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ab129732 – Resazurin Cell Viability Assay

View Resazurin Cell Viability Assay datasheet:

www.abcam.com/ab129732

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For the measurement of cellular/mitochondrial viability in high throughput

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Table of Contents

1. Introduction 1
2. Materials Supplied and Storage 3
3. Materials Required, Not Supplied 3
4. General guidelines, precautions, and troubleshooting 4
5. Reagent Preparation 4
6. Sample Preparation 5
7. Assay Procedure 6
8. Data Analysis 6
9. FAQs / Troubleshooting 7
10. Typical Data 10

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1. Introduction

Principle:

ab129732, the Resazurin Cell Viability Kit contains a fluorometric/colorimetric dye used to determine cellular metabolic activity and mitochondrial viability of live cells in a high throughput format. It uses Resazurin as an indicator dye (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) to measure oxidation-reduction reactions which principally occur in live cells. When reduced by metabolically active cells, the blue nonfluorescent resazurin reagent is reduced to highly fluorescent pink resorufin with absorbance at 570nm and red-fluorescent properties (560±10Ex/590Em) at neutral pH. The dye can be measured in absorbance or fluorescence mode. However, fluorescence mode measurement offers greater assay linearity, reproducibility, robustness and sensitivity.

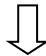
Background:

Resazurin cell viability assay is widely used in drug discovery for the study of cytokines, growth factors, and cytotoxic agents. The Resazurin cell viability assay provides high throughput screening tool, which can be used to assess mammalian cell toxicity, viability, migration, and invasion in both early drug discovery compound screening and subsequent drug safety and toxicity studies. It is reliable, sensitive, simple, and cost-efficient. In addition, it keeps cells intact, which allows other parallel analyses, such as mRNA.

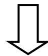
Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. Oxido-reductase reactions happen principally in life cells. The reduction of resazurin therefore, correlates with the number of live cells. The transference of electrons from NADPH to resazurin, will reduce the blue Resazurin to a pink fluorescent counterpart, resorufin. The level of reduction can be quantified by spectrophotometers using appropriate filters, since resazurin exhibits an absorption peak at 600ηm and resorufin at 570ηm wavelengths. The level of reduction can also be measured in fluorescence units (RFU) using a fluorometer (Ex=530-570 nm, Em=590-620 nm)

This assay can also be used to discriminate between compounds which affect overall cellular metabolism and viability from those that specifically affect mitochondria since oxido-reductase reactions are carried out mostly by mitochondrial enzymes in the cell. This can be achieved by growing cells in media supplemented with two different carbon sources. When cells are cultured in vitro using glucose rich media, known to be toxic to mitochondria and in particular, the electron transport chain (ETC) will affect minimally cellular viability even at very high concentrations. This is because the cells will continue to generate ATP through glycolysis, preventing cell death from occurring. However, when cells are cultured in galactose and glutamine, the cellular metabolism remains dependent upon mitochondrial function and therefore toxic compounds to the ETC will rapidly deplete ATP leading to cell death.

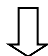
Seed cells in a microwell culture plate.



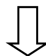
Carry out experimental conditions under which cellular/mitochondrial viability is to be assessed



Once the time course of experimental conditions has been completed, add stain to the culture media



Incubate for 4 hours at 37˚C.



Read by fluorescence of absorbance

2. Materials Supplied and Storage

Store kit at 4°C in the dark immediately on receipt and check below for storage for individual components. Kit can be stored for 6 months from receipt, if components have not been diluted.

Aliquot components in working volumes before storing at the recommended temperature.

Reagent is provided for more than 2000 assays on 96-well plates.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
20X Cell Viability Stain	24 mL	4°C	4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- A standard spectrophotometer capable of 570 nm endpoint reading or a fluorescent reader capable of 550 nm excitation and 590 nm emission
- Dark wall 96 or 384-well plate(s).
- Cell culture reagents.
- Multi and single channel pipettes.

