Product Manual

OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Green Fluorescence)

Catalog Number

STA-347	96 assays
STA-347-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are well-established molecules responsible for the deleterious effects of oxidative stress. Accumulation of free radicals coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease states. The role of oxidative stress in vascular diseases, diabetes, renal ischemia, atherosclerosis, pulmonary pathological states, inflammatory diseases, cancer, as well as ageing has been well established. Free radicals and other reactive species are constantly generated *in vivo* and cause oxidative damage to biomolecules, a process held in check by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lipids. Measuring the effect of antioxidant therapies and ROS/RNS activity is crucial to suppressing or treating oxidative stress inducers.

The OxiSelect™ *In Vitro* ROS/RNS Assay Kit is an assay for measuring the total free radical presence of a sample. The assay employs a proprietary quenched fluorogenic probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS probe that is based on similar chemistry to the popular 2', 7'-dichlorodihydrofluorescein diacetate. The DCFH-DiOxyQ probe is first primed with a quench removal reagent, and subsequently stabilized in the highly reactive DCFH form. In this reactive state, ROS and RNS species can react with DCFH, which is rapidly oxidized to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) (Figure 1). Fluorescence intensity is proportional to the total ROS/RNS levels within the sample. The DCFH-DiOxyQ probe can react with hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), nitric oxide (NO), and peroxynitrite anion ($ONOO^-$). These free radical molecules are representative of both ROS and RNS, thus allowing for measurement of the total free radical population within a sample. OxiSelect™ *In Vitro* ROS/RNS Assay Kit can also be used to evaluate antioxidant's effect on free radicals. The kit has a detection sensitivity limit of 10 pM for DCF and 40 nM for H_2O_2 respectively. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

Assay Principle

The OxiSelect™ *In Vitro* ROS/RNS Assay Kit is an *in vitro* assay for measuring total ROS/RNS free radical activity. Unknown ROS or RNS samples or standards are added to the wells with a catalyst that helps accelerate the oxidative reaction. After a brief incubation, the prepared DCFH probe is added to all wells and the oxidation reaction is allowed to proceed (Figure 1). Samples are measured fluorometrically against a hydrogen peroxide or DCF standard. The assay is performed in a 96-well fluorescence plate format that can be read on a standard fluorescence plate reader. The free radical content in unknown samples is determined by comparison with the predetermined DCF or hydrogen peroxide standard curve.

