ROS / RNS Assay Kit

ROS / RNS Assay Kit is a detection kit for the quantification of total ROS (reactive oxygen species) and RNS (reactive nitrogen species) in serum, plasma, cell culture supernatants, urine and cell / tissue lysates.

Catalog number: ARG82769

Package: 96 assays

For research use only. Not for use in diagnostic procedures.

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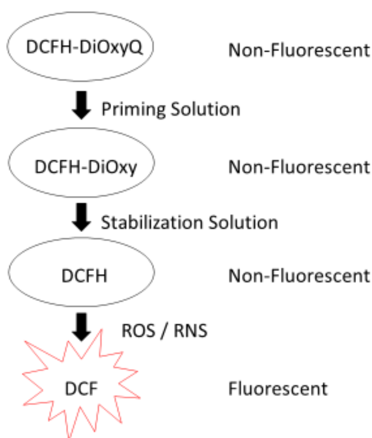
INTRODUCTION

Reactive oxygen species (ROS) are highly reactive chemical molecules formed due to the electron receptivity of O_2. Examples of ROS include peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen. [Provide by Wikipedia: Reactive oxygen species]

Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide (•NO) and superoxide ($O_2^{\bullet-}$) produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase respectively. NOS2 is expressed primarily in macrophages after induction by cytokines and microbial products, notably interferon-gamma (IFN-γ) and lipopolysaccharide (LPS). [Provide by Wikipedia: Reactive nitrogen species]

PRINCIPLE OF THE ASSAY

This ROS / RNS Assay Kit employs a convenient fluorescence method for the detection of total ROS / RNS free radical activity from serum, plasma, cell culture supernatants, urine and cell / tissue lysate samples. The samples or Standards are added to a 96 well fluorescence microplate with a catalyst that helps accelerate the oxidative reaction. After a brief incubation, the prepared DCFH probe is added to all wells. (See REAGENT PREPARATION section) Samples are measured fluorescence signal against a hydrogen peroxide or DCF Standards. The free radical content in samples is determined by comparison with the predetermined DCF or hydrogen peroxide standard curve.



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'-di-chloro-di-hydro-fluorescein 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’-di-chloro-di-hydro-fluorescein (DCF). Fluorescence intensity is proportional to the total ROS / RNS levels within the sample. The DCFH-DiOxyQ probe can react with hydrogen peroxide (H2O2), 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.

MATERIALS PROVIDED & STORAGE INFORMATION

Upon received, Store the DCF-DiOxyQ and DCF Standard at -20°C. Store the other components at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Priming Solution	250 µL	4°C
10X Stabilization Solution	1.5 mL	4°C
250X Catalyst	20 µL	4°C
DCF-DiOxyQ	50 µL	-20°C (Protect from light)
DCF Standards	100 µL	-20°C (Protect from light)
Hydrogen Peroxide (8.821M)	100 µL	4°C (Protect from light)

MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescent microplate reader capable of reading 480 nm (excitation) and 530 nm (emission)
- Deionized or Distilled water
- 96-well black or fluorescence microplate
- 1X PBS
- Pipettes and pipette tips
- Multichannel micropipette reservoir

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon receipt, store the DCF-DiOxyQ and DCF Standard at -20°C. Avoid multiple freeze/thaw cycles. Store all other components at 4°C.
- Due to light-induced auto-oxidation, the stock DCF-DiOxyQ solution and all subsequent DCF-DiOxy and DCFH solutions must be protected from light.
- The concentrations can vary over a wide range depending on the different samples. For unknown samples, we recommend doing a pilot experiment & testing several doses to ensure the readings are within the standard curve range.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.

- It is highly recommended assaying the Standards and samples in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

All samples should be assayed immediately or aliquoted and stored at-80°C for up to 1-2 months. Avoid repeated freeze-thaw cycles. 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.

Cell or Tissues: Re-suspend 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 minutes. The homogenate can be assayed directly or aliquoted and stored at -80°C 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 aliquoted and stored at-80°C as necessary.

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%

REAGENT PREPARATION

- **1X Stabilization Solution:** Dilute 10X Stabilization Solution into deionized water to yield 1X Stabilization Solution. (E.g., add 1.5 mL of 10X Stabilization Solution into 13.5 mL of deionized water to a final volume of 15 mL) Stir or vortex to homogeneity. Store the solution at 4°C.

- **1X Catalyst:** Prior to use, Dilute 250X Catalyst into 1X PBS to yield 1X Catalyst. (E.g., add 10 µL of 250X Catalyst into 2.49 mL of 1X PBS for 50 wells) Prepare only enough for immediate use.

