DOJINDO

Oxidative Stress

Research Protocol

2nd Revised Edition

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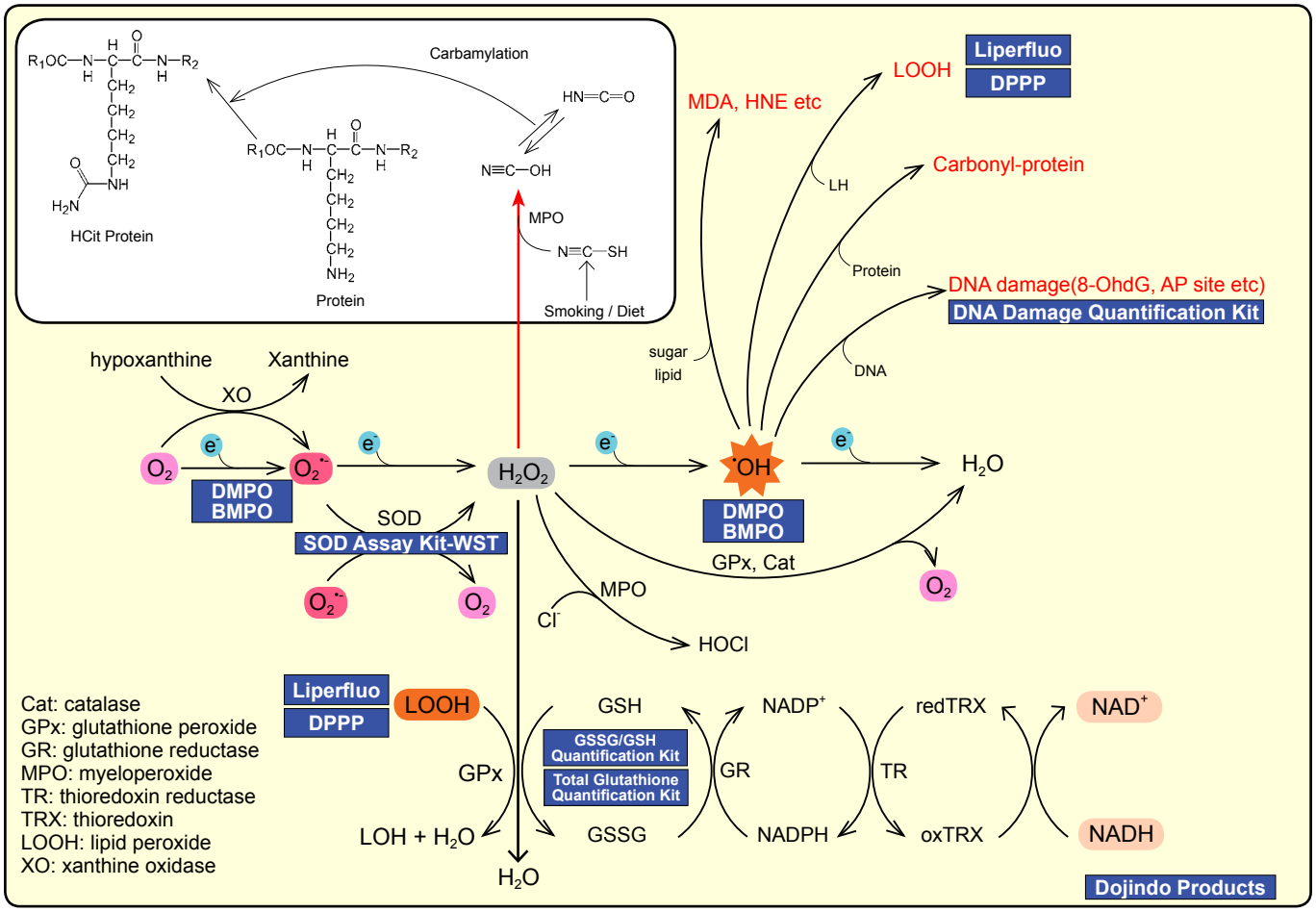
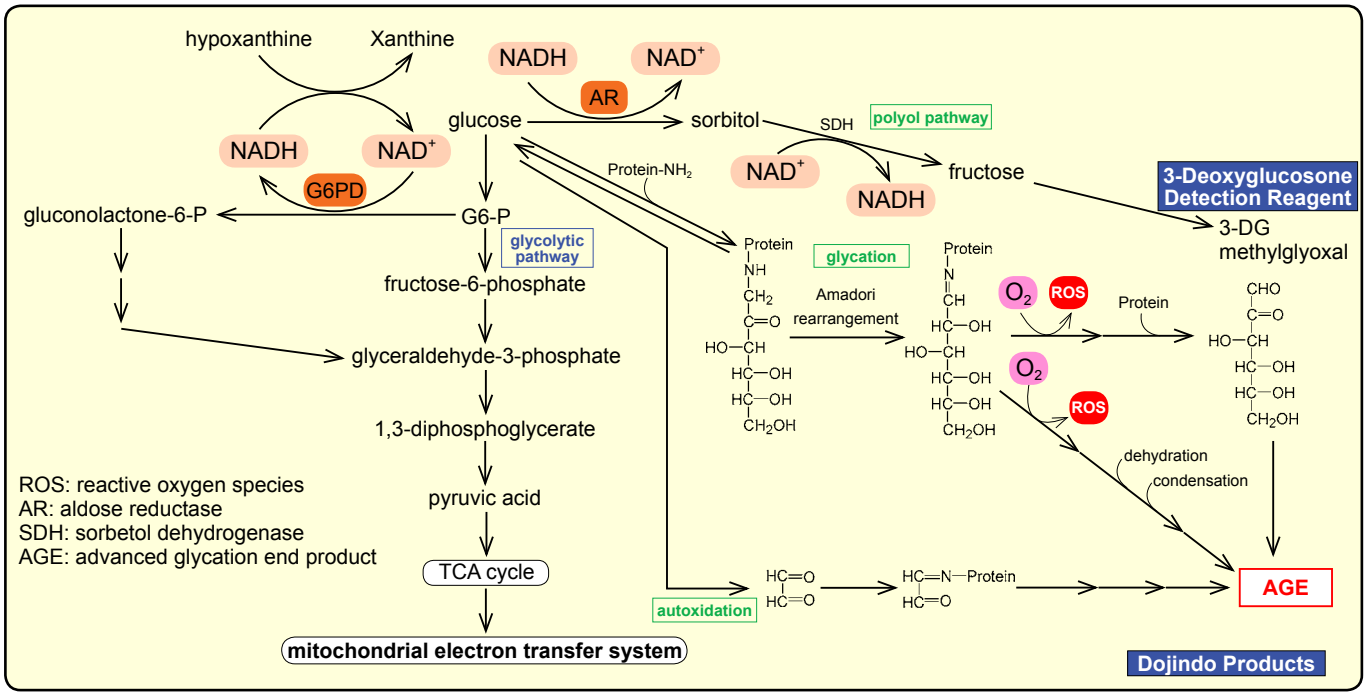
Oxidative Stress Research Products - Index