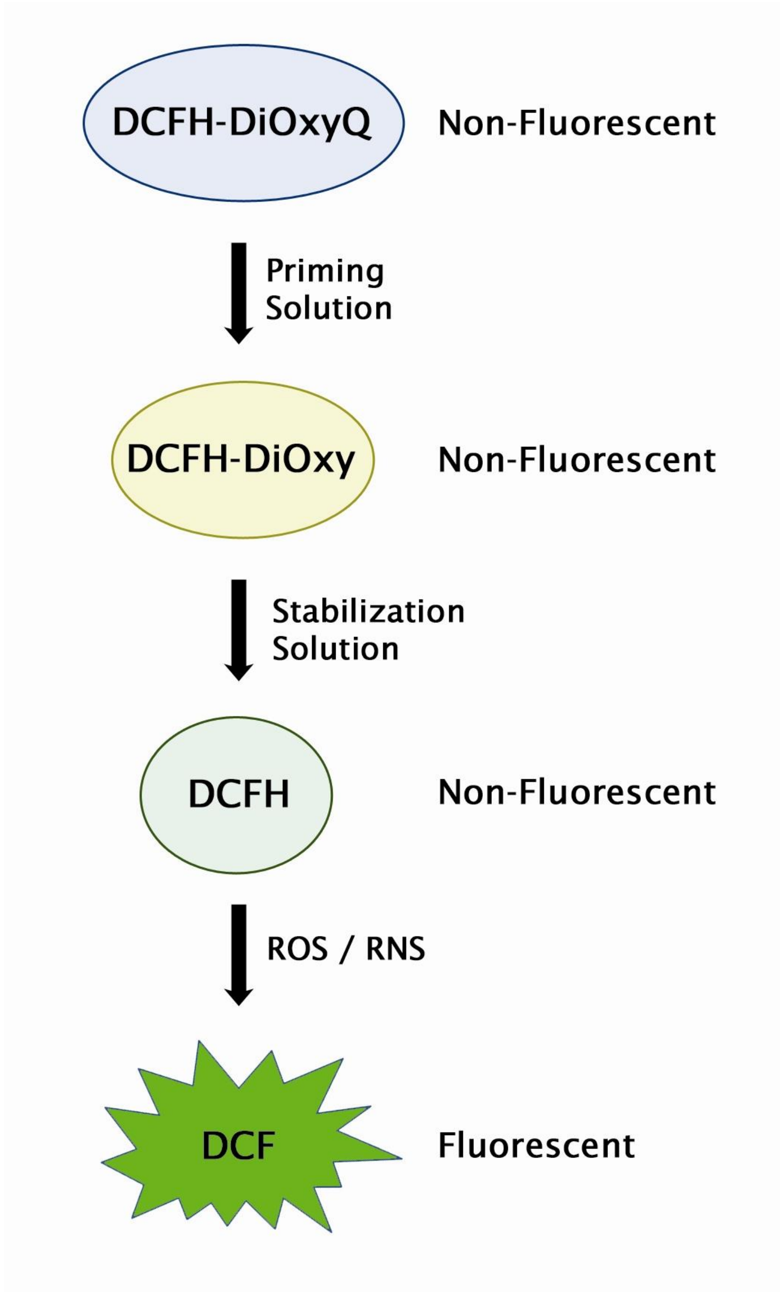

Figure 1. Mechanism of *In Vitro* ROS/RNS Assay.

Related Products

1. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
2. STA-340: OxiSelect™ Superoxide Dismutase Activity Assay
3. STA-341: OxiSelect™ Catalase Activity Assay Kit
4. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)

5. STA-832: OxiSelect™ MDA Adduct Competitive ELISA Kit
6. STA-838: OxiSelect™ HNE Adduct Competitive ELISA Kit

Kit Components

1. Priming Reagent (Part No. 234701): One 250 µL tube of solution.
2. Stabilization Solution (10X) (Part No. 234702): One 1.5 mL tube of solution.
3. Catalyst (250X) (Part No. 234703): One 20 µL tube of solution.
4. DCF-DiOxyQ (Part No. 234704): One 50 µL amber tube of solution in methanol.
5. DCF Standard (Part No. 234202): One 100 µL amber tube of a 1 mM solution in DMSO.
6. Hydrogen Peroxide (Part No. 234102): One 100 µL amber tube of an 8.821 M solution.

Materials Not Supplied

1. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
2. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
3. Multichannel micropipette reservoir
4. Phosphate Buffered Saline for sample preparations and dilutions
5. 96-well black or fluorescence microtiter plate
6. Fluorescent microplate reader capable of reading 480 nm (excitation) and 530 nm (emission)

Storage

Upon receipt, store the DCF-DiOxyQ and DCF Standard at -20ºC. Avoid multiple freeze/thaw cycles. Store all other components at 4ºC.

Preparation of Reagents

- 1X Stabilization Solution: Dilute the 10X Stabilization Solution 1:10 by adding 1.5 mL of solution to 13.5 mL of deionized water. Stir or vortex to homogeneity. Store the solution at 4ºC.
- 1X Catalyst: Prior to use, dilute the 250X Catalyst 1:250 in PBS. Vortex thoroughly. Prepare only enough for immediate applications (eg. add 10 µL of Catalyst to 2.49 mL PBS for 50 wells).
- DCFH Solution: Prepare only enough DCFH Solution for immediate applications in an amber tube or aluminum foil covered tube. Prepare DCFH Solution by diluting the stock solution of DCF-DiOxyQ 1:5 with Priming Reagent (eg. for 50 assays, add 25 µL DCF-DiOxyQ to 100 µL Priming Reagent). Vortex to homogeneity. Incubate the solution for 30 minutes at room temperature. Next, dilute the reaction 1:40 with 1X Stabilization Solution (eg. for 50 assays, add 125 µL DCF-DiOxyQ/ Priming Reagent reaction to 4.875 mL of Stabilization Solution). Vortex to homogeneity. Protect the solution from light. This solution is now stable in the DCFH form and ready to use. The solution may be stored at -20ºC for up to one week when protected from light.

  Note: Due to light-induced auto-oxidation, the stock DCF-DiOxyQ solution and all subsequent DCF-DiOxy and DCFH solutions must be protected from light.

Preparation of Samples

All samples should be assayed immediately or stored at -80°C for up to 1-2 months. The assay may be used on cell or tissue lysates, cell culture supernatants, serum, plasma, urine, and other biological fluids. Always run a standard curve with samples. Use PBS for dilution and preparation of samples.