- **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 (E.g., 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 (E.g., 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 -20°C 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.

- **Hydrogen Peroxide Standards:** 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 (E.g. Add 5 µL of Hydrogen Peroxide to 22 mL deionized water). This solution has a concentration of **2 mM**. Use the 2 mM H_2O_2 solution to prepare standards in the concentration range of **0 µM – 20 µM** by further diluting in **1X PBS**.

Standard #	Final H_2O_2 Standards conc. (µM)	Volume of 1X PBS (µL)	Volume of 2mM H_2O_2 Standards (µL)
S1	20	990	10 of H_2O_2 Standards stock
S2	10	500	500 of S1
S3	5	500	500 of S2
S4	2.5	500	500 of S3
S5	1.25	500	500 of S4
S6	0.625	500	500 of S5
S7	0.313	500	500 of S6
S8	0.156	500	500 of S7
S9	0.078	500	500 of S8
S10	0.039	500	500 of S9
S11	0	500	0

- **DCF Standards:** 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 below).

Standard #	Final DCF Standards conc. (nM)	Volume of 1X PBS (µL)	Volume of DCF Standards (µL)
S1	10000	990	10 of 1mM DCF Standards stock
S2	1000	900	100 of S1
S3	100	900	100 of S2
S4	10	900	100 of S3
S5	1	900	100 of S4
S6	0	1000	0

- Transfer 200 µL of each DCF standard to a 96-well plate suitable for fluorescence measurement.

- Read the relative fluorescence with a fluorescence plate reader at 480 nm excitation / 530 nm emission.

- The DCF standard is used to get a general measurement of all free radicals in a sample and the hydrogen peroxide standard is used to measure the hydrogen peroxide levels in a sample. It is recommended to run the hydrogen peroxide standard curve to confirm that the assay is working properly.

- The DCF standard curve is optional and is used to ensure that the plate reader is working properly and that the dye can be detected at various concentrations; however the DCF standards do not go through the assay protocol and cannot give any indication of free radicals in a sample.

- If using the DCF standard it is not necessary to add either the catalyst or DCFH solution to those wells. Once the DCF standard is prepared, it is complete and can be read immediately. The catalyst and DCFH solution should be added to the wells containing the H_2O_2 standard and all the wells with your samples.

ASSAY PROCEDURE

Prepare and mix all reagents thoroughly before use. Each sample and Standards should be assayed in duplicate or triplicate.

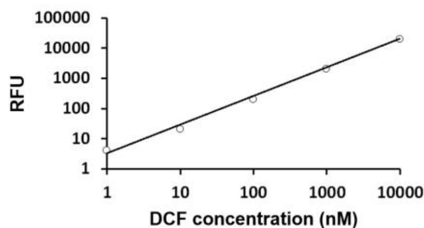
1. Add **50 µL** of **samples** or **Hydrogen Peroxide Standards** into the appropriate wells of 96-Well fluorescence microplate.
2. Adding **50 µL** of **Catalyst** to each well. Mix well and incubate for **5 minutes** at **room temperature**.
3. Add **100 µL** of **DCFH Solution** to each well. Cover the plate to protect reaction wells from light and incubate for **15-45 minutes** at **room temperature**.
4. Read the fluorescence with a fluorescence microplate reader at **480 nm excitation** / **530 nm emission** immediately.

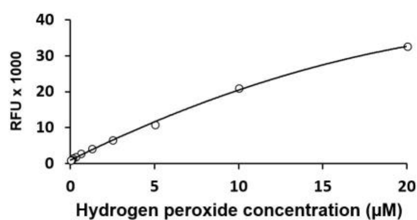
CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of Standards (hydrogen peroxide standard curve) and samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with fluorescence value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Use the mean fluorescence value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU can be calculated automatically using a linear curve fit.
5. The hydrogen peroxide standard curve is more useful because it is used to measure the hydrogen peroxide levels in the sample, which is the predominant form of ROS and will be the majority of the signal detected in the sample.
6. It is fine to use the DCF standard curve if you are interested in presenting your results as relative comparisons between samples. The DCF standard curve does not give any indication of free radicals in a sample and a DCF value is not meaningful, so it is probably not a good idea to present your result values as DCF (nM). If you have to present your results as absolute values, you should use the hydrogen peroxide standard curve, which will reflect the amount of hydrogen peroxide in the samples.

EXAMPLE OF TYPICAL STANDARD CURVE

The following figures demonstrate typical results with ROS / RNS Assay Kit. One should use the data below for reference only. This data should not be used to interpret actual results.





QUALITY ASSURANCE

Sensitivity

DCF: 10 pM

H_2O_2: 40 nM