Oxidative Stress Map

H_2O_2 Related Oxidative Stress

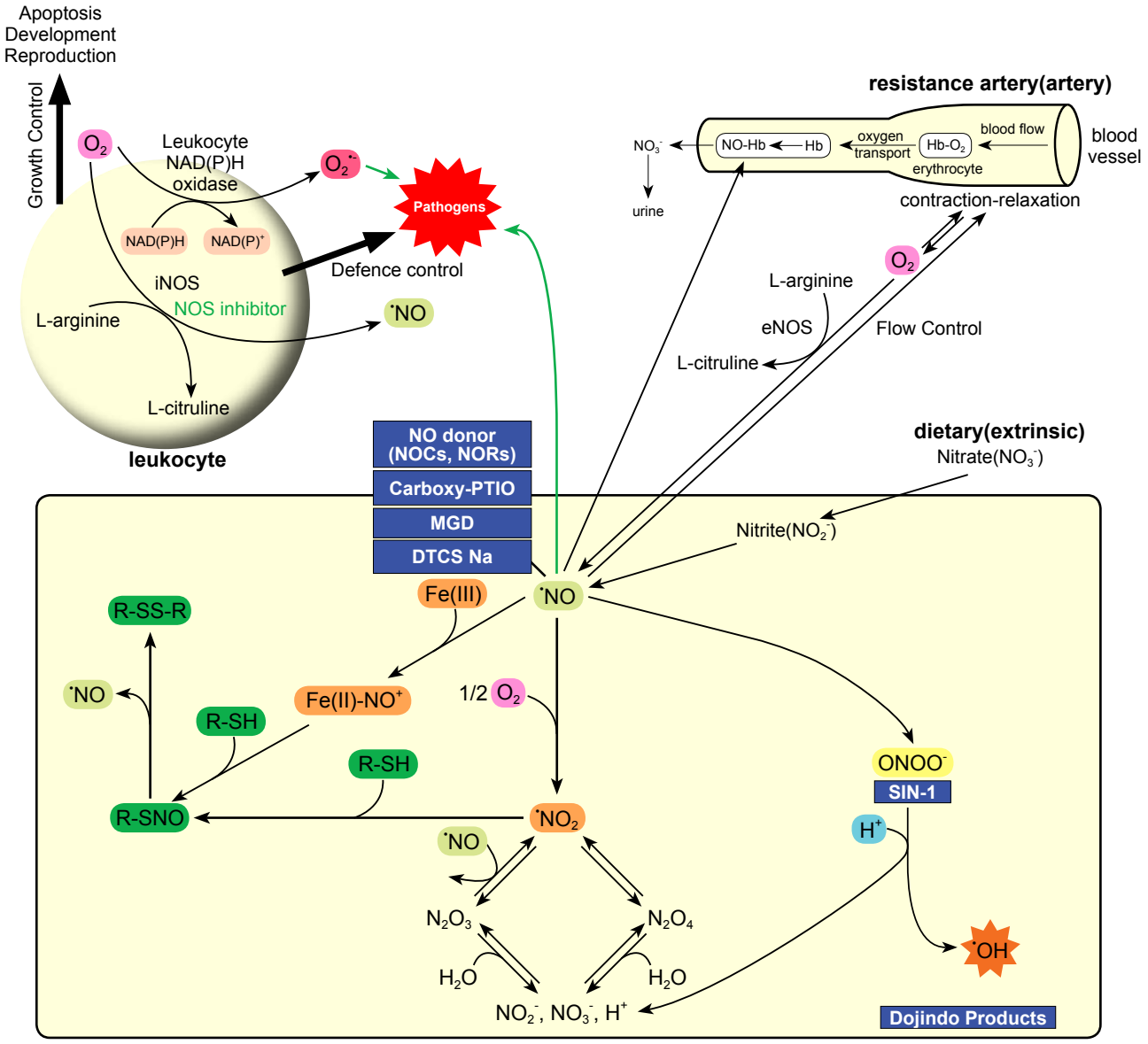


Diabetes and Oxidative Stress



Oxidative Stress Map

Nitric Oxide (NO) Related Stress

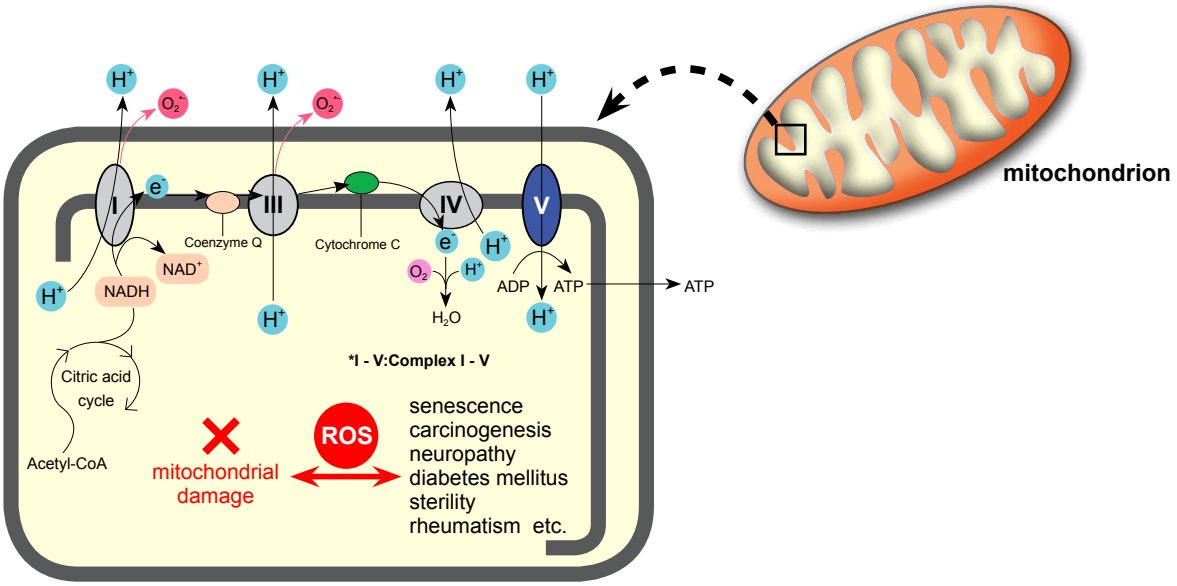


Property of NADPH Oxide (NOX) Family

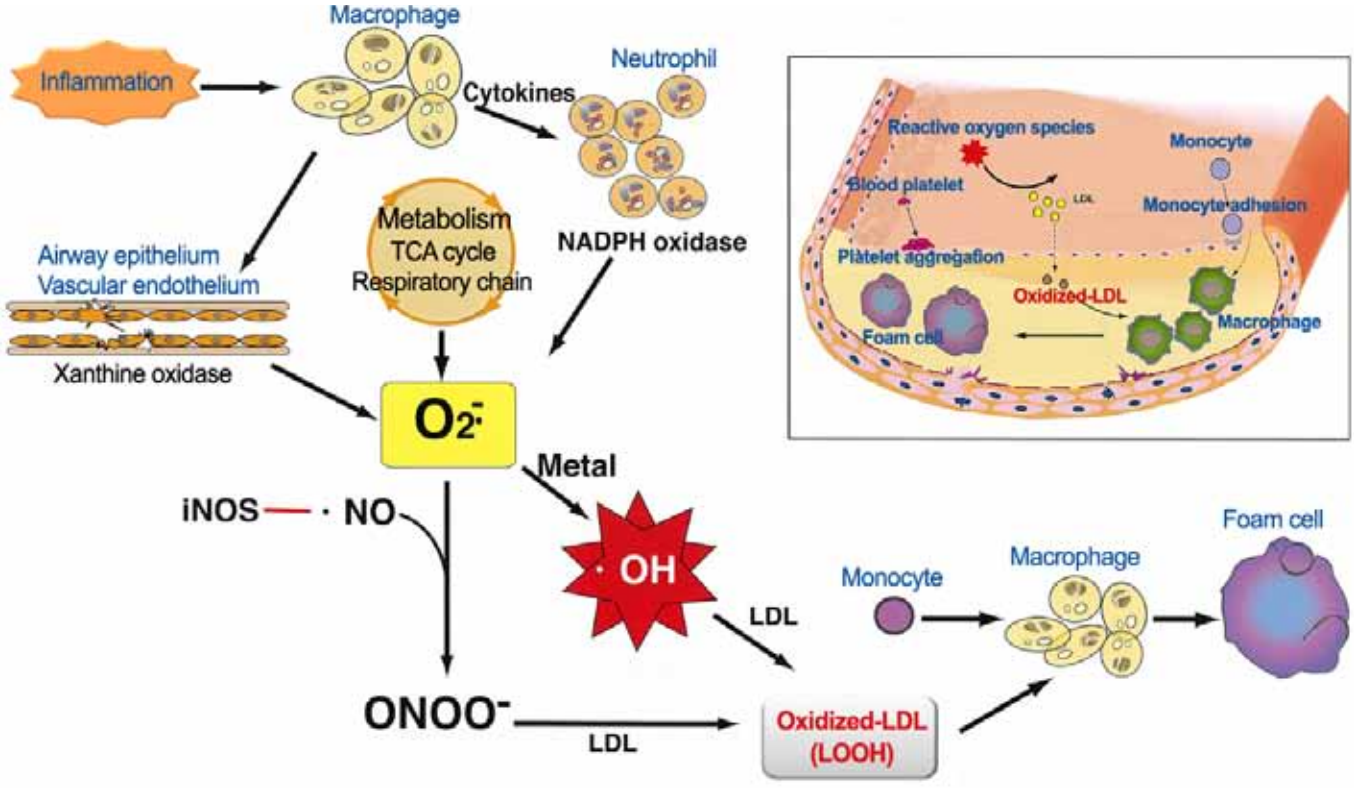
mammals Nox family oxidase			
	gene locus (human)	amino acid number (human)	tissue of high expression (function)
Nox1	Xq22	564	colonic epithelium, vascular smooth muscle (blood pressure elevation of Ang II)
Nox2/gp91^{phox}	Xp21.1	570	phagocyte, B lymphocyte (phylaxis)
Nox3	6q25.1-26	568	inner ear, fetal kidneys (otolith formation)
Nox4	11q14.2-21	578	renal tublar, vascular endothelial
Nox5	15q22.31	737	spermatozoon, spleen, lymphocyte
Duox1	15q21	1,551	thyroid, bronchial
Duox2	15q21	1,548	thyroid, large intestine (synthetic thyroid hormone)

Oxidative Stress Map

Mitochondrial Oxidative Stress



Reactive Oxygen Species Related in Atherosclerosis



* Signaling and metabolic pathways were edited under the supervision of Dr. Keizo Sato/Kyushu University of Health and Welfare.