Some common detergents and denaturants have been tested for compatibility in the assay (below table). Dilution of samples, and interfering substances, may be necessary for assay compatibility.

Substance	Compatible Concentration
Triton X-100	≤1%
NP-40	≤1%
SDS	≤0.1%
Deoxycholate	≤1%
Tween-20	≤0.1%
EDTA	≤10 mM
EGTA	≤10 mM
Glycerol	≤10%

Table 1. Substance Compatibility Table

- Cells or Tissues: Resuspend cells at 1-2 x 10^7 cells/mL or tissues at 10-50 mg/mL in PBS. Homogenize or sonicate on ice. To remove insoluble particles, spin at 10,000 g for 5 min. The homogenate can be assayed directly or stored at -80ºC as necessary.
- Serum, Plasma, Urine or Cell Culture Supernatants: To remove insoluble particles, spin at 10,000 g for 5 min. The supernatant can be assayed directly or stored at -80°C as necessary.

Preparation of the DCF Standard Curve

1. Prepare a 1:10 dilution series of DCF standards in the concentration range of 0 µM – 10 µM by diluting the 1mM DCF stock in 1X PBS (see Table 2).

Standard Tubes	DCF Standard (µL)	PBS (µL)	DCF (nM)
1	10	990	10,000
2	100 of Tube #1	900	1000
3	100 of Tube #2	900	100
4	100 of Tube #3	900	10
5	100 of Tube #4	900	1
6	0	1000	0

Table 2. Preparation of DCF Standards

2. Transfer 200 µL of each DCF standard to a 96-well plate suitable for fluorescence measurement.
3. Read the relative fluorescence with a fluorescence plate reader at 480 nm excitation / 530 nm emission.

Preparation of the H_2O_2 Standard Curve

1. To prepare the Hydrogen Peroxide standards, first perform a 1:4400 dilution of the stock Hydrogen Peroxide in deionized water. Use only enough for immediate applications (eg. Add 5 µL of Hydrogen Peroxide to 22 mL deionized water). This solution has a concentration of 2 mM.
2. Use the 2 mM H_2O_2 solution to prepare standards in the concentration range of 0 µM – 20 µM by further diluting in PBS (see Table 3). H_2O_2 diluted solutions and standards should be prepared fresh. Use the table below as a reference guide only. The volumes and concentrations of the standard may be adjusted by the user.

Standard Tubes	2 mM H_2O_2 Standard (µL)	PBS (µL)	H_2O_2 (µM)
1	10	990	20
2	500 of Tube #1	500	10
3	500 of Tube #2	500	5
4	500 of Tube #3	500	2.5
5	500 of Tube #4	500	1.25
6	500 of Tube #5	500	0.625
7	500 of Tube #6	500	0.313
8	500 of Tube #7	500	0.156
9	500 of Tube #8	500	0.078
10	500 of Tube #9	500	0.039
11	0	1000	0

Table 3. Preparation of H_2O_2 Standards

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknown(s) and standard(s), should be assayed in duplicate or triplicate.
2. Add 50 µL of unknown sample or hydrogen peroxide standard to wells of a 96-well plate suitable for fluorescence measurement.
3. Add 50 µL of Catalyst to each well. Mix well and incubate 5 minutes at room temperature.
4. Add 100 µL of DCFH solution to each well. Cover the plate reaction wells to protect them from light and incubate at room temperature for 15-45 minutes.
5. Read the fluorescence with a fluorescence plate reader at 480 nm excitation / 530 nm emission.

Example of Results

The following figures demonstrate typical Free Radical ROS/RNS Assay results. Fluorescence measurement was performed on SpectraMax Gemini XS Fluorometer (Molecular Devices) with a

485/538 nm filter set and 530 nm cutoff. One should use the data below for reference only. This data should not be used to interpret actual results.

