ROS-Glo™ H_2O_2 Assay - Novel Luminescence-Based Assay for ROS Measurement

Gediminas Vidugiris[1], Sarah Duellman[1], John Shultz[1], Jolanta Vidugiriene[1], Hui Wang[2], Jean Osterman[2], Wenhui Zhou[2], Poncho Meisenheimer[2] and James J. Cali[1]

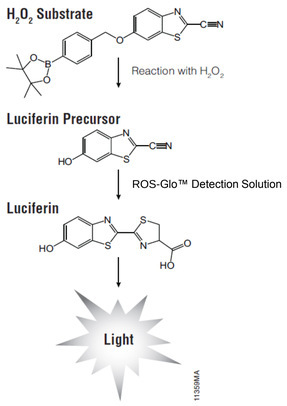
[1]Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711; [2]Promega Biosciences LLC, 277 Granada Dr, San Luis Obispo, CA 93401

Abstract #257

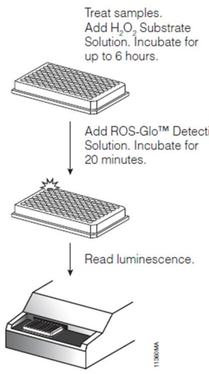
1. Introduction

H_2O_2 is a reactive oxygen species [ROS] that is measured in cells as a marker of oxidative stress. It is also measured as a marker of enzyme activities that either consume or produce H_2O_2. It is desirable to screen chemical compounds for their capacity to alter H_2O_2 levels in cultured cells or for their effects on H_2O_2 levels in enzyme reactions. Current fluorescent assay formats are prone to false hit rates that are too high for efficient screening applications. We developed a novel luminescent H_2O_2 assay (ROS-Glo™) that detects H_2O_2 directly, minimizes false hit rate and provides simple formats for cell-based and enzymatic assays.

Since various ROS are interconverted to H_2O_2 in the cell and H_2O_2 is the longest lived ROS, an increase in H_2O_2 can reflect a general increase in the ROS level. Our method for measuring hydrogen peroxide utilizes the H_2O_2 Substrate, which directly reacts with H_2O_2 to produce a luciferin precursor. Addition of the ROS-Glo™ detection solution converts the precursor to luciferin and provides luciferase and other components to produce a light signal proportional to the level of H_2O_2.



2. Simple add-mix-read assay in cell culture wells



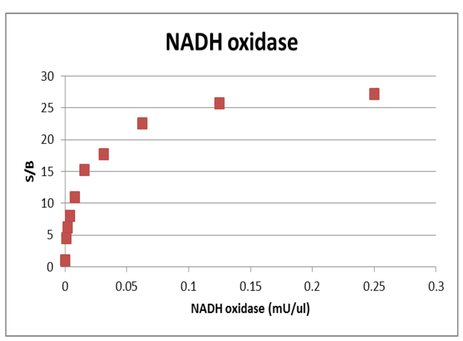
The ROS-Glo™ H_2O_2 Substrate is added to the wells of cultured cells with test compounds.

The cells are then incubated under normal mammalian cell culture conditions.

After incubation, ROS-Glo™ Detection Solution is added and the plate is incubated for 20 min prior to reading luminescence.

3. Measuring activity of H_2O_2-generating enzymes

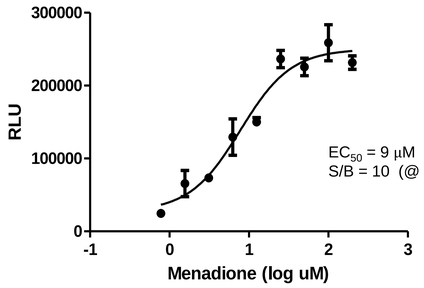
$$NADH + O_2 \xrightarrow{\text{NADH Oxidase}} NAD^+ + H_2O_2$$



The ROS-Glo™ H_2O_2 Assay can also be used to measure the activity of enzymes that generate or eliminate H_2O_2. The signal-to-background ratios from various concentrations of NADH Oxidase is shown in the graph. Once the appropriate level of NADH Oxidase is determined, the assay can then be used to identify inhibitors of the enzyme in a chemical library.

The ROS-Glo™ H_2O_2 Assay limit of detection for H_2O_2 is about 0.1 µM.

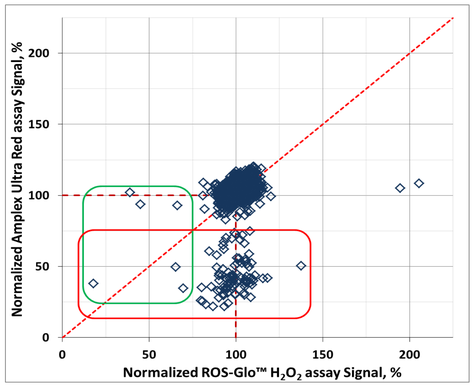
4. ROS induction in cultured cells



EC_{50} = 9 µM
S/B = 10 (@ 200 µM menadione / vehicle control)

Menadione is a compound that interrupts the electron transport chain in the mitochondrion, producing large amounts of ROS in cultured cells. In the graph above, K562 cells were treated with menadione and the ROS-Glo™ H_2O_2 Assay was used to determine ROS production. Menadione resulted in a concentration-dependent ROS increase.