Introduction

Oxygen is a very important molecule for the synthesis of biologically active materials such as hormones and ATP. Acquisition of the ability to utilize oxygen was a significant driving force for the evolution of life. Oxygen activates various enzymes in cells and activated oxygen species are involved in the operation of cell functions. Although oxygen itself is an essential element of life, molecules in cells, such as DNA and proteins, are sometimes damaged by reactive oxygen species (ROS) in what is called oxidative stress. ROS can be created by metabolism, ionizing radiation, and carcinogenic compounds that directly interact with DNA. During metabolism, a small portion of oxygen is converted to superoxide anion by one electron reduction; superoxide anion is then converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is reduced to water by catalase or glutathione peroxidase. However, if hydrogen peroxide is not completely reduced by these enzymes, it can generate an extremely reactive hydroxy radical when oxidized by iron (Fenton reaction). Hydroxy radical is also generated by UV irradiation or directly from water by ionizing radiation. Hydroxy radical reacts with lipid to generate lipid peroxide. However, not all ROS are unwanted. Hypochlorite ion, an ROS derived from hydrogen peroxide by myeloperoxidase in neutrophils, has germicidal activity. Nitric oxide, also known as endothelial-derived relaxation factor, is generated by NO synthetase. However, NO and superoxide anion may react to generate peroxynitrite, which is cytotoxic.

The ROS and reactive nitrogen compounds have many different activities in biological systems. In response, aerobic organisms created defense mechanisms to avoid oxidative stress. Oxidative stress has recently become the focus of many studies seeking to understand these defense mechanisms and the relationships between oxidative damage and disease or aging processes. To this end, many assay methods have been developed for the detection of ROS-related or ROS-derived substances such as superoxide anion, superoxide dismutase, glutathione, glutathione reductase, glutathione peroxidase, DNA lesions, 8-oxoguanine, 8-nitroguanosine, and protein carbonyl.

Nitric oxide (NO) has been identified as an endothelial-derived relaxation factor and antiplatelet substance. It serves as a neurotransmitter when derived from a neutrophil, and as a cytotoxic substance when derived from an activated macrophage. NO reacts with superoxide anion to generate highly toxic peroxynitrite. The reaction rate of NO with superoxide is three times that of SOD. In some cases, NO also activates cyclooxygenase. The most important role of NO is thought to be the activation of guanylate cyclase. Recently, published NO research has reported many contradictory results, which are due to NO's unique chemical properties. Since NO is a free radical, it is very reactive and unstable. NO changes its form in a complex manner immediately after appearing in a biological environment. Each of NO's metabolites might have different bioactivities from NO itself. For this reason, it is vital to separately investigate each function of the NO-related metabolites.

Measurement of superoxide dismutase activity

SOD Assay Kit-WST

Product Code: S311

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye, and the interaction with the reduced form of xanthine oxidase.

SOD Assay Kit-WST allows a very convenient and highly sensitive SOD assay by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion (Fig. 1). The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD (see figure below). Therefore, the IC_{50} (50% inhibition concentration) of SOD or SOD-like materials can be determined using colorimetric methods.

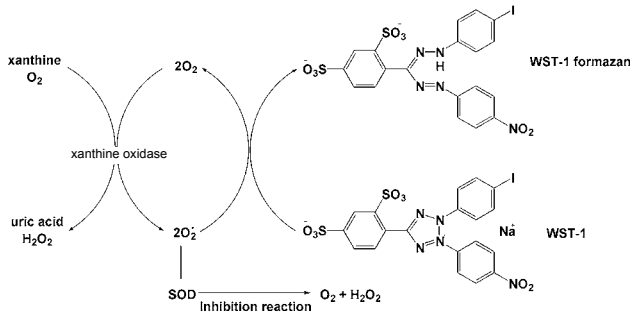


Fig.1 SOD inhibition assay mechanism

1. Preparation of Sample Solutions

► Erythrocytes or Plasma

1. Centrifuge 2-3 ml of anticoagulant-treated blood (such as heparin 10 U/ml with final concentration) at 600 xg for 10 minutes at 4°C.
2. Remove the supernatant and dilute it with saline to use as a plasma sample. Add saline to the pellet to prepare the same volume, and suspend the pellet.
3. Centrifuge the pellet suspension at 600 xg for 10 minutes at 4°C, and discard the supernatant.
4. Add the same volume of saline, and repeat Step 3 twice.
5. Suspend the pellet with 4 ml distilled water, then add 1 ml ethanol and 0.6 ml chloroform.
6. Shake the mixture vigorously with a shaker for 15 minutes at 4°C.
7. Centrifuge the mixture at 600 xg for 10 minutes at 4°C and transfer the upper water-ethanol phase to a new tube.
8. Mix 0.1 ml of the upper phase with 0.7 ml of distilled water, and dilute with 0.25% ethanol to prepare sample solution.

► Tissue(100 mg)

1. Wash the tissue with saline to remove as much blood as possible. Blot the tissue with paper towels and then measure its weight.
2. Add 400-900 µl sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) and homogenize the sample using Teflon homogenizer. If necessary, sonicate the homogenized sample on an ice bath (60W with 0.5 second intervals for 15 minutes).
3. Centrifuge the homogenized sample at 10,000 xg for 60 minutes at 4°C, and transfer the supernatant to a new tube.
4. Dilute the supernatant with distilled water to prepare sample solution.

2. Preparations of Solutions (for one 96-well plate)

► WST working solution
Dilute 1 ml of WST Solution with 19 ml of Buffer Solution

► Enzyme working solution
Centrifuge the Enzyme Solution tube for 5 seconds. Mix by pipetting and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

► Sample solution
Dilute sample solution prepared with Dilution Buffer or Saline.
e.g.) dilution rate: x1(no dilution), x1/5, x1/5^2, x1/5^3, x1/5^4, x1/5^5, x1/5^6

Contents of the Kit

	500 tests
WST Solution	5 ml x 1
Enzyme Solution	100 µl x 1
Buffer Solution	100 ml x 1
Dilution Buffer	50 ml x 1

Required Equipment & Materials

Microplate Reader (450 nm filter)
96-well microplate
2-20 µl & 20-200 µl multi-channel pipettes
Incubator(37°C)

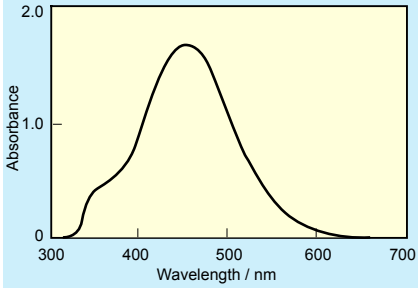


Fig. 2 Absorption spectrum of WST-1 formazan

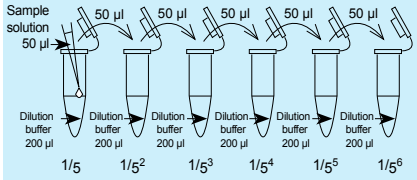


Fig. 3 Serial dilution process

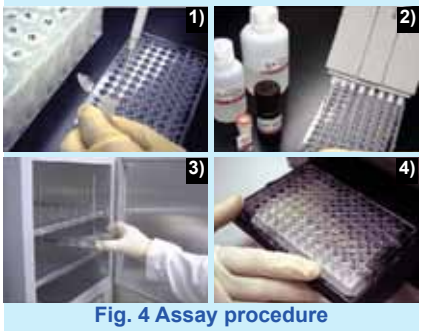


Fig. 4 Assay procedure

3. General Protocol (refer to Table 1, Fig. 4 and Fig. 5)

1. Add 20 µl of sample solution to each sample well and blank 2 well, and add 20 µl of ddH_2O(double-distilled water) to each blank 1 and blank 3 well.
2. Add 200 µl of WST Working Solution to each well, and mix by pipetting.
3. Add 20 µl of Dilution Buffer to each blank 2 and blank 3 well.
4. Add 20 µl of Enzyme Working Solution to each sample and blank 1 well.
5. Incubate the plate at 37ºC for 20 minutes.
6. Read the absorbance at 450 nm using a microplate reader.
7. Calculate the SOD activity(inhibition rate %) using the following equation.

SOD activity(inhibition rate %) = $\{[(A_{blank\ 1}-A_{blank\ 3})-(A_{sample}-A_{blank\ 2})]/(A_{blank\ 1}-A_{blank\ 3})\}$x100

Table 1 Solution and buffer volumes in each well

	sample	blank 1	blank 2	blank 3
Sample Solution	20 µl	-	20 µl	-
ddH_2O	-	20 µl	-	20 µl
WST Working Solution	200 µl	200 µl	200 µl	200 µl
Dilution Buffer	-	-	20 µl	20 µl
Enzyme Working Solution	20 µl	20 µl	-	-

4. Inhibition Curve

As Fig. 6 shows, SOD Assay Kit-WST can measure 100% inhibition because WST-1 does not react with the reduced form of xanthine oxidase(XO).