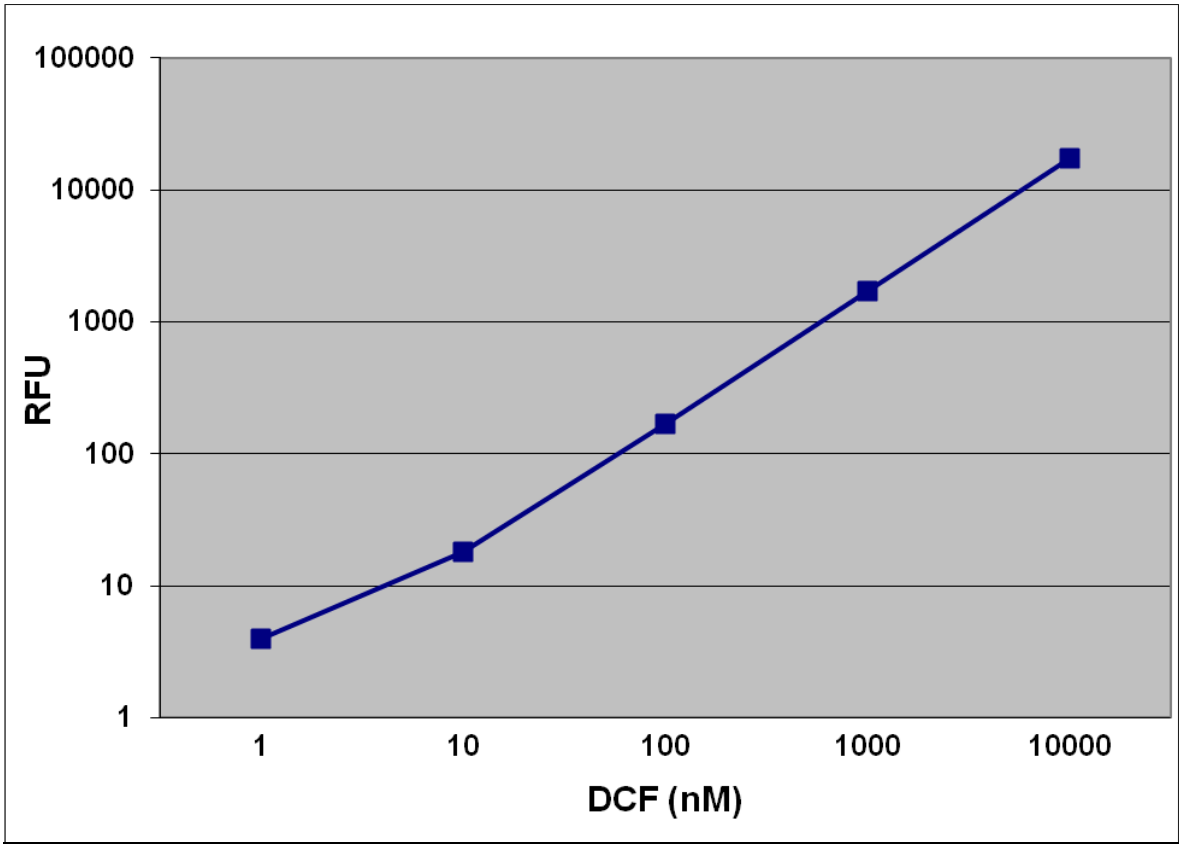

Figure 2. DCF Standard Curve.

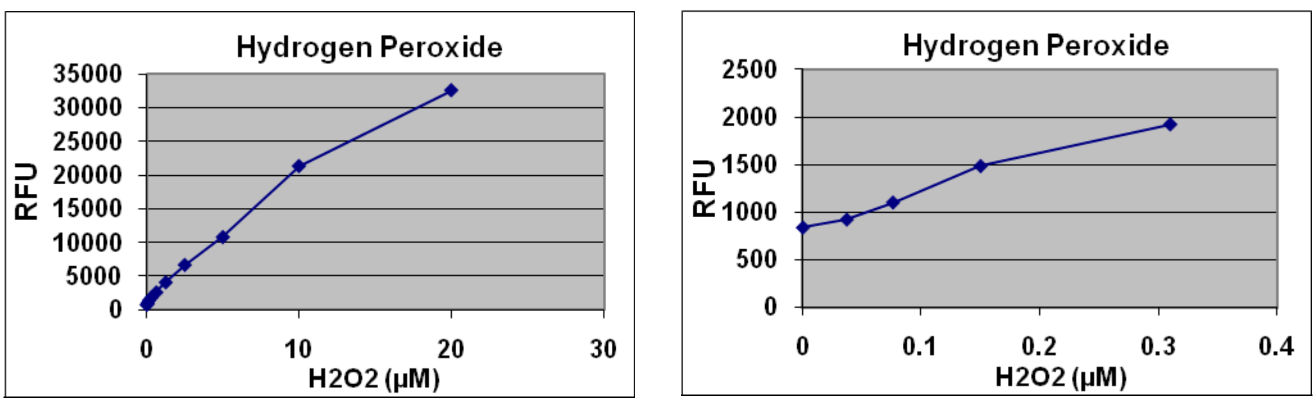

Figure 3. Hydrogen Peroxide Standard Curve.