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Reagent Preparation

5.1 Equilibrate stain to room temperature.
Note: We recommend thawing for 10 mins in a 37°C water bath to accelerate the melting process

5.2 Optional: Prepare a 2X cell viability stain solution by diluting the 20x stock 10 fold (i.e. for 1 96-well plate dilute 1mL of 20X cell viability stain in 9mL of growth media). Mix well. Store in the dark at room temperature. Reagent is provided for at least 2000 tests, each carried out in a total volume of 200 µL per well.
Note: Adding 100 µL per well of a 2X solution will increase reproducibility of the assay over addition of 5 µL of a 20X stock solution.

6. Sample Preparation

General sample information:

We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

We recommend that you use fresh samples for the most reproducible assay.

Note: The protocol below is described for a 96-well plate. If performing the assay on a 384-well plate, adjust volumes accordingly. This assay has been optimized for use on adherent and suspension cells.

6.1 Seed cells directly into a dark walled 96-well plate. If seeding adherent cells allow attachment for several hours to overnight. It is advisable to seed in a 100 µL volume of the same media used to maintain the cells in bulk culture. The optimal cell seeding density is dependent on cell type and duration of experimental time course. Experimental examples and cell seeding are shown in the data analysis section below. Details about galactose/glutamine cultured conditions are shown in FAQs section.

6.2 Ensure that there will be at least one well per experimental condition to which cells but no dye and also one well per plate with dye, to assess background signal.

7. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

7.1 At the end of the treatment or experimental condition time course, overlay 100 µL of 2X cell viability stain on each well containing 100 µL of media and incubate under sterile conditions at 37˚C for 4 hours.
Note = 4 hour incubation is long enough to give a robust and reproducible signal, yet is short enough to prevent added toxic cellular effects due to the dye itself.

7.2 Read the plate by absorbance at 570nm or by fluorescence and set the instrument with 550nm excitation and 590nm emission.

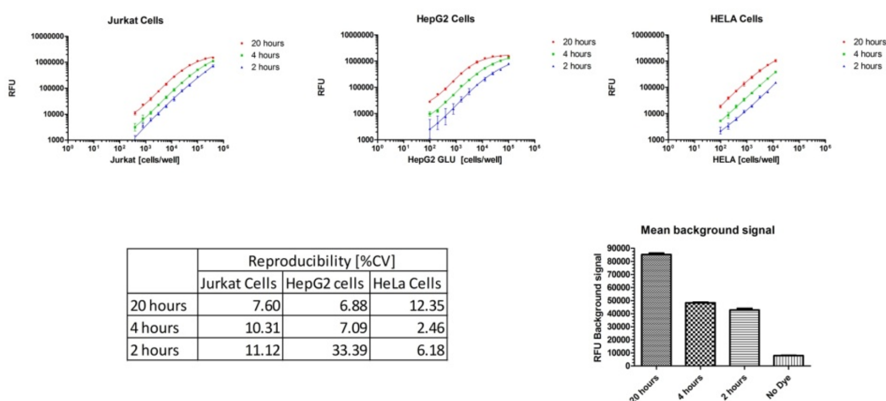
8. Data Analysis

Subtract media/experimental condition background (test wells without dye) from the actual test measurements prior to subtracting background (empty wells with dye) from all the measurements.

9. Typical Data

Data provided **for demonstration purposes only**.

Effect of cell viability stain incubation time on fluorescent signal formation – The cell viability stain has been typically used after incubation from 2 to 48 hours. Optimal reproducibility with the lowest background is obtained after 4 hours of incubation in most cell lines. Caution must be exercised when changing the incubation conditions specified in this protocol, since longer incubation times have shown to induce oxidative stress by the dye itself in certain cell lines.



	Reproducibility [%CV]		
	Jurkat Cells	HepG2 cells	HeLa Cells
20 hours	7.60	6.88	12.35
4 hours	10.31	7.09	2.46
2 hours	11.12	33.39	6.18

Figure 1. Effect of stain incubation time on assay reproducibility and background. Amine coated dark wall plates were seeded in a titration series of each cell line in a final volume of 100 µL of media. 2X cell viability stain (diluted in the cell line specific growth media) was added to each well at specified time points. Cartesian (XY) graph data is shown after media and background subtraction. Bar graph shows mean background signal at different time points. 4 hour incubation of stain results in optimal reproducibility with minimal increase of background over the 2 hour incubation. Reproducibility was calculated for Jurkat cells, HepG2 cells and HeLa cells. %CV after 4 hours of incubation was 10%, 7% and 2.4% respectively, whereas %CV after 2 hours of incubation was 11%, 33% and 6.1% respectively.