5. Definition of Unit(U)

One unit is defined as a point where a 20 µl of sample solution gives 50% inhibition of a colorimetric reaction between WST-1 and superoxide anion.

* Unit definition differ from the unit definition of Cytochrome C assay.

6. Calculate Unit(U)

1. Calculate a dilution ratio where the inhibition curve gives 50% inhibition.
2. SOD unit in original sample can be calculated by multiplying the dilution rate.

7. Example of Calculating Unit(U): Erythrocytes(x108 dilution sample)

1. Calculate a dilution ratio from the point of IC_{50} in the inhibition curve. Fig. 7 gives the dilution rate at IC_{50} of 1/1.8.
2. According to the definition of unit, 20 µl of this sample is calculated 1.8 U.
3. SOD unit per 1ml of this sample solution can be calculated by the following equation, 1.8 / 0.02 = 90.0 U/ml.
4. Original erythrocytes sample was diluted 108 times during the sample preparation. To calculate the SOD unit in the original, multiply 90.0 U/ml by 108. The SOD unit in the original sample is 9,720 U/ml of blood.

* SOD unit can be calculated as U/gram or U/mg.

8. Distinguish Mn-SOD from Cu/Zn-SOD and EC-SOD

Mn-SOD can be measured by blocking the Cu/Zn-SOD and EC-SOD activity using potassium cyanide(KCN) or Diethyldithiocarbamate(DDC).

9. Interference

Reducing agents, such as ascorbic acids and reduced forms of glutathiones, interfere with the SOD assay. Table 2 shows the concentrations of materials that cause 10% increase in the O.D. value. If sample contains these materials, please dilute the sample to avoid the interfere.

10. References

1. J. M. McCord, *et al.*, An Enzymic Function for Erythrocuprein(hemocuprein). *J Biol Chem.* 1969;**244**:6049-6055.
2. B. L. Geller, *et al.*, A Method for Distinguishing Cu,Zn- and Mn-Containing Superoxide Dismutases. *Anal Biochem.* 1983;**128**:86-92.
3. S. Goldstein, *et al.*, Comparison Between Different Assays for Superoxide Dismutase-like Activity. *Free Rad Res Commun.* 1991;**12**:5-10.
4. R. H. Burdon, *et al.*, Reduction of a Tetrazolium Salt and Superoxide Generation in Human Tumor Cells (HeLa). *Free Rad Res Commun.* 1993;**18**:369-380.
5. M. W. Sutherland, *et al.*, The Tetrazolium Dyes MTS and XTT Provide New Quantitative Assays for Superoxide and Superoxide Dismutase. *Free Radic Res.* 1997;**27**:283-289.
6. H. Ukeda, *et al.*, Flow-Injection Assay of Superoxide Dismutase Based on the Reduction of Highly Water-Soluble Tetrazolium. *Anal Sci.* 1999;**15**:353-357.
7. H. Ukeda, *et al.*, Spectrophotometric Assay for Superoxide Dismutase Based on the Reduction of Highly Water-soluble Tetrazolium Salts by Xanthine-Xanthine Oxidase. *Biosci Biotechnol Biochem.* 1999;**63**:485-488.
8. H.Ukeda, *et al.*, Spectrophotometric Assay of Superoxide Anion Formed in Maillard Reaction Based on Highly Water-soluble Tetrazolium Salt. *Anal Sci.* 2002;**18**:1151-1154.
9. N. Tsuji, *et al.*, Enhancement of Tolerance to Heavy Metals and Oxidative Stress in *Dunaliella Tertiolecta* by Zn-induced Phytochelatin Synthesis. *Biochem Biophys Res Commun.* 2002;**293**:653-659.

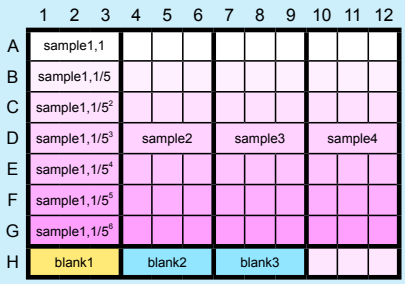


Fig. 5 Sample and blank arrangement on a 96-well microplate

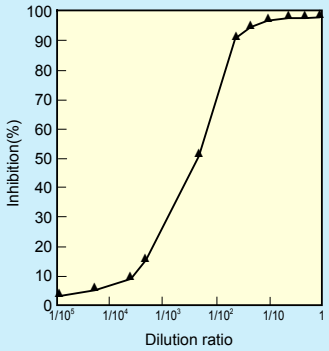


Fig. 6 Inhibition curve of Cu/Zn-SOD

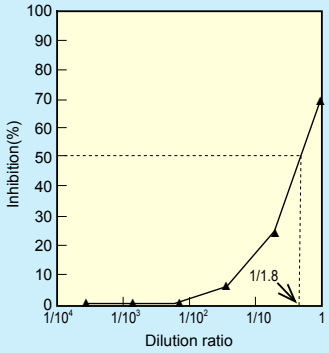


Fig. 7 Inhibition curve on erythrocytes sample

Table 2 Minimum Concentrations of Interfering Substances

Detergents	SDS	0.05%
	Tween 20	0.5%
	NP-40	0.5%
	Triton X-100	0.2%
Solvents	Ethanol	25%
	DMSO	5%
Reducing agents	Glutathione, reduced form	1.25 mmol/l
	Ascorbic acid	0.1 mmol/l
Other	EDTA	2 mmol/l
	BSA	1%w/v

Table 3 Measurement Examples

Total SOD	
erythrocyte	9,720 U/ml of blood
serum	355 U/ml of blood
heart(rat)	15,712 U/g (wet)
liver(rat)	142,907 U/g (wet)
HeLa cell	73 U/1x10^7 cells
HL60 cell	226 U/1x10^8 cells

11. Recent Publications

Title	Reference
Amyotrophic Lateral Sclerosis Model Derived from Human Embryonic Stem Cells Overexpressing Mutant Superoxide Dismutase 1	T. Wada, *et al*., *Stem Cells Trans Med*. 2012; **1**: 396
Copper chelation by tetrathiomolybdate inhibits lipopolysaccharide-induced inflammatory responses in vivo	H. Wei, *et al., Am J Physiol Heart Circ Physiol*. 2011; **301**: H712
A New Enteral Diet, MHN-02, Which Contains Abundant Antioxidants and Whey Peptide, Protects Against Carbon Tetrachloride–Induced Hepatitis	T. Takayanagi, *et al., JPEN J Parenter Enteral Nutr*. 2011; **35**: 516
Effects of dietary supplementation of methionine and its hydroxy analog DL-2-hydroxy-4-methylthiobutanoic acid on growth performance, plasma hormone levels, and the redox status of broiler chickens exposed to high temperatures	H. Willemsen, *et al., Poult. Sci*., 2011; **90**: 2311
Raf Kinase Inhibitor Protein RKIP Enhances Signaling by Glycogen Synthase Kinase-3β	F. Al-Mulla, *et al., Cancer Res*. 2011; **71**: 1334
Temporal changes in the expression of mRNA of NADPH oxidase subunits in renal epithelial cells exposed to oxalate or calcium oxalate crystals	Saeed R. Khan, *et al., Nephrol. Dial. Transplant*. 2011; **26**: 1778
Profiling of superoxide dismutase isoenzymes in compartments of the developing bovine antral follicles	C. Combelles, *et al., Reproduction*, 2010; **139**: 871
Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice	H. Sugimoto, *et al., Am J Physiol Gastrointest Liver Physiol*. 2010; **298**: G283
Gallium Disrupts Iron Uptake by Intracellular and Extracellular Francisella Strains and Exhibits Therapeutic Efficacy in a Murine Pulmonary Infection Model	O. Olakanmi, *et al., Antimicrob. Agents Chemother.* 2010; **54**: 244
Iron restriction improves type 2 diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats	Y. Minamiyama, *et al., Am J Physiol Endocrinol Metab*, 2010; **298**: E1140

12. FAQ

►What is the definition of a Unit?
One unit is defined as a point where a sample gives 50% inhibition of a colorimetric reaction between reactive dye (such as cytochrome C, WST-1, nitro-tetrazolium blue or XTT) and superoxide anion. For example, if the O.D. of "Blank 1" that does not contain any SOD is 1.0, the sample that gives 0.5 O.D. is defined as having 1 unit of SOD activity. You can use this unit to determine the SOD activity of your sample. Therefore, SOD activities determined using different dyes or methods are not comparable with each other.

