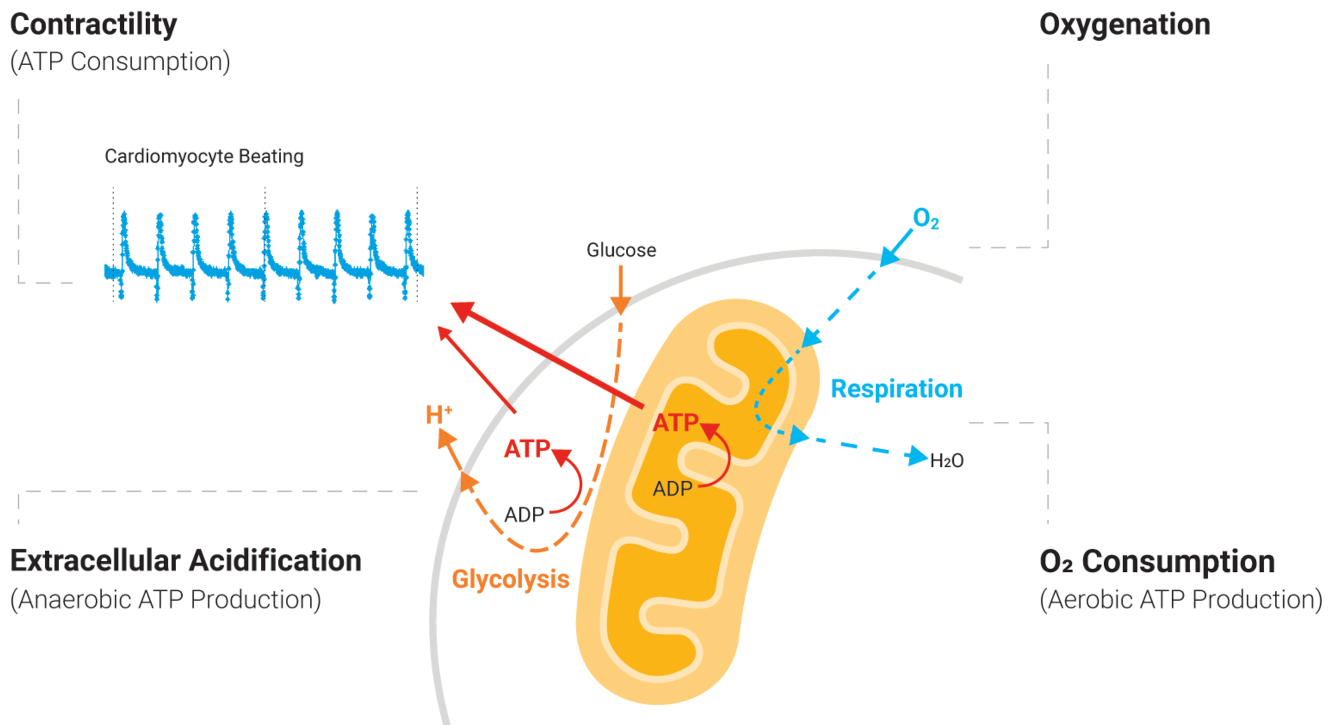


Figure 1. A simplified schematic of the inter-relationship between cardiomyocyte metabolism and beating activity. OXPHOS produces most of the ATP needed, with pyruvate and Acyl CoA being the main respiratory substrates. By measuring beating, OXPHOS (via O_2 consumption), glycolytic flux (via extracellular acidification), and cellular oxygenation a more complete picture of cardiomyocyte function can be established.

