

ROS detection in a cell-based format using the Promega ROS-Glo™ assay

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- The VANTASTAR® offers automatic crosstalk reduction for optimal detection of luminescent signals
- MARS data analysis software enables evaluation of EC₅₀ values
- Promega's homogeneous ROS-Glo™ assay detects Reactive Oxygen Species in cell-based or enzymatic reactions

Introduction

Reactive oxygen species (ROS) are chemically reactive derivatives of molecular oxygen that arise from aerobic metabolism¹. ROS play an important role in cell signaling and gene expression². However, if ROS accumulate at high levels inside the cell, they can also damage DNA, RNA, proteins and lipids (fig. 1). High levels of ROS can occur due to oxidative stress and pose an overall threat to cellular health³. ROS are also associated with aging, apoptosis or necrosis, and are implicated in diabetes, vascular diseases, pulmonary disorders, inflammatory diseases, and cancer⁴.

There are many intracellular sources of ROS, including mitochondria and NADPH oxidases⁵. ROS detection assays are used in drug screening campaigns to determine the effects of compounds from chemical libraries on enzymatic reactions (e.g. NADPH oxidase) and to study the effects of antioxidant therapies. ROS typically have a short half-life. H₂O₂ is an exception and has the longest half-life of all known ROS, which makes it a good marker of oxidative stress and well suited for ROS detection.

Menadione is a compound that interrupts the electron transport chain in mitochondria, producing large amounts of ROS in cultured cells (fig. 1). Hence, it is often used as a control reagent in ROS detection. N-Acetyl-L-cysteine (NAC) functions as an antioxidant and shows an inhibitory effect on ROS generation.

solution, the D-cysteine contained in the solution enables the conversion of the luciferin precursor to luciferin. Finally, this luciferin is converted by the Ultra-Glo™ luciferase and a luminescence signal is generated for ROS detection.

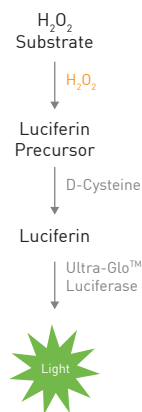


Fig. 2: Assay Principle of the ROS-Glo assay.

Materials & Methods

- 384-well plate, tissue culture (TC)-treated, transparent (#CLS4681, Corning)
- HeLa cell line (ACC 57, DSMZ)
- DMEM phenol red-free, high glucose, with 10% FBS, 2 mM glutamine, and 1% pen/strep (all Thermo Fisher)
- Incubator with 37°C, 5% CO₂ and humidity control
- ROS-Glo [#G8820, Promega]
- 384-well plate, white, med.-binding [#781075, Greiner Bio-One]
- VANTASTAR microplate reader (BMG LABTECH)

Experimental procedure

ROS induction with menadione

Cells were seeded in transparent 384-well plates with 2,000 cells per well in 80 µL medium (six replicates each). After cells were attached overnight, 48.4 µL medium were aspirated and 31.6 µL medium remained in the wells. Menadione (10, 50 or 100 µM) or vehicle and ROS-Glo substrate solution were added in a total volume of 8.4 µL to the remaining medium. Cells, ROS-inducer and substrate solution were incubated for 2 h at 37°C and 5% CO₂.

40 µL supernatant were transferred to a white 384-well plate for ROS detection with the ROS-Glo assay. The ROS-Glo detection solution was prepared according to the manufacturer's instructions and 40 µL were added to each well and incubated for 20 min at room temperature until luminescence was read for ROS detection.

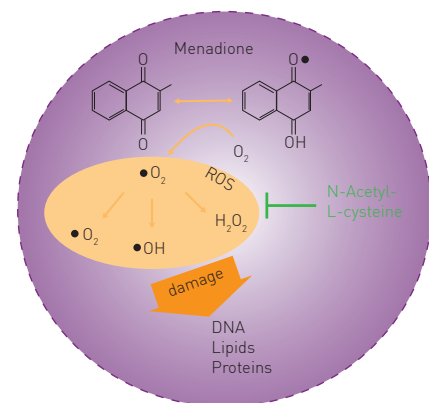


Fig. 1: Effects of menadione and N-acetyl-L-cysteine on cellular ROS generation.