►Can I use standard SOD to determine SOD activity in sample solutions?
Yes, you can. Prepare a inhibition curve (typical inhibition curve, and determine SOD activity in the sample solution. SOD bovine erythrocytes (CAS# 9054-89-1, EC 1.15.1.1) can be purchased from Sigma (catalog# S7571).

►Can I use a kinetic method to determine SOD activity?
Yes, you can use a kinetic method for SOD assay. Since the rate of the color development remains the same for up to 20 minutes, measure the slope for 5 minutes during this linear phase.

►The sample has color. Can I still use this sample?
Yes, you can still use the sample. Diluting the sample will minimize the interference. Subtract the O.D. of blank 2 from the O.D. of the sample to cancel out the background color. However, if the SOD activity in the sample is low, it may not be measurable.

►How do I prepare more Dilution Buffer?
Dilution Buffer is PBS. Please prepare the Dilution buffer with following concentrations; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4.

►Can I determine Mn-SOD and Cu/Zn-SOD independently using this kit?
Yes. In order to measure Mn-SOD activity, it is necessary to block the Cu/Zn-SOD activity using potassium cyanide(KCN). Adding 1 mM KCN to samples can block Cu/Zn-SOD activity completely. To measure Cu/Zn-SOD activity, measure the total SOD activity with and without KCN, and then subtract the Mn- SOD activity from total SOD activity.

►How long can I store the sample?
A sample stored in a freezer at -80ºC is stable for 1 month.

►Can I measure the levels of superoxide anion using this kit?
No. However, you could simply use WST-1, instead of this kit, to measure superoxide. You would need a standard to determine the amount of superoxide in sample solution. Since superoxide is not stable and reacts with various materials, it might be difficult to determine the total amount of superoxide generated in the system. The xanthine-xanthine oxidase system in this kit can be used as a standard for measuring the relative amount of superoxide production in each sample.

Quantification of total glutathione

Total Glutathione Quantification Kit

Product Code: T419

Glutathione (GSH) is the most abundant thiol compound in animal tissues, plant tissues, bacteria, and yeast. GSH has many different roles, including protection against reactive oxygen species and the maintenance of protein thiol groups. During these processes, GSH is converted into its oxidized form, glutathione disulfide (GSSG). Since GSSG is then enzymatically reduced by glutathione reductase, GSH is the dominant form in organisms.
DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed to detect thiol compounds. In 1985, Dr. M. E. Anderson suggested that the glutathione recycling system involving DTNB and glutathione reductase could be used as a highly sensitive glutathione detection method. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSSG (Fig. 1). Since 2-nitro-5-thiobenzoic acid is yellow, the GSH concentration in a sample solution can be determined by O.D. measurement at 412 nm absorbance (Fig. 2). GSH is regenerated from GSSG by glutathione reductase and will again react with DTNB to produce 2-nitro-5-thiobenzoic acid. This recycling reaction improves the sensitivity of total glutathione detection.

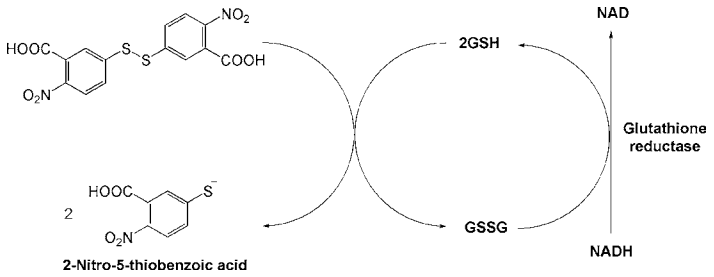


Fig.1 Principal of total glutathione detection

1. Preparation of Sample Solutions

► Cell(adhesive cell: 5 x 10^5 cells, leukocyte cell: 1 x 10^6 cells)
1. Collect cells by centrifugation at 200 g for 10 minutes at 4°C. Discard the supernatant.
2. Wash the cells with 300 µl PBS and centrifuge at 200 g for 10 minutes at 4°C. Discard the supernatant.
3. Add 80 µl 10 mM HCl, and lyse the cells by freezing and thawing twice.
4. Add 20 µl 5% SSA and centrifuge at 8,000 g for 10 minutes.
5. Transfer the supernatant to a new tube, and use it for the assay. If the final concentration of SSA is over 1%, add ddH_2O to reduce the concentration of SSA from 0.5 to 1%.

► Tissue(100 mg)
1. Homogenize the tissue in 0.5-1.0 ml 5% SSA.
2. Centrifuge the homogenized tissue sample at 8,000 g for 10 minutes.
3. Transfer the supernatant to a new tube and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay.

► Plasma
1. Centrifuge anticoagulant-treated blood at 1,000 g for 10 minutes at 4°C.
2. Transfer the top plasma layer to a new tube and add 5% SSA equivalent to half of the volume of the plasma.
3. Centrifuge at 8,000 g for 10 minutes at 4°C
4. Transfer the supernatant to a new tube, and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay.

► Erythrocyte
1. Centrifuge anticoagulant-treated blood at 1,000 g for 10 minutes at 4°C.
2. Discard the supernatant and the white buffy layer.
3. Lyse the erythrocytes with 5% SSA equivalent to 4 times the volume of the erythrocytes.
4. Centrifuge at 8,000 g for 10 minutes at 4°C.
5. Transfer the supernatant to a new tube, and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay. Erythrocytes can be isolated from the remaining sample solution after the plasma sample isolation.

*Preparation of 5% 5-Sulfosalicylic Acid (SSA) Solution
Note: SSA is not included in this kit.
1. Dissolve 1 g SSA in 19 ml water.
2. Store the solution at 4°C (stable for 6 months at 4°C).

Contents of the Kit

Substrate(DTNB)	2 vials
Enzyme Solution	50 µl x 1
Coenzyme(lyophilized)	2 vials
Standard GSH (lyophilized)	1 vial
Buffer Solution	50 ml x 1

Required Equipment & Materials

Microplate Reader (405 or 415 nm filter)
96-well microplate
20-200 µl multi-channel pipettes
Incubator (37°C)
5-sulfosalicylic acid(SSA)

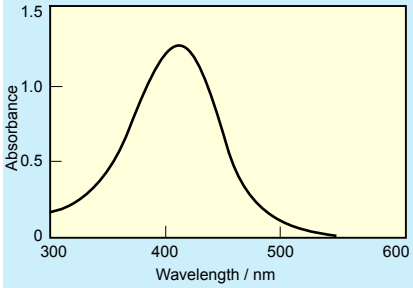


Fig. 2 Absorption spectrum of 5-Mercapto-2-nitrobenzoic acid

2. Preparations of Solutions

► Substrate working solution
Add 1.2 ml of Buffer Solution to one vial of Substrate, and dissolve.
Store the solution at -20°C (stable for 2 months).

► Enzyme working solution
Mix Enzyme Solution using pipette. Take out 20 µl of Enzyme Solution, and mix it with 4 ml of Buffer Solution.
Store the solution at 4°C (stable for 2 months).

► Coenzyme working solution
Add 1.2 ml of ddH_2O to the Coenzyme vial and dissolve. The Coenzyme vial is decompressed. Use a syringe to add ddH_2O, then open the vial.
Store the solution at -20°C(stable for 2 months).

► GSH standard solution
Add 2 ml of 0.5% SSA to Standard GSH vial, and dissolve to prepare 200 µM of GSH standard solution. The Standard GSH vial is decompressed. Use a syringe to add 0.5% SSA, then open the vial.
Store the solution at -20°C(stable for 2 months).
Dilute 100 µl of 200 µM GSH standard solution by serial dilution with 100 ul of 0.5% SSA in plastic tubes as indicated Fig. 4.

3. General Protocol (refer to Fig. 3)

1. Add 20 µl of Enzyme working solution, 20 µl of Coenzyme working solution and 120 µl of Buffer Solution to each well.
2. Incubate the plate at 37°C for 5 minutes.
3. Add 20 µl of GSH standard solution and 20 µl of sample solution to each well.
4. Incubate the plate at 37°C for 10 minutes.
5. Add 20 µl of Substrate working solution, and incubate the plate at room temperature for 10 minutes.
6. Read the absorbance at 405 nm or 415 nm using a microplate reader.
7. Determin concentrations of GSH in the sample solutions using a calibration curve. Since the colorimetric reaction is stable and the O. D. increases linearly over 30 min. A time course of the colorimetric reaction is shown Fig. 5. Typical calibration curves prepared using the pseudo-endpoint method is indicated in Fig. 6.

4. Calculation of total glutathione (GSH and GSSG) concentration

Determine the total glutathione concentration[a)] in a sample solution using the following equations.

