Human ROS ELISA Kit

Catalog #: BG-HUM20964 (96 wells)

User Manual

This kit is designed to quantitatively detect the levels of Human ROS in serum/ plasma, cell lysate, cell culture supernatant and other suitable sample solution.

Manufactured and Distributed by:

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Important notes

Before using this product, please read this manual carefully; after reading the subsequent contents of this manual, please note the following specially:

- The operation should be carried out in strict accordance with the provided instructions.
- Store the unused strips in a sealed foil bag at 2-8°C.
- Always avoid foaming when mixing or reconstituting protein solutions.
- Pipette reagents and samples into the center of each well, avoid bubbles.
- The samples should be transferred into the assay wells within 15 minutes of dilution.
- We recommend that all standards, testing samples are tested in duplicate.
- Using serial diluted sample is recommended for first test to get the best dilution factor.
- If the blue color develops too light after 15 minutes incubation with the substrate, it may be appropriate to extend the incubation time (Do not over-develop).
- Avoid cross-contamination by changing tips, using separate reservoirs for each reagent.
- Avoid using the suction head without extensive wash.
- Do not mix the reagents from different batches.
- Stop Solution should be added in the same order of the Substrate Solution.
- TMB developing agent is light-sensitive. Avoid prolonged exposure to the light.

ALWAYS REFER TO LOT SPECIFIC PROTCOL PROVIDED WITH EACH KIT FOR INSTRUCTIONS.

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Scientific Background

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen.

In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. The production of ROS is strongly influenced by stress factor responses in plants, these factors that increase ROS production include drought, salinity, chilling, nutrient deficiency, metal toxicity and UV-B radiation. ROS are also generated by exogenous sources such as ionizing radiation.

Reactive oxygen species are implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels, in ototoxicity of drugs such as cisplatin, and in congenital deafness in both animals and humans. ROS are also implicated in mediation of apoptosis or programmed cell death and ischaemic injury. Specific examples include stroke and heart attack.

In general, harmful effects of reactive oxygen species on the cell are most often:[14]

1. damage of DNA or RNA
2. oxidations of polyunsaturated fatty acids in lipids (lipid peroxidation)
3. oxidations of amino acids in proteins
4. oxidative deactivation of specific enzymes by oxidation of co-factors

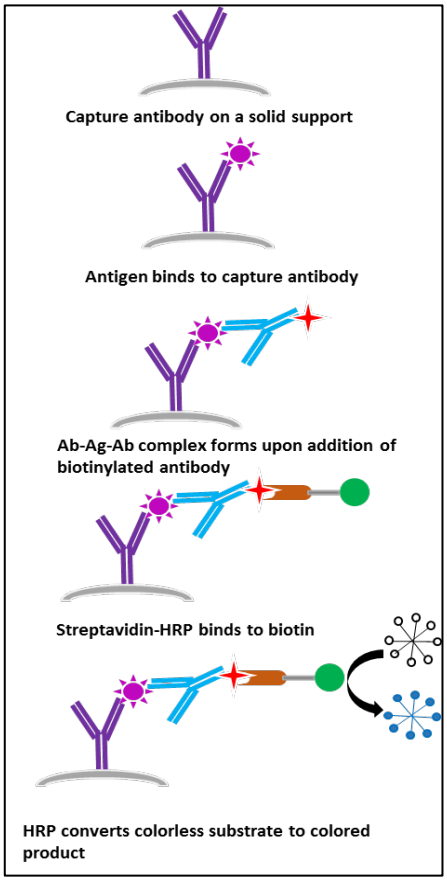
Intended use

The kit is used to quantify the Human ROS in serum/ plasma, cell culture supernatant and other suitable sample solution.

Standard range	31.2 – 2000 pg/ml
Assay time	3.5 hours
Validity	Six months
Store at	2-8 °C

Assay principle

The Human ROS ELISA Kit is based on standard sandwich enzyme-linked immunosorbent assay technology. Anti-Human ROS specific antibody has been pre-coated onto 96-well plate. Human ROS present in the standards/ samples bind to the capture antibody. Subsequently, biotinylated anti-Human ROS detection antibody is added to form an Ab-Ag-Ab sandwich. After a washing step, streptavidin-HRP is added and the unbound conjugate is removed with wash buffer. Next, addition of HRP substrate, TMB, results in the production of a blue colored product that changes to yellow after the addition of acidic Stop Solution. The density of yellow color is directly proportional to the amount of Human ROS captured on plate.