Mitochondrial dysfunction and contractility

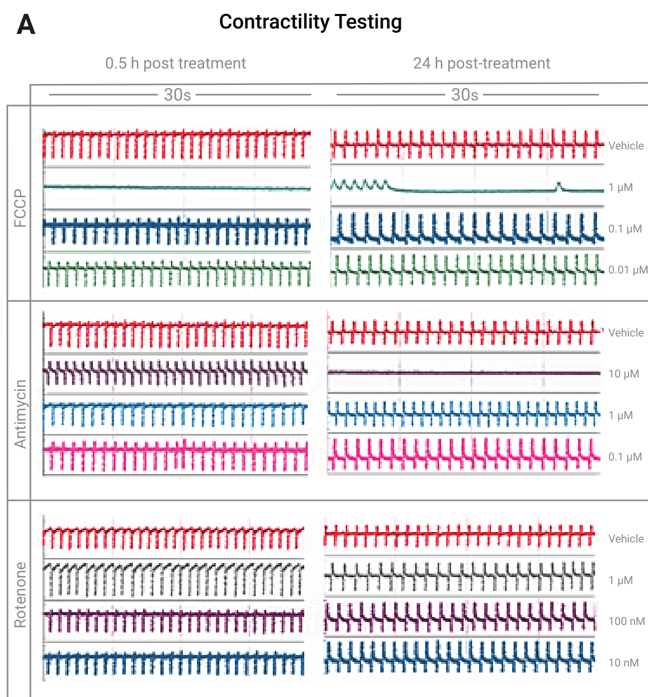
Contractility is measured by culturing iPS cardiomyocytes on E-Plate Cardio 96 and measuring them on the Agilent xCELLigence RTCA Cardio system in real time. The E-Plate has interdigitated impedance (IMP) microelectrode arrays on the bottom of each well. IMP electrodes measure cellular impedance, which is affected by the number of cells covering the electrode, the morphology of the cells, and the degree of the cell attachment. The fast sampling rate of IMP measurement (12.9 ms/77 Hz) allows capturing temporal rhythmic changes in cell morphology and degree of cell attachment to the plate associated with contraction of cardiomyocytes. Therefore, the Cardio system is used to predict drug-induced proarrhythmia, contractile liability, and chronic toxicity of drugs under development.

Cell metabolism is measured using the Agilent MitoXpress Xtra oxygen consumption assay to assess mitochondrial function and the Agilent pH-Xtra glycolysis assay, which uses extracellular acidification (ECA) to assess glycolytic function. Soluble metabolic sensor reagents show a change in fluorescence signal in response to changes in oxygen or acidification as a result of energy production. Both reagents can be measured using dual-read TR-F (time resolved fluorescence) detection.[3] This allows measurement on E-Plates such that, if necessary, metabolism and contractility can be measured sequentially on the same test plate. Furthermore, cellular oxygenation measurements with the Agilent MitoXpress Intra intracellular oxygen assay can be conducted between xCELLigence RTCA time points (in parallel but on plate reader platform) if desired.

Results and discussion

iPS cardiomyocytes maintain beat rates in the presence of mitochondrial inhibitors.

To assess the effects of metabolism on beat rate, cardiomyocytes were treated with mitochondrial modulators on an E-Plate. Beat rates were assessed 0.5 and 24 hours post-treatment (Figure 2A). Interestingly 1 µM FCCP influenced the beat rate at both time points suggesting that cardiomyocytes cannot recover following mitochondrial uncoupling (Figure 2A). Lower concentrations did not reduce the beat rate.



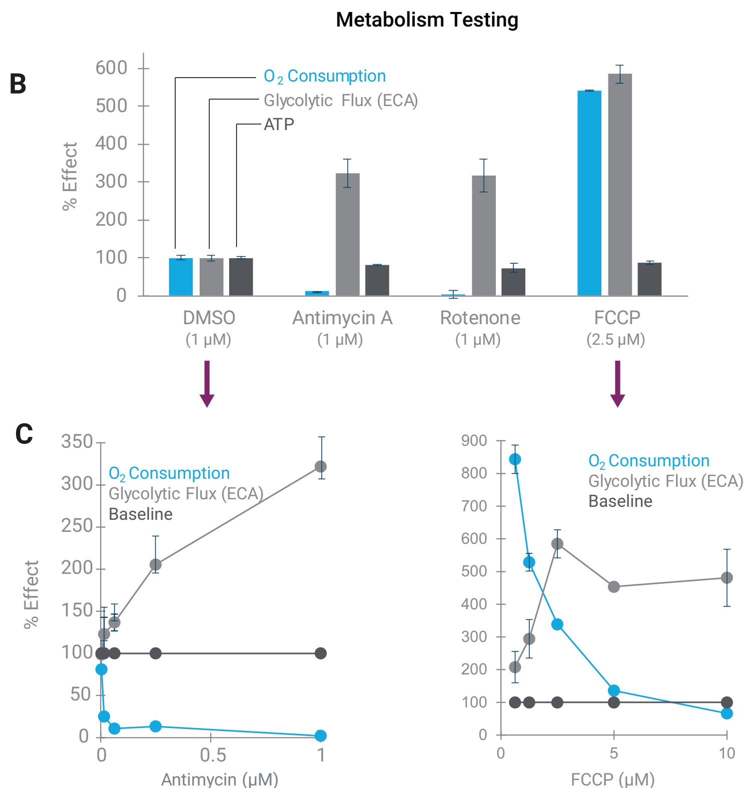


Figure 2. The impact of mitochondrial impairment on cardiomyocyte beating. Beating is maintained in the presence of mitochondrial inhibitors through increased glycolytic ATP supply. 30 s xCELLigence traces at 0.5 and 24 hours post-treatment (A). O_2 consumption, extracellular acidification, and ATP were measured at fixed concentrations (B). O_2 consumption, extracellular acidification dose responses for antimycin (C) and FCCP (D). Data presented relative to untreated control.