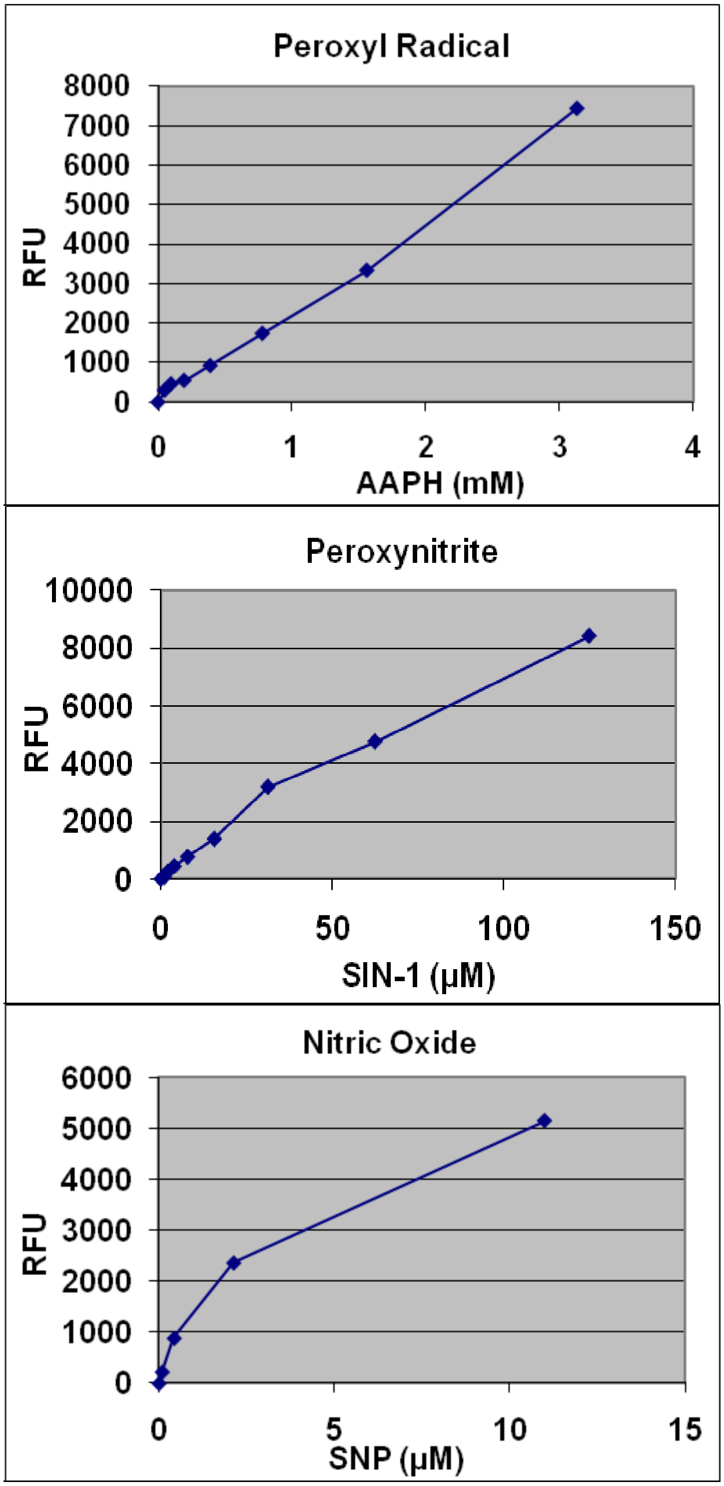

Figure 4. Detection of various free radical species using OxiSelect™ *In Vitro* ROS/RNS Assay Kit. DCF Fluorescence curves for AAPH (peroxyl radical generator, top), SIN-1 (peroxynitrite generator, center), and SNP (nitric oxide generator, bottom).

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Recent Product Citations

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Contact Information

Cell Biolabs, Inc.
7758 Arjons Drive
San Diego, CA 92126
Worldwide: +1 858-271-6500
USA Toll-Free: 1-888-CBL-0505
E-mail: tech@cellbiolabs.com
www.cellbiolabs.com