Materials supplied

1. Human ROS standard:	2000 pg ×2
2. 96-well plate pre-coated with anti-Human ROS Ab:	1
3. Sample Diluent buffer :	10 ml × 2
4. Detection antibody:	1 vial, dilution 1:100
5. Streptavidin-HRP:	1 vial dilution 1:100
6. Antibody Diluent buffer:	10 ml
7. Streptavidin HRP diluent buffer:	10 ml
8. TMB substrate:	10 ml
9. Stop Solution:	10 ml
10. 20 × Wash Buffer:	25 ml
11. Plate sealers	2
12. Package insert	1

Materials required but not supplied

- 1x PBS.
- Standard plate reader capable of measuring absorbance at 450 nm.
- Adjustable pipettes and disposable pipette tips.
- Multi-channel pipettes, manifold dispenser or automated microplate washer.
- Distilled water.
- Absorbent paper.
- Materials used for sample preparation.

Sample Preparation and storage

Isolate the test samples soon after collecting, then, analyze immediately (within 2 hours). Or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.

- Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
- Plasma: Collect plasma using EDTA-Na2 as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
- Tissue homogenates: For general information, hemolysis blood may affect the result, so you should rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS (the volume depends on the weight of the tissue. 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitor is recommended to add into the PBS.) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then spun for 5minutes at 5000×g to get the supernatant.
- Cell culture supernatant: Centrifuge supernatant for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8°C. Collect the clear supernatant and carry out the assay immediately.
- Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8°C. Collect the supernatant and carry out the assay immediately.

Reagent Preparation

Standard

- 2000pg/ml of standard solution: Add 1 ml of Sample / Standard dilution buffer into one Standard tube, keep the tube at room temperature for 10 min and mix thoroughly

- 1000pg/ml→31.2pg/ml of standard solutions: Label 6 Eppendorf tubes with 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, respectively. Aliquot 0.3 ml of the Sample / Standard dilution buffer into each tube. Add 0.3 ml of the above 2000pg/ml standard solution into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube and mix thoroughly, and so on.

Note: *The standard solutions are best used within 2 hours.*

Preparation of detection anti-Human ROS antibody working solution

- The stock solution is stable at 2-8 ^{o}C for up to 1 month. After opening the vial use within 1 month. For long-term storage, please aliquot and store at -20 ^{o}C. Avoid freeze-thaw cycles.
- The working solution should be prepared no more than 2 hours prior to the experiment
- The reagent is supplied as 100X concentrate. Empty the total contents in to 10 ml of Antibody Diluent Buffer or prepare the solution separately in a volume as needed. The solution should be mixed thoroughly.
- The total volume should be: 0.1 ml/well x the number of wells (Allowing 0.1-0.2 ml more than total volume.

Preparation of Streptavidin-HRP working solution

- The solution should be prepared no more than 1 hour prior to the experiment.
- The total volume should be: 0.1 ml/well x the number of wells (allowing 0.1-0.2 ml more than total volume).
- Streptavidin-HRP should be diluted 1:100 with Streptavidin-HRP Diluent buffer and mixed thoroughly.

Wash Buffer

- If crystals have formed in the 20X wash buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
- Dilute 25 ml Wash Buffer Concentrate (20X) to a total volume of 500 ml with distilled water.

Assay Procedure

Bring all reagents to room temperature before use. Human ROS standard curve should be prepared for each experiment. The user will decide sample dilution factor by rough estimation of Human ROS concentration in samples.

1. 1. Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions. It is recommend to measure each standard and sample in duplicate. **Wash plate 2 times before adding standard, sample and control (zero) wells!**
2. Aliquot standard solutions into the standard wells.
3. Add 0.1 ml of Sample / Standard dilution buffer into the control (zero) well.
4. Add 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) into test sample wells. Add 100 µl of the Detection Antibody

working solution to each well. Cover with a new adhesive strip and incubate at room temperature for 2 hours with shaking at 400 rpm.