Inhibitory concentrations of antimycin A and rotenone (1 µM) did not have a significant impact on beat rates at both time points (Figure 2A). This suggests that cardiomyocytes can still generate ATP. High concentrations of antimycin A did reduce beat rates after 24 hours.

Measuring oxygen consumption rates using MitoXpress Xtra confirmed that antimycin A and rotenone decrease mitochondrial respiration as oxygen consumption decreases acutely upon treatment (Figure 2B). FCCP was shown to increase oxygen consumption but as mitochondria are uncoupled, they are unable to generate ATP (Figure 2B).

Analysis of the extracellular acidification using pH-Xtra glycolysis assay shows that when mitochondria are inhibited or uncoupled, glycolysis is increased (Figure 2B). There is a clear concentration-dependent increase in acidification (Figure 2C) suggesting that ATP depletion is ameliorated through increased glycolysis in cardiomyocytes.

Together, this suggests that increased glycolysis supplies the cells with enough ATP to facilitate cardiomyocyte beating despite the lack of mitochondrial ATP from oxidative phosphorylation. This is consistent with previous observations on specific cell lines.[4]

Cell metabolism is tightly coupled to contractile activity

The β-adrenoreceptor agonist, isoproterenol is used for the treatment of bradycardia (slow heart rate). Figure 3A shows beat rate traces of cardiomyocytes using the xCELLigence RTCA, treatment with isoproterenol increased the beat rate by ~45% compared to control 30 minutes post drug addition (Figure 3A). Isoproterenol also caused a similar increase in oxygen consumption (Figure 3B).

These data suggest that when the beat rate is elevated, the increased ATP demand is met by increasing aerobic ATP production through mitochondrial respiration (Figure 3B). An antimycin A control was included to measure non-mitochondrial oxygen consumption. Acidification rates did not increase (data not shown) suggesting that OXPHOS rather than glycolysis is supplying the additional ATP required.

Changes in cellular oxygenation were measured using MitoXpress Intra. Figure 4 demonstrates that untreated cardiomyocytes under these conditions experience ~14% oxygen, ~7% less than ambient oxygen due to respiration and other non-mitochondrial background oxygen-consuming processes. When cells are treated with antimycin A, experienced oxygen increases to around ambient levels (~21%) as aerobic ATP production has been inhibited.

Conversely, cells treated with isoproterenol were shown to have an increased beat rate, and therefore oxygen consumption experience as low as 6% oxygen as a result of the increased oxygen consumption. This causes a significant but temporary reduction in oxygen availability with values of ~6% observed for >15 minutes despite cells being cultured and measured at 21% O_2.

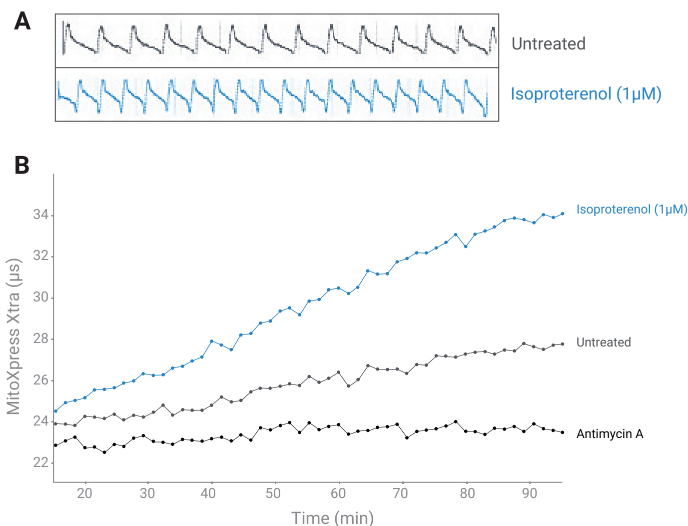


Figure 3. Impact of isoproterenol on cardiomyocyte beat rate measured on an Agilent xCELLigence RTCA Cardio system (A) and cardiomyocyte metabolism (B) measured on an advanced TR-F detection compatible fluorescence plate reader. Increased oxygen consumption caused more rapid oxygen depletion.