Assay Principle

The Promega ROS-Glo™ assay can be used for ROS detection in biochemical and cell-based applications. Cells are therefore incubated under mammalian cell culture conditions with the test compounds and the ROS-Glo H₂O₂ substrate. The substrate reacts with available H₂O₂ and generates a luciferin precursor for ROS detection (fig. 2). After addition of the ROS detection

ROS inhibition with N-acetyl-L-cysteine

ROS inhibition studies were performed using, the same steps described for the ROS induction experiments. In addition to 50 μM menadione, different concentrations of NAC (12.5, 25, 50, 100, 200 or 400 μM) or vehicle were administered with the substrate solution. Luminescence was measured at the following settings for ROS detection:

Instrument settings

Optic settings	Luminescence, endpoint	
	No filter	
General settings	Interval time	1.0 s
	Settling time	0.1 s
	Focal height	11.5
	Dynamic range	EDR

The Enhanced Dynamic Range (EDR) function available on the VANTaStar ensures that each well is read with optimal gain to allow the maximum dynamic range and highest sensitivity. EDR also avoids signal saturation.

Furthermore, the VANTaStar automatically applies the ideal focal height and moves the aperture spoon into the light path. This reduces crosstalk light from non-target wells during luminescence-based ROS detection.

Results & Discussion

ROS induction with menadione

Menadione treatment of HeLa cells resulted in a concentration-dependent increase in ROS (fig. 3). Cell-free assays, which contained solely medium and the ROS inducer menadione also showed a concentration-dependant increase in ROS, which was however lower compared to ROS detection for experiments with cells. This difference arises since some compounds undergo reactions in cell culture medium to produce H_2O_2 , independent of cells [abiotic ROS production]. In addition, cultured cells have a strong capacity to eliminate H_2O_2 . Thus, ROS production in the preparations with cells was assumed to be higher than the final values obtained in ROS detection since part of the produced H_2O_2 was already degraded by the cells during the incubation period.

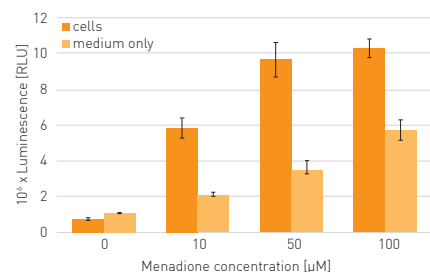


Fig. 3: Cellular and abiotic ROS generation after treatment with different concentrations of menadione for 2 h. ROS detection was performed with the ROS-Glo assay.

ROS inhibition with N-acetyl-L-cysteine

The ROS-inducing effect of the menadione treatment was

eliminated at increasing concentrations of NAC (fig. 4). The luminescence-based ROS detection signal, which reports ROS detection, was reduced to less than the control value (cells with medium only) with the addition of 400 μM NAC. This suggests further inhibition of cellular ROS production by NAC to levels less than those that cells would normally generate without a menadione treatment.

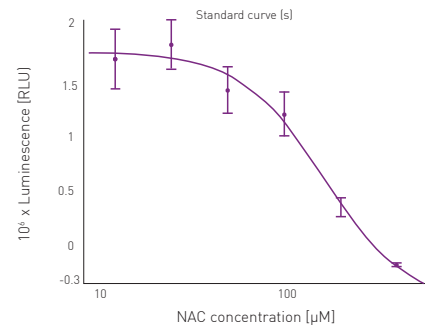


Fig. 4: Cellular ROS generation after menadione treatment and application of different concentrations of the antioxidant N-acetyl-cysteine for 2 h. ROS detection was performed with the ROS-Glo assay. Control values (cells with medium only) were subtracted from all intensities before plotting.

An EC_{50} of 161.5 μM was determined for NAC using BMG LABTECH's MARS data analysis software.

Conclusion

The VANTaStar delivers automatic crosstalk reduction by means of an aperture spoon and automatic adjustment to the optimal focal height for ease of use. In addition, the EDR applies the ideal gain to each well and ensures the maximum dynamic range and highest sensitivity while avoiding detector saturation.

ROS detection was successfully performed with the VANTaStar. The high sensitivity of the ROS-Glo assay allowed the ROS detection procedure to be miniaturized for use on 384-well microplates. The homogeneous format of the assay enables easy handling for ROS detection even in cell-based experiments.

References

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