5. Seal the plate with a cover and incubate at 37 °C for 90 min.

6. Remove the cover and discard the plate content, clap the plate on the absorbent filter papers or other absorbent material. **DO NOT let the wells completely dry at any time. Do Not Wash Plate!**

7. Add 0.1 ml of Biotin-detection antibody working solution into the above wells (standard, test sample & zero wells). Add the solution at the bottom of each well without touching the side wall.

8. Seal the plate with a cover and incubate at 37°C for 60 min.

9. Remove the cover, and wash plate 3 times with Wash buffer.

10. Add 0.1 ml of SABC working solution into each well, cover the plate and incubate at 37°Cfor 30 min.

11. Remove the cover and wash plate 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.

12. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°Cin dark within 15-30 min. (Note: This incubation time is for reference use only, the optimal time should be determined by end user.) And the shades of blue can be seen in the first 3-4 wells (with most concentrated ROS standard solutions), the other wells show no obvious color.

13. Add 50 µl of Stop solution into each well and mix thoroughly. The color changes into yellow immediately. 14. Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

Result calculation

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Human ROS concentration of the samples can be interpolated from the standard curve.

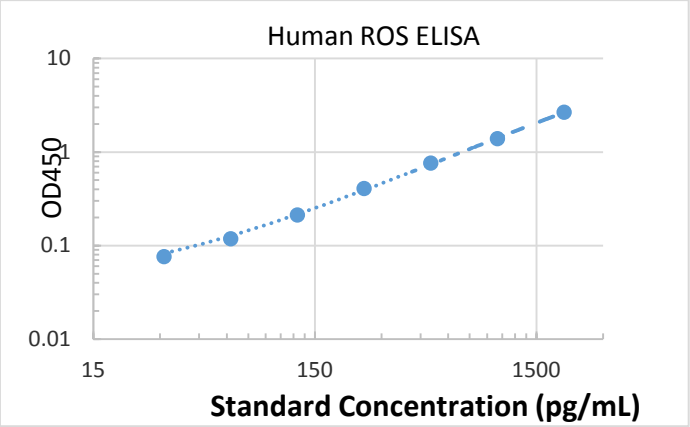
Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution

Assay summary

1. Wash plate 2 times before adding standard, sample and control (zero) wells!
2. Add 100µL standard or sample to each well for 90 minutes at 37°C
3. Add 100µL Biotin-detection antibody working solution to each well for 60 minutes at 37°C
4. Aspirate and wash 3 times
5. Add 100µLSABCworking solution to each well. Incubate for 30 minutes at 37°C
6. Aspirate and wash 5 times
7. Add 90µLTMB substrate. Incubate 15 -30 minutes at 37°C
8. Add 50µL Stop Solution. Read at 450nm immediately
9. Calculation of results

Typical data

This standard curve was generated at the Novatein Biosciences laboratory for demonstration purpose only. A standard curve must be run with each assay.



Sensitivity

The sensitivity or minimum detectable dose (MDD) of Human ROS was determined to be 20 pg/ml. MDD is defined as the Human ROS concentration resulting in an $O.D._{450}$ value that is 2 standard deviations higher than blank.

Spiking and Recovery

Matrices listed below were spiked with certain level of ROS and the recovery rates were calculated by comparing the measured value to the expected amount of ROS in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=16)	91-105	100
EDTA plasma(n=16)	89-101	99
heparin plasma(n=16)	92-103	98

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration ofROS and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16
serum(n=16)	95-104%	98-103%	89-95%	90-102%
EDTA plasma(n=16)	93-95%	92-102%	88-98%	88-101%

heparin plasma(n=16)	92-98%	93-100%	91-96%	89-93%

Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level ROS were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level ROS were tested on 3 different plates, 8 replicates in each plate.
CV(%) = SD/mean X 100
Intra-Assay: CV < 7.6%
Inter-Assay: CV < 9.7%

Specificity

This kit recognizes both natural and recombinant Human ROS. This assay has high sensitivity and excellent specificity for detection of ROS . No significant cross reactivity or interference between ROS and analogues was observed.