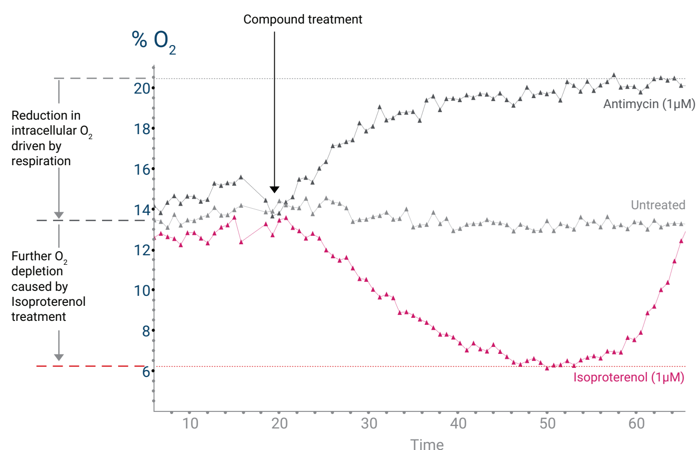


Figure 4. Impact of isoproterenol on cardiomyocyte oxygenation measured using advanced TR-F detection fluorescence plate reader with atmospheric control.

Contractility can be perturbed using several compounds such as nifedipine or E-4031. Nifedipine is used to treat and manage angina, high blood pressure, and several other conditions, it acts as an L-type Ca^{2+} channel antagonist. Figure 5A demonstrates the dose-dependent effects of nifedipine on contractile force, while Figure 5B illustrates a dose-dependent decrease in cardiomyocyte O_2 consumption. Extracellular acidification was also reduced (data not shown). The hERG channel inhibitor E-4031 causes an irregular beat rate pattern (Figure 6A), which also causes a decrease in oxygen consumption and a minor decrease in acidification rates (Figure 6B). Suggesting that with a decrease in ATP demand the cell responds by decreasing both ATP generating pathways.

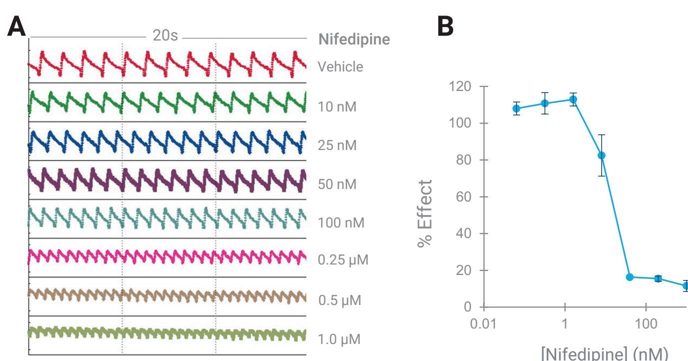


Figure 5. The impact of nifedipine on the beat rate (A) and metabolism (B). Beating was measured 30 minutes post-treatment. A range of concentrations from 10 nM to 1 µM were assayed. Metabolism data presented as oxygen consumption rate as a percentage of untreated control.

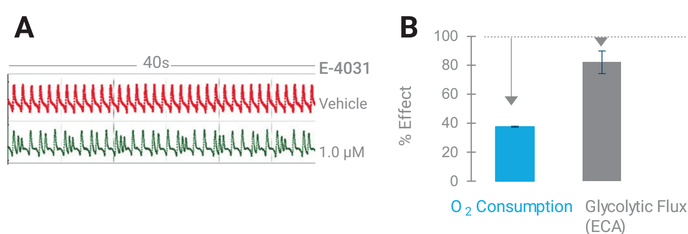


Figure 6. The impact of E-4031 on the beat rate (A) and metabolism (B). Beating was measured 30 minutes post-treatment. A single concentration 1 µM of E-4031 was used. Metabolism data presented as oxygen consumption rate and ECA as a percentage of untreated control.

Materials and methods

Cell culture

Induced pluripotent stem cells cardiomyocytes were supplied by NCARDIA. Cells were plated onto fibronectin-coated E-Plate Cardio 96 and placed in culture for 2 to 3 days, performing media changes as per the manufacturer's instructions. Cells were plated at 4 to 5 $\times 10^4$ cells/well for pH-Xtra and MitoXpress-Xtra assays.

Oxygen consumption assay

Fresh media containing the MitoXpress Xtra reagent, 150 µL/well was added before measurement. Compounds were added directly, then all wells were sealed with prewarmed HS oil. Plates were measured kinetically for 2.5 to 3.0 hours at 37 °C (Ex 380 nm, Em 650 nm, and Advanced dual-read TR-F plate reader detection)

Glycolysis assay

The sample plate is placed in CO_2 free incubator 3 hours before measurement, to remove CO_2. Samples were washed three times using respiration buffer (1 mM phosphate) prepared using the buffer tablet provided. 150 µL of respiration buffer containing the pH-Xtra reagent was added to sample wells. Compounds were added directly, and the plate was measured kinetically for 2.5 hours at 37 °C (Ex 380 nm, Em 615 nm, and Advanced dual-read TR-F plate reader detection).