Suitability of the cell viability assay for high-throughput screening – Fluorescent and colorimetric readout Z factors were assessed in Jurkat and HepG2 cells, respectively. Average Z factors for both readouts was greater than 0.7.

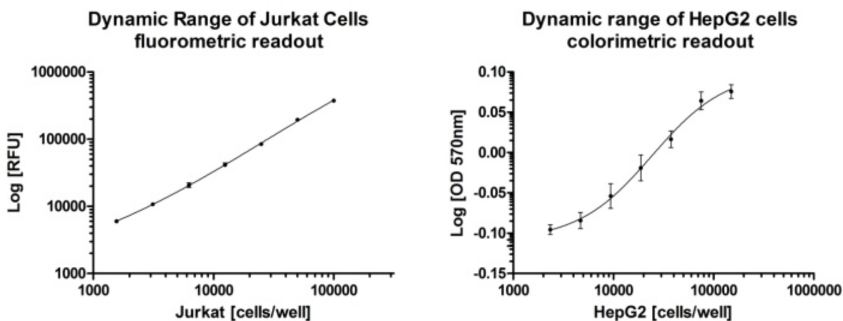


Figure 2. Dynamic range and suitability of the cell viability assay for high throughput screening. Cells were seeded in a titration series with n=11 for each data point. Fluorometric readout gave an average Z factor of 0.82 on Jurkat cells seeded from 1,500 – 100,000 cells per well on a 96-well plate. Colorimetric readout gave an average Z factor of 0.72 on HepG2 cells seeded from 40,000 – 150,000 cells per well.

Cell viability stain as an indicator of cellular viability – This assay was validated as an indicator of cellular viability after dose-response treatment of Jurkat cells with idarubicin and staurosporin. Idaribicin is an antileukemic drug which inserts itself into DNA and interferes with topoisomerase II, inducing rapid cell death. Staurosporin is a non-specific kinase inhibitor that is known to induce apoptosis in many cell lines.

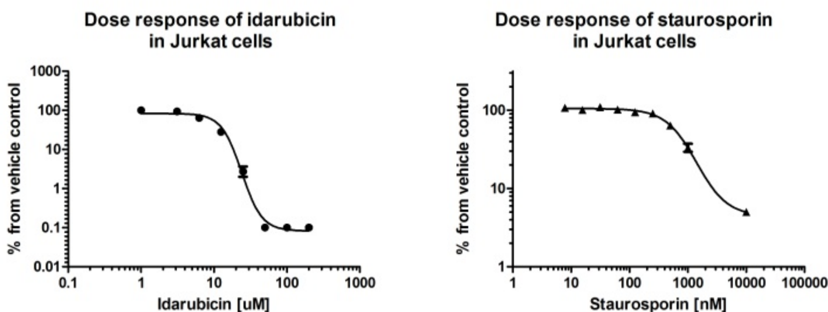


Figure 3. Jurkat cells were seeded at 25,000 cells per well in a 50 µL volume and were immediately overlay with a 2X concentration of either Idarubicin or Staurosporin for a final volume of 100 µL. Cells were incubated for 2 hours with Idarubicin and for 4 hours with Staurosporin prior to the addition of 2X stain diluted in RPMI media. After 4 hours of further incubation with stain, fluorescence was measured. IC50 for Idarubicin was found at 22 – 25 µM and IC50 for staurosporin was found at 500nM.

Mitochondrial viability stain as an indicator of the mitochondrial metabolic state – This assay was further validated in the context of mitochondrial toxicity, comparing fluorescent readout after 72 hour rotenone treatment in HepG2 cells cultured in glucose versus galactose/glutamine.