► pseudo-end point method

$$\textbf{Total glutathione (GSH+GSSG)}=(O.D._{sample} - O.D._{blank})/slope^{b)}$$

► kinetic method

$$\textbf{Total glutathione (GSH+GSSG)}=(Slope^{c)}_{sample} - Slope^{c)}_{blank})/slope^{b)}$$

a) Since the values obtained by these equations are the amount of total glutathione in treated sample solutions, further calculations are necessary if the actual concentrations of glutathione in samples need to be determined.
b) slope of the calibration curve
c) slope of the kinetic reaction

5. Interference

Reducing agents such as ascorbic acid, β-mercaptoethanol, dithiothreitol (DTT) and cysteine, or thiol reactive compounds such as maleimide compounds, interfere with the glutathione assay. Therefore, SH compounds, reducing agents and SH reactive materials should be avoided during the sample preparation.

6. Notes

- The kit is stable for 6 months at 0 to 5°C.
- Use the reagents in the kit after the reagents temperature are equilibrated to the room temperature.
- Triplicate measurements per sample is recommended to obtain accurate data.
- Since the colorimetric reaction starts immediately after the addition of Substrate working solution to a well, use a multichannel pipette to avoid the reaction time lag of each well.
- If the concentration range of total glutathione in a sample is unknown, prepare multi-diluted sample solutions.
- This kit is not for GSSG/GSH ratio determination. For GSSG/GSH ratio determination, please refer to GSSG/GSH Quantification Kit.

7. References

1. G. L. Ellman, Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;**82**:70-77.
2. O. W. Griffith, Determination of Glutathione and Glutathione Disulfide Using Glutathione Reductase and 2-Vinylpyridine. *Anal Biochem*. 1980;**106**:207-212.
3. M. E. Anderson, Determination of Glutathione and Glutathione Disulfide in Biological Samples. *Methods Enzymol*. 1985;**113**:548-555.
4. M. A. Baker, *et al*., Microtiter Plate Assay for the Measurement of Glutathione and Glutathione Disulfide in Large Numbers of Biological Samples. *Anal Biochem*. 1990;**190**:360-365.

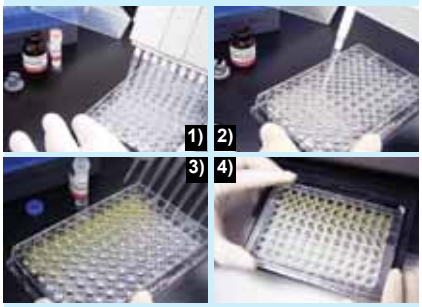


Fig. 3 Assay procedure

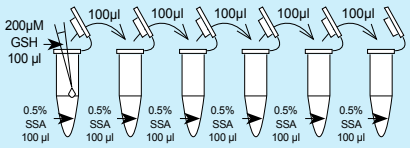


Fig. 4 Serial dilution process

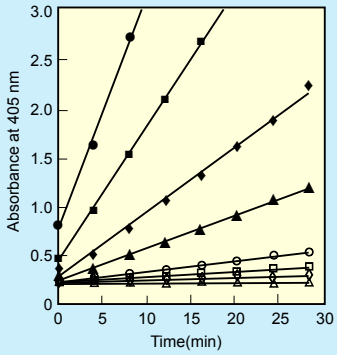


Fig. 5 Time-dependent Abs. increase on eight diferent samples

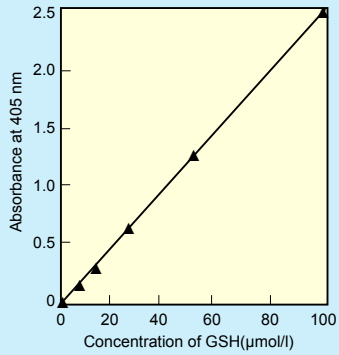


Fig. 6 Calibration curve prepared using pseudo-endpoint method(10 min incubation at room temperature)

8. Recent Publications

Title	Reference
Copper Chaperone-Dependent and -Independent Activation of Three Copper-Zinc Superoxide Dismutase Homologs Localized in Different Cellular Compartments in Arabidopsis	C. Huang, *et al. Plant Physiology.* 2012; **158**: 737 - 746
IRE1α activation protects mice against acetaminophen-induced hepatotoxicity	K. Yeon, *et al., J. Exp. Med.* 2012; **209**: 307 - 318
The Nitric Oxide Prodrug JS-K Is Effective against Non–Small-Cell Lung Cancer Cells In Vitro and In Vivo: Involvement of Reactive Oxygen Species	A.. Maciag, *et al., J. Pharmacol. Exp. Ther.*, 2011; **336**: 313 - 320
Mutations in PNKD causing paroxysmal dyskinesia alters protein cleavage and stability	Y. Shen, *et al., Hum. Mol. Genet.*, 2011; **20**: 2322 - 2332
Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species	S. Suzuki, *et al., PNAS*, 2010; **107**: 7461 - 7466
Oxidative Stress and Sodium Methyldithiocarbamate–Induced Modulation of the Macrophage Response to Lipopolysaccharide In Vivo	S. Pruett, *et al., Toxicol. Sci.* 2009; **109**: 237 - 246
Nrf2 Enhances Cell Proliferation and Resistance to Anticancer Drugs in Human Lung Cancer	S. Homma, *et al., Clin. Cancer Res.* 2009; **15**: 3423 - 3432
Inhibition of hepatic Niemann-Pick C1-like 1 improves hepatic insulin resistance	K. Irie, *et al., Journal of Dental Research*. 2008; **87**: 456 - 460
3-Morpholinopropyl isothiocyanate is a novel synthetic isothiocyanate that strongly induces the antioxidant response element-dependent Nrf2-mediated detoxifying/antioxidant enzymes in vitro and in vivo	Y. Keum, *et al., Carcinogenesis.* 2008; **29**: 594 - 599
Cells Deficient in the FANC/BRCA Pathway Are Hypersensitive to Plasma Levels of Formaldehyde	J. Ridpath, *et al., Cancer Res.* 2007; **67**: 11117 - 11122

9. FAQ

►Do I have to dilute the sample solution prior to the assay?
If you do not know the total glutathione level of your sample, multiple dilutions may be necessary. If the total glutathione level of your sample is less than 100 µM, no dilution is necessary.

►What interferes with the assay?
Reducing agents (such as ascorbic acid, beta-mercaptoethanol, dithiothreitol, and cysteine) and thiol reactive compounds (such as maleimides) interfere with the glutathione assay. Therefore, reducing agents and thiol reactive compounds should be avoided during the sample preparation.

Distinguish Measurement of Glutathione

GSSG/GSH Quantification Kit

Product Code: G257

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a tripeptide present in the body, and it is involved in antioxidation, drug metabolism, and other as enzyme substrate of glutathione peroxidase, glutathione *S*-transferase, and thiol transferase, etc. Glutathione is usually present as reduced form (GSH), but GSH is converted into its oxidized form (GSSG) by stimulation such as oxidative stress. Therefore, the ratio of GSH and GSSG has been noted as index of oxidative stress.
The GSSG/GSH Quantification kit contains Masking Reagent of GSH. The GSH can be deactivated in the sample by adding the Masking Reagent. Therefore, only the GSSG is detected by measuring the absorption (λmax = 412nm) of DTNB (5,5'-dithiobis (2-nitrobenzoic acid) using the enzymatic recycling system. Also, GSH can be determined the quantity by subtracting GSSG from the total amount of glutathione.
The kit can be limited to quantify GSH/GSSG concentration from 0.5 µmol/l to 50 µmol/l and GSSG concentration from 0.5 µmol/l to 25 µmol/l.

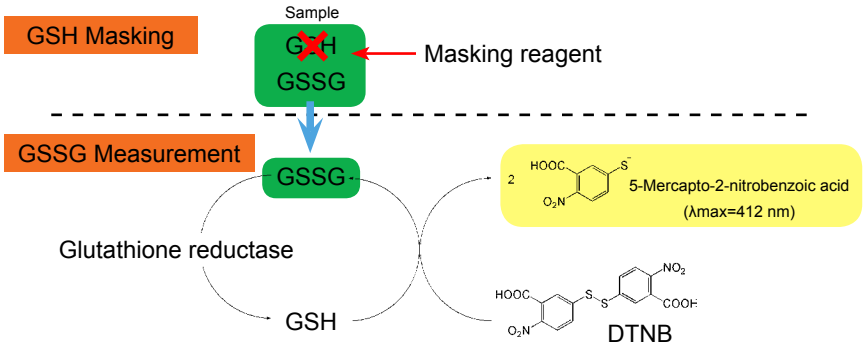


Fig.1 Principal of GSSG/GSH detection

1. General Protocol

► Preparation of Sample Solution
Please refer to Total Glutathione Quantification, page 5.