Sample Dilution

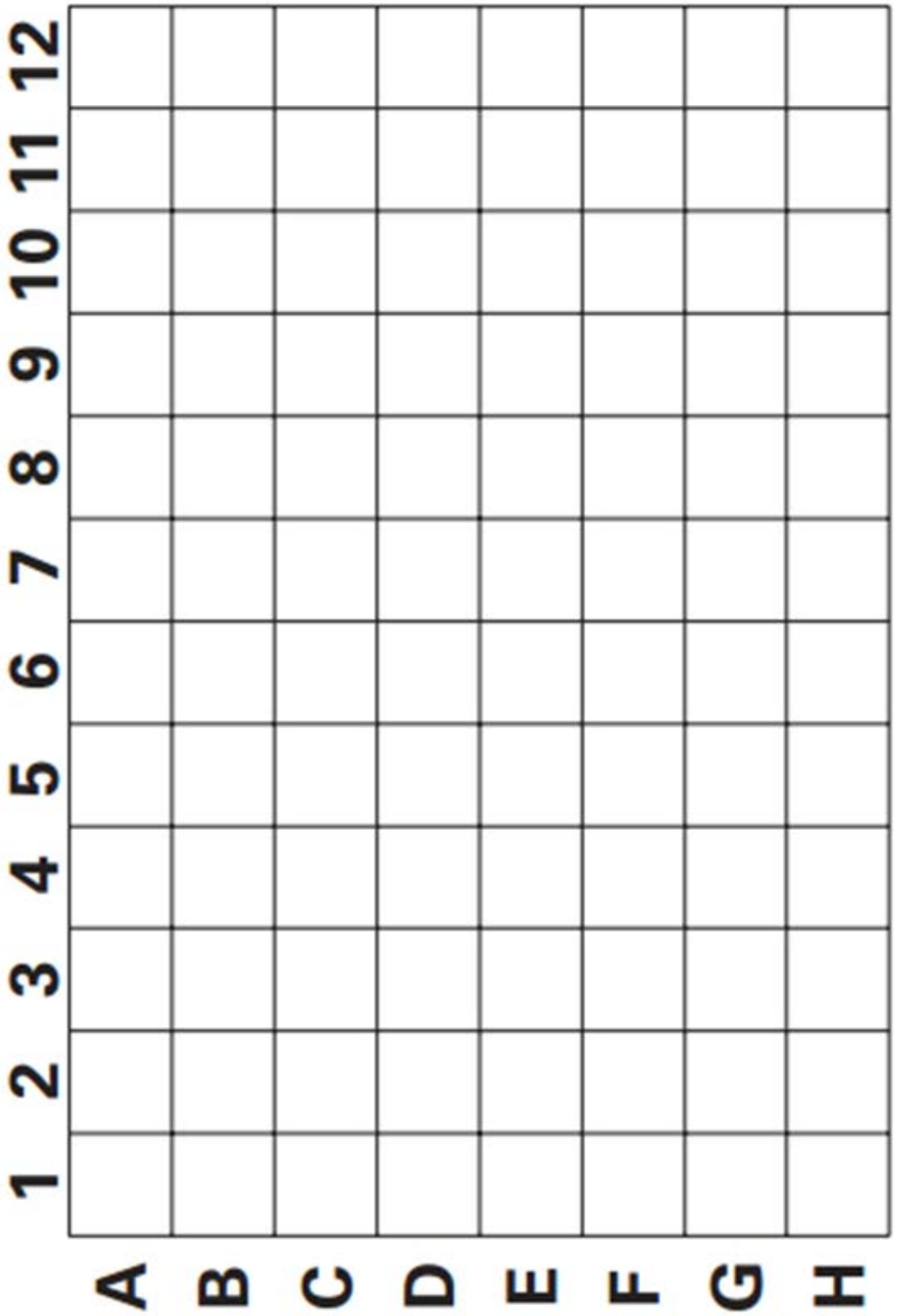
The user may need to determine the dilution factor in a preliminary experiment. If required, samples should be diluted in sample diluent buffer. Dilute the sample with the provided dilution buffer, and several trials may be necessary in practice. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be make in pre-experiment.