5. Small molecule screening – "Hits" correlation results for ROS-Glo™ H_2O_2 Assay vs HRP-based assay



The graph on the left compares two small molecule screens using the Library of 1280 Pharmacologically Active Compounds (LOPAC1280; Sigma-Aldrich):
ROS-Glo™ H_2O_2 Assay vs. Amplex Red (HRP-based) assay.

Compounds were screened at 10 µM.

The assay was set up with 10 µM H_2O_2 (negative control: no H_2O_2).

Red box = HRP inhibitors

Green box = ROS-Glo™ inhibitors

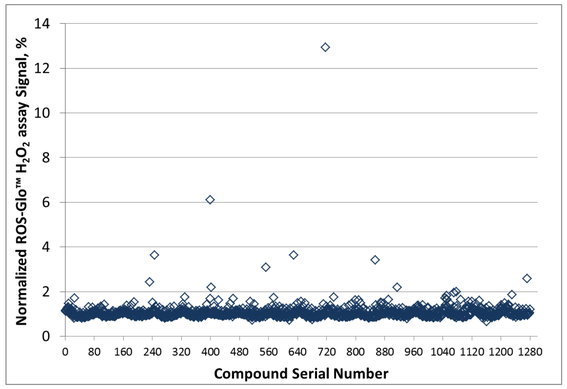
6. Significantly lower false hit rate with ROS-Glo™ compared to HRP-based method

Compound Summary	ROS-Glo™ Assay		HRP Method	
	# Compounds	% LOPAC library	# Compounds	% LOPAC library
Total compounds screened	1280	100	1280	100
Inhibitors ≤75% activity	6	0.5	91	7.1
Inhibitors ≤50% activity	3	0.2	67	5.2
Activators ≥150% activity	2	0.2	0	0

The Amplex Red assay (HRP method) uses a fluorogenic substrate to detect H_2O_2 in a reaction with horseradish peroxidase (HRP). HRP reacts with numerous compounds, which appears to result in a reduction in H_2O_2 but instead these are false hits.

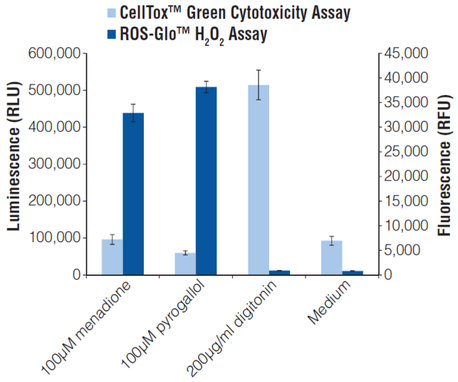
The ROS-Glo™ H_2O_2 Assay uses a luminogenic probe that reacts directly with H_2O_2 and thereby obviates the interferences associated with HRP. The signal inducers found in the ROS-Glo screen are natural ROS generators that are missed due to HRP inhibition with Amplex Red.

7. Screening of LOPAC library using the ROS-Glo™ H_2O_2 Assay – Hep G2 cells



The ROS-Glo™ H_2O_2 Assay was used to screen the LOPAC library using Hep G2 cells in complete media.
The compounds that increase the ROS-Glo™ signal are compounds in the LOPAC library that generate ROS in cultured mammalian cells (true hits).

8. Multiplex ROS-Glo™ H_2O_2 Assay with CellTox™ Green



This multiplex allows determination of ROS production and cytotoxicity. It also allows total cell number analysis for normalization.

ROS-Glo™ can be multiplexed with CellTox Green by co-applying the H_2O_2 Substrate and CellTox Green dye to the cells during treatment. The fluorescent CellTox Green signal is read first, then addition of the ROS-Glo™ Detection Solution allows detection of the luminescence signal which correlates with ROS levels. The ROS-Glo™ Detection Solution also lyses the cells so another fluorescent read of the CellTox Green signal will determine the total cell number and allow normalization.

9. Summary

- The ROS-Glo™ H_2O_2 Assay measures changes in the level of ROS in cultured mammalian cells. The method does not rely on a reaction catalyzed by HRP, has a low false hit rate and can easily be performed in multi-well plates.
- ROS-Glo can be multiplexed with a variety of assays, allowing more data to be obtained per well.
- The assay can also be used to measure the activity of enzymes that generate or eliminate H_2O_2. This allows chemical libraries to be screened for compounds that affect the activity of such enzymes as NADH Oxidase.

gediminas.vidugiris@promega.com