► Determination of GSSG concentration

1. Add 4 µl of Masking Solution to sample solution and 200 µl of GSSG standard solution diluted with 0.5% SSA respectively, then transfer 40 µl of the solution to each well.
2. Add 120 µl of Buffer Solution to each well and incubate for 1 hour at 37°C.
3. Add 20 µl of Substrate working solution to each well, then add 20 µl of Coenzyme working solution and Enzyme working solution to each well respectively.
4. Incubate for 10 minutes at 37°C and read the absorbance at 405 or 415 nm using a microplate reader.
5. Determine concentrations of GSSG in the sample solution using a GSSG calibration curve (Fig. 2).

► Determination of total glutathione concentration

1. Add sample solution and 40 µl of GSH standard solution diluted with 0.5% SSA to each well.
2. Add 120 µl of Buffer solution to each well and incubate for 1 hour at 37°C.
3. Add 20 µl of Substrate working solution to each well, then add 20 µl of Coenzyme working solution and Enzyme working solution to each well respectively.
4. Incubate for 10 minutes at 37°C and read the absorbance at 405 or 415 nm using a microplate reader.
5. Determine concentrations of total glutathione in the sample solution using a GSH calibration curve(Fig. 3).

► Calculating the concentration of GSH
GSH(conc.) = total Glutathione(conc.) - 2 x GSSG(conc.)

2. Recent Publications

Title	Reference
Diurnal Variation of cadmium-induced mortality in mice	N. Miura, and T. Hasegawa, *et al., J. Toxicol. Sci*. 2012; **37**: 191

Contents of the Kit

Enzyme Solution	50 µl x 1
Coenzyme	2 vials
Buffer Solution	60 ml x 1
Substrate (DTNB)	4 vials
Standard GSH	1 vial
Standard GSSG	1 vial
Masking Reagent	20 µl x 1

Required Equipment & Materials

Microplate Reader (405 or 415 nm filter)
96-well microplate
20-200 µl multi-channel pipettes
Incubator (37°C)
5-sulfosalicylic acid (SSA)
Ethanol

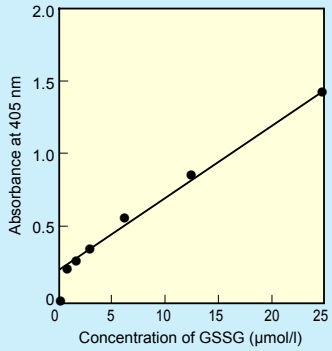


Fig. 2 Determination of the concentration of GSSG

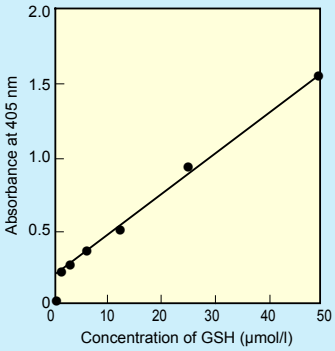


Fig. 3 Determination of the concentration of total glutathione

Quantification of damaged base in genomic DNA

DNA Damage Quantification Kit-AP Site Counting-

Product Code: DK02

Oxidative damage to DNA is a result of its interaction with reactive oxygen species (ROS), in particular, the hydroxy radical. Hydroxy radicals, which are produced from superoxide anion and hydrogen peroxide by the Fenton reaction, produce multiple modifications in DNA. Oxidative attacks by hydroxy radicals on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites (AP sites). In fact, AP sites are one of the major types of DNA damage generated by ROS.

Aldehyde Reactive Probe (ARP; N'-aminooxymethylcarbonylhydrazin-D-biotin) reacts specifically with an aldehyde group present on the open ring form of the AP sites (Fig. 1). This reaction makes it possible to detect DNA modifications that result in the formation of an aldehyde group. After treatment with excess ARP reagent, all of the AP sites on DNA are tagged with a biotin residue. These biotin-tagged AP sites can be quantified using the avidin-biotin assay, followed by colorimetric detection with either peroxidase or alkaline phosphatase conjugated to the avidin. DNA Damage Quantification Kit contains all the necessary solutions for detecting between 1 to 40 AP sites per 1 x 10^5 base pairs.

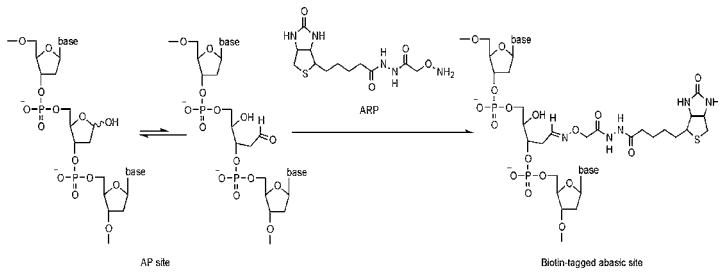


Fig. 1 Reaction of AP site with ARP

1. Purification of genomic DNA

Several different methods and products are available for the isolation of genomic DNA from samples such as membrane binding method, guanidine/detergent lysis method, and polyelectrolyte precipitation method. Among these methods, the guanidine/detergent lysis method is simple, and it gives highly purified genomic DNA for the ARP-based abasic sites detection. During the purification process, avoid heating of the DNA solution. Determine the concentration and purity of the purified genomic DNA using the spectrophotometer* and agarose gel electrophoresis. Dissolve the genomic DNA in TE at the concentration of 100 µg/ml. It is important for an accurate assay that the DNA concentration is adjusted exactly to 100 µg/ml.

* 1 $OD_{260\,nm}$ = 50 µg/ml. The ratio of $OD_{260\,nm}/OD_{280\,nm}$ of highly purified DNA solution is 1.8 or higher. Protein contamination in the sample solution may cause a positive error.

2. General Protocol

► ARP reaction

1. Mix 10 µl of purified genomic DNA solution(100 µg/ml) and 10 µl of ARP Solution in a 0.5 ml tube, and incubate at 37°C for 1 hour.
2. Wash the inside of the Filtration Tube with 100 µl of TE twice.
3. Add 380 µl of TE to the reaction solution, and transfer the solution to the Filtration Tube.
4. Centrifuge the Filtration Tube at 2,500 xg for 15 minutes, and discard the filtrate solution.
5. Add 400 µl of TE to the Filtration Tube and resuspend the DNA on the filter with a pipette.
6. Centrifuge the Filtration Tube at 2,500 xg for 15 minutes.[a)]
7. Add 200 µl of TE to the Filtration Tube to resuspend the DNA on the filter with a pipette.
8. Transfer the DNA solution to the 1.5 ml tube, and add 200 µl of TE again to the Filtration Tube to transfer the ARP-labeled DNA on the filter completely to the 1.5 ml tube.[b)]
9. Store the ARP-labeled genomic DNA solution at 0 to 5°C.

a) If the DNA solution still remains on the filter after the centrifugation, spin for another 5 minutes.

b) Recovery rate of DNA using the filtration tube is 90%, so the concentration of the ARP-labeled DNA is 2.25 µg/ml. For more accurate determination of the number of abasic sites in the sample DNA, we recommend measuring the DNA concentration.

Contents of the Kit

5 samples

ARP Solution (10mM ARP)	100 µl x 1
ARP-DNA Standard Soln.*	250 µl ea.
(0, 2.5, 5, 10, 20, 40 AP sites/100,000 bp)	
DNA Binding Solution	10 ml x 1
Substrate Solution	10 ml x 1
TE Buffer	15 ml x 1
HRP-Streptavidin	25 µl x 1
Washing Buffer	1 pack
Filtration Tube	5 tubes
96-well Microplate/ U bottom	1 plate

20 samples

ARP Solution (10mM ARP)	250 µl x 1
ARP-DNA Standard Soln.*	250 µl ea.
(0, 2.5, 5, 10, 20, 40 AP sites/100,000 bp)	
DNA Binding Solution	10 ml x 1
Substrate Solution	10 ml x 1
TE Buffer	40 ml x 1
HRP-Streptavidin	25 µl x 1
Washing Buffer	1 pack
Filtration Tube	20 tubes
96-well Microplate/ U bottom	1 plate

Required Equipment & Materials

Microplate Reader (650 nm filter)
10 µl, 100-200 µl and 1 ml pipettes
50-250 µl multi-channel pipettes
Incubator(37°C)
0.5 ml and 1.5 ml tube
Centrifuge
Paper Towel

► Determination of the number of AP site in DNA

Day 1

1. Dilute 90 µl of the ARP-labeled genomic DNA with 310 µl of TE.
2. Add 60 µl of ARP-DNA Standard Solution per well. Use three wells per 1 standard solution.
3. Add 60 µl of the diluted ARP-labeled genomic DNA solution per well. Use at least three wells per 1 sample.
4. Add 100 µl of the DNA Binding Solution to each well, then allow the plate to remain at room temperature overnight.