- High target protein concentration (20000-200000pg/ml): Dilution: 1:100. (i.e. Add 1µl of sample into 99 µl of Sample / Standard dilution buffer.)
- Medium target protein concentration (2000-20000pg/ml): Dilution: 1:10.(i.e. Add 10 µl of sample into 90 µl of Sample / Standard dilution buffer.)
- Low target protein concentration (31.2-2000pg/ml): Dilution: 1:2.(i.e. Add 50 µl of sample into 50 µl of Sample / Standard dilution buffer.)
- Very low target protein concentration (≤31.2pg/ml): Unnecessary to dilute, or dilute at 1:2.

For trouble shooting information please visit the following website:
http://www.novateinbio.com/en/content/15-tech-info OR

Email us at techsupport@novateinbio.com

Plate Layout



Troubleshooting Information

High Background

Probable Cause:	Solution/ Action
High incubation temperature:	Incubate at room temperature (25 ^{o}C) throughout the procedure
Insufficient washing of the plate:	Fill the wells with wash buffer and aspirate completely for the next wash Increase the number of washes Add soak time (20-30 seconds) in between the washes Use automated plate washer, if available and check that all the channels are operating properly
Concentrated streptavidin-HRP	Streptavidin-HRP was not diluted properly Dilute the streptavidin-HRP as mentioned in the manual
Light exposure during substrate incubation	The TMB substrate is light sensitive and turns to blue color in the presence of light. The incubation must be carried out in dark.
Stop solution not added	Color will continue to develop if stop solution is not added
Diluents came with the kit were not used	Standards/ sample, detection antibody and streptavidin-HRP must be diluted in the respective buffers came with the kit. Do not use buffers from other kits
Contaminated solutions	Prepare fresh working solutions

Poor Standard Curve

Probable Cause:	Solution/ Action
Improper standard reconstitution:	Spin the vial briefly before opening Reconstitute the standard as mentioned in the manual. After reconstitution, leave it atleast for 10 minutes at room temperature Do not store and reuse diluted standards
Curve fitting problem:	Log transform the values on both axes Use 4-PL/ 5-PL curve fitting programs
Incubation temperature/ time	Use the recommended standard incubation conditions
Poor dilutions	Pipetting error. Check pipetting technique and calculations. Use calibrated pipettes.

No Signal

Probable Cause:	Solution/ Action
Omission of reagent(s):	Read the manual entirely. Check that all the reagents are added in the correct order as stated in the manual
Incorrect detection antibody was used:	Use the detection antibody came with the kit
Chromogen solutions were mixed improperly	Use the recommended procedure to prepare the TMB substrate
HRP inhibitor in sample/ buffers	Check that the samples/ buffers do not have sodium azide as it will inhibit peroxidase reaction.
Vigorous washing	If the washing is done manually, pipette the wash buffer gently.
Dried wells	Do not allow the wells to dry out during the assay. Seal with the supplied adhesive cover during incubations
Improper plate reader settings	Check the wavelength and read the plate again

Erratic duplicate OD values

Probable Cause:	Solution/ Action
Insufficient washing of the plate	Fill the wells with wash buffer and aspirate completely for the next wash Increase number of washes Add soak time (20-30 seconds) in between the washes Use automated plate washer, is available and check that all the channels are functioning properly
Poor dilutions	Pipetting error. Check pipetting technique and calculations. Use calibrated pipettes.
Improper mixing of samples/ buffers	Mix the samples well before pipetting Thoroughly mix the working solutions of detection antibody/ streptavidin-HRP
Contamination from other wells	Do not reuse the adhesive covers from previous assay setups Change pipette tips during reagent addition. If same pipette tip is being used to dispense reagents, care should be taken, not to touch the solution in the well
Precipitates in the samples/ buffer	If precipitates are visible in wash buffer concentrate, keep it at 37 ^{o}C for 10-15 minutes until no precipitates are visible

	Centrifuge the samples to remove particulate matter
Dried wells	Do not allow the wells to dry out during the assay. Seal with the supplied adhesive cover during incubations