Cellular oxygenation assay

Cells were loaded with MitoXpress-Intra reagent overnight (14 hours) in a E-Plate Cardio 96 the day before measurement. Cells were washed twice and 150 µL of fresh media was added. The plate was measured kinetically at 37 °C. (Ex 380 nm, Em 650 nm, and Advanced dual-read TR-F plate reader detection).

Contractile assay

iPS-cardiomyocytes were plated on 96 well E-Plates and impedance measurements were recorded at selected time points (60 seconds sweep at a sampling rate of 77 Hz). Drug treatment was initiated once the culture showed 40 to 60 synchronic beats/min. The data were normalized to baseline.

Conclusion

The combination of Agilent MitoXpress Xtra, MitoXpress Intra, and pH-Xtra metabolic assays with the xCELLigence RTCA Cardio system and E-Plate Cardio 96 enabled the sequential measurement of metabolism and contractility from the same sample using the same plate. Using the dual-read TR-F measurement approach on conventional TR-F plate readers informs on oxygen consumption and ECA. The combined use of microplate-based contractility and metabolism measurements has been demonstrated to generate a more complete picture of cardiomyocyte response to drug treatment and allows the delineation of inter-relationships between cardiomyocyte beating and the underlying bioenergetic processes. This multiparametric workflow helps to improve data density per well of sample.

Complete impairment of OXPHOS through treatment with electron transport inhibitors did not immediately impair cardiomyocyte beating. Increased ECA suggests that ATP supply is maintained through increased glycolytic flux allowing beating to continue for >24 hours post-treatment.

The β-adrenoreceptor agonist isoproterenol increased beat rate and caused a significant increase in O_2 consumption but little change in ECA. This suggests that increased ATP demand is being met through OXPHOS rather than glycolysis. The L-type Ca^{2+} channel antagonist nifedipine reduced contractile force and caused a dose-dependent reduction in both oxygen consumption and ECA, indicative of reduced OXPHOS and glycolytic activity in response to treatment. This combined analysis of critical cardiomyocyte functions therefore delivers a more holistic and informative *in vitro* cardiotoxicity screen in that it related cellular function to the metabolic activity driving that function. In so doing, it provides additional mechanistic information as to the cause of observed alterations in cardiomyocyte metabolism or contractility.

These highly informative workflows allow users to interrogate metabolic modulators of cardiomyocyte function. As better *in vitro* cardiac models are developed, knowing the metabolic phenotype is essential to ensure that assays appropriately reflect mature cardiomyocyte biology. Reliance on glycolysis or OXPHOS shapes how these cells will respond to drugs and how they will survive in environments that they can be exposed to such as nutrient deprivation or hypoxia. These workflows allow for assessment of contractility followed by metabolic interrogation with the same biomaterial without having to re-plate or potentially differentiate additional cardiomyocytes for parallel measurements. This saves on cell consumption while improving data density and delivering multiparameter outputs from single samples. The flexibility of these workflows makes them well-positioned to characterize both metabolism and cardiomyocyte function under a range of conditions including drug screening, nutrient deprivation, hypoxia, and ischemia/reperfusion. Integrating these Agilent Cell Analysis technologies offers a complete solution for assessing cardio-metabolism.

References

1. Lawrence, C. L. *et al. In Vitro* Models of Proarrhythmia. *Br. J. Pharmacol.* **2008** Aug, *154(7)*, 1516–2
2. Dykens J. A.; Will Y. The Significance of Mitochondrial Toxicity Testing in Drug Development. *Drug Discov. Today* **2007** Sep, *12(17–18)*, 777–85.
3. Hynes J. *et al.* A High-Throughput Dual Parameter Assay for Assessing Drug-Induced Mitochondrial Dysfunction Provides Additional Predictivity Over Two Established Mitochondrial Toxicity Assays. *Toxicol. In Vitro* **2013** Mar, *27(2)*, 560–9.
4. Marroquin L. D. *et al.* Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of Hepg2 Cells to Mitochondrial Toxicants. *Toxicol. Sci.* **2007** Jun, *97(2)*, 539–47.

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

DE44202.2461458333

This information is subject to change without notice.

© Agilent Technologies, Inc. 2021

Printed in the USA, February 1, 2021
5994-2987EN

Agilent
Trusted Answers