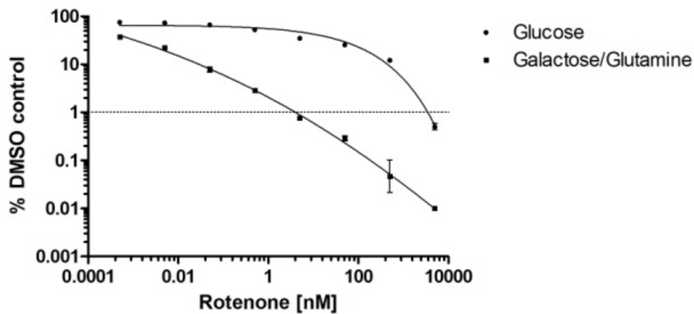


Figure 4. Mitochondrial viability stain in long term toxicity. HepG2 cells previously cultured in glucose or galactose/glutamine supplemented media were seeded at 10,000 cells per well and allowed to adhere overnight. Media was replaced for the specific culture media in the presence of a titration series of Rotenone (5 µM – 0.5 pM). Cells were treated for 72 hours. Cells were incubated with 2X stain for 4 hours before fluorescent readout analysis.

10. FAQs/Troubleshooting

10.1 I want to use a cell line other than the cell lines shown in section 10 of this protocol, how do I know how many cells to seed per well?

The cell seeding density is dependent on the cell type, the time course in which experimental conditions will be evaluated and instrument capabilities. As a rule of thumb, highly metabolic cells (i.e. HepG2) require lower seeding than low metabolic cells (i.e. Jurkat). Furthermore, fluorometric reading allows for greater flexibility in seeding due to a wider dynamic range and increased sensitivity over colorimetric reading (which can easily saturate at the high end of the assay). To optimize for all these variables, we suggest seeding a two-fold dilution series of cells (12 points) starting from 100 – 200k/well and down to zero. Incubate the cells for the same duration as the proposed treatment then stain as described. Once this data is analyzed, the experimental plate should be seeded near the top of the linear range.

10.2 Can I incubate stain for a different amount of time?

Yes, staining is typically observed between 2 and 48 hours of incubation. However, we have found that 2 hours of incubation leads to low reproducibility. Any metabolic cell staining procedure will affect the cells therefore we do not recommend treatments of longer than 6 hours. Long treatments alone may trigger cellular reactive oxygen species leading to mitochondrial dysfunction, reduced proliferation and autophagy.

10.3 How do I treat cells with Galactose media?

Culture the cell line of interest in the following media: DMEM without glucose containing 5mM D-Galactose, 6mM L-glutamine, 1mM sodium pyruvate, 44mM sodium bicarbonate, 1X RPMI non-essential amino acids, 10% Dialyzed FCS and 1X Penicillin/streptomycin. It is advisable to generate a stock solution of 1M D-Galactose in media without glucose prior to generating the complete galactose media. The 1M D-Galactose stock solution may require warming at 37˚C for complete solubility. The galactose stock can be stored long term at -20°C. Cells cultured in galactose may decrease in doubling time compared to glucose counterparts. Ensure that the galactose/glutamine cultured cells are split at a maximum of 1:2, to maintain a healthy culture.

10.4 My cells do not adhere well to the standard tissue culture treated 96-well plate?

To improve on adherence of cells, it may be necessary to seed cells on plates with specialized coating surfaces, such as collagen, Poly-L-Lysine, amine or carboxyl surfaces, however this assay can also be performed on suspension cells

10.5 I need to determine mitochondria health, should I use the ATP assay or should I use the mitochondrial viability stain assay?

The mitochondrial viability stain when used with the galactose/glutamine media is particularly useful for determining mitochondrial health after a prolonged/chronic treatment (i.e >48 hours). If short incubation times/acute effects are to be measured (i.e. less than 6 hours) we recommend measuring ATP assay (ab113849).

Problem	Cause	Solution
High background	Product has been exposed to light for extended periods of time	Do not expose the dye to direct light
Saturation of signal by fluorescence reading	Plate reader lamp intensity is too high	Decrease the lamp intensity or decrease seeding of cells and ensure to work within the dynamic range of the assay
High CV	Staining time too brief	Increase stain incubation to 4 hours
Low Signal	Staining time too brief	Increase stain incubation to 4 hours
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation

Technical Support

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