Day 2

1. Prepare stock solutions
   - » Washing Buffer: Dissolve the contents of the Washing Buffer packet in 1 L of deionized or distilled water. Store this Washing Buffer solution at room temperature.
   - » HRP-Streptavidin solution: Dilute HRP-Streptavidin with Washing Buffer to prepare 1/4000 diluted working solution.*
   - » 1/4000 dilution preparation: Centrifuge HRP-Streptavidin tube for 30 seconds. Add 10 µl of HRP-Streptavidin into 40 ml of Washing Buffer solution, and mix well.

   * Since this working solution is not stable, always use freshly prepared solution.
2. Discard the DNA Binding Solution in the wells, and wash the well with 250 µl Washing Buffer 5 times.
3. Add 150 µl of diluted HRP-Streptavidin solution to each well, and incubate the plate at 37°C for 1 hour.
4. Discard the solution in the well, and wash the well with 250 µl Washing Buffer 5 times.[b)]
5. Add 100 µl of Substrate Solution to each well, and incubate at 37°C for 1 hour.
6. Measure the O.D. at 650 nm within 1 hour after the incubation is finished, and prepare a calibration curve using the data obtained with ARP-DNA Standard solutions.
7. Determine the number of abasic sites in the genomic DNA using the calibration curve.

3. Notes

1. Please store the kit at 0-5°C. Do not freeze. Store Washing Buffer solution at room temperature.
2. AP-DNA is not stable. Please treat it with ARP and purify with Filtration Tube after the isolation of genomic DNA from a sample.
3. Purified ARP-DNA solution in TE buffer is stable over one year at 0-5°C storage.
4. After the spinning of Filtration Tube for ARP-labeled DNA purification, add 200 µl TE immediately. If the DNA stays in Filtration Tube for more than 30 minutes after the spinning, the DNA recovery ratio may decline.
5. γ-Ray-sterilized tubes may cause DNA binding on the surface of the tube during the mixing of the DNA solution with DNA Binding Solution. If you prefer to mix ARP-DNA solution with DNA Binding Solution in a tube rather than mixing them in a well, please avoid using g-ray-sterilized tubes.
6. If the 650 nm filter is not available for the measurement of O.D. after the color development, transfer 50 µl of the solution in each well to a well of a new plate (not provided). Then, add 50 µl of 1 M sulfuric acid, and measure the O.D. at 450 nm.
7. Remaining solution in a well may cause error, so please remove the solution thoroughly by tapping the plate on a paper towel in each step.

4. References

1. T. Lindahl, *et al*., Rate of Depurination of Native Deoxyribonucleic Acid. *Biochemistry*. 1972;**11**:3610-3618.
2. M. Liuzzi, *et al*., A New Approach to the Study of the Base-excision Repair Pathway Using Methoxyamine. *J Biol Chem*. 1985;**260**:5252-5258.
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5. B. X. Chen, *et al*., Properties of a Monoclonal Antibody for the Detection of Abasic Sites, a Common DNA Lesion. *Mutat Res*. 1992;**273**:253-261.
6. J. A. Gralnick, *et al*., The YggX Protein of Salmonella enterica Is Involoved in Fe(II) Trafficking and Minimizes the DNA Damage Cause by Hydroxyl Radicals:Residue CYS-7 is Essential for YggX Function. *J Biol Chem*. 2003;**278**:20708-20715.

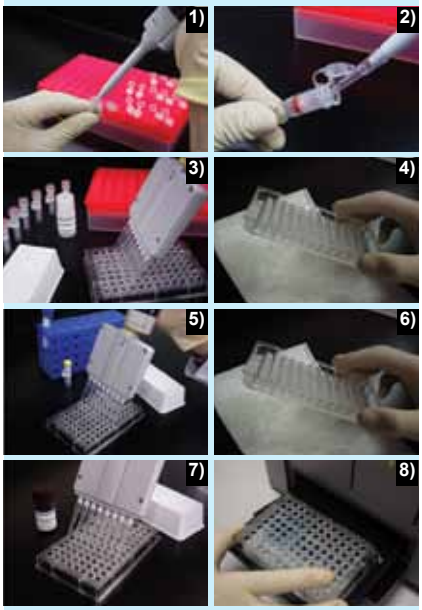


Fig. 2 Assay procedure

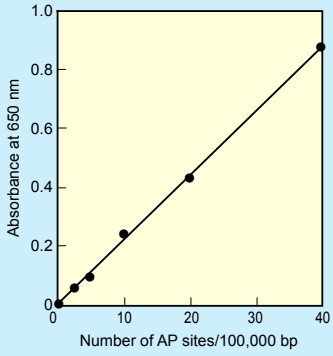


Fig. 3 Typical calibration curve of DNA Damage Quantification Kit

5. Recent Publications

Title	Reference
Novel Role of Base Excision Repair in Mediating Cisplatin Cytotoxicity	A. Kothandapani, and S. M. Patrick, *et al., J. Biol. Chem*. 2011; **286**: 14564 - 14574
Cadmium affects metabolic responses to prolonged anoxia and reoxygenation in eastern oysters (Crassostrea virginica)	I. O. Kurochkin, and I. M. Sokolova, *et al., Am J Physiol Regulatory Integrative Comp Physiol*. 2009; **297**: R1262 - R1272
Impairment of APE1 Function Enhances Cellular Sensitivity to Clinically Relevant Alkylators and Antimetabolites	Daniel R., and D. M. Wilson, *et al., III Mol. Cancer Res*. 2009; **7**: 897 - 906
Temporary Pretreatment With the Angiotensin II Type 1 Receptor Blocker, Valsartan, Prevents Ischemic Brain Damage Through an Increase in Capillary Density	Jian-Mei Li, and M. Horiuchi, *et al., Stroke*. 2008; **39**: 2029 - 2036
Bcl2 Inhibits Abasic Site Repair by Down-regulating APE1 Endonuclease Activity	J. Zhao, and X. Deng, *et al., J. Biol. Chem.* 2008; **283**: 9925 - 9932
Cockayne syndrome B protein stimulates apurinic endonuclease 1 activity and protects against agents that introduce base excision repair intermediates	H. Wong, and D. M. Wilson, *et al., III Nucleic Acids Res.* 2007; **35**: 4103 - 4113
Angiotensin II-Induced Neural Differentiation via Angiotensin II Type 2 (AT2) Receptor-MMS2 Cascade Involving Interaction between AT2 Receptor-Interacting Protein and Src Homology 2 Domain-Containing Protein-Tyrosine Phosphatase 1	Jian-Mei Li, and M. Horiuchi, *et al., Mol. Endocrinol.* 2007; **21**: 499 - 511
A Dominant-Negative Form of the Major Human Abasic Endonuclease Enhances Cellular Sensitivity to Laboratory and Clinical DNA-Damaging Agents	Daniel R. McNeill and David M. Wilson, *III Mol. Cancer Res*. 2007; **5**: 61 - 70
Bcl2 Suppresses DNA Repair by Enhancing c-Myc Transcriptional Activity	Z. Jin, and X. Deng, *et al., J. Biol. Chem.* 2006; **281**: 14446 - 14456
Folate Deficiency Increases Postischemic Brain Injury	M. Endres, and K. Gertz, *et al., Stroke*. 2005; **36**: 321 - 325

6. FAQ

►Can I use single-stranded DNA or RNA?
No, you cannot use this kit to determine the number of abasic sites in single-stranded DNA or RNA. The O.D. reading of single-stranded DNA will be nearly twice that of double-stranded DNA because of the binding efficiency on the microplate.

►How should genomic DNA be stored?
Prepare a DNA pellet and store at -20°C or -80°C if the DNA cannot be labeled with ARP immediately after isolation. After ARP labeling, the sample can be stored at 4°C in TE Buffer for several months.

►How should I prepare the DNA?
You can use general protocols or commercially available DNA isolation kits. Between 2 to 4 abasic sites per 1 x 10^5 base pairs will be created during the DNA isolation process. Therefore, use the same isolation method to prepare each DNA sample.

►How can I determine the number of abasic sites if there are more than 40 per 1 x 10^5 base pairs?
Simply dilute the ARP-labeled sample DNA with 0.5 µg per ml double-stranded genomic DNA, such as calf thymus or salmon sperm DNA, using TE Buffer.

►What should I do if the sample DNA concentration is less than 100 µg per ml?
You can either use a filtration tube to concentrate your sample DNA or ethanol precipitation to recover DNA as a pellet and then re-dissolve it to prepare a 100 µg per ml solution.

►What should I do if the sample DNA is less than 1 µg?
Add the same volume of ARP Solution and follow the manual. The recovery of the ARP-labeled DNA may be lower than the usual reactions, so measure the ARP-labeled DNA solution. The average recovery rate of the 0.5 µg DNA and 0.25 µg DNA is 70% and 50%, respectively.

Nitrated base of DNA and RNA detection

Anti-Nitroguanosine Antibodies

8-Nitroguanine is a nitrated base of DNA and RNA. It is formed by peroxynitrite, which is generated from nitric oxide and superoxide anion radical. It is known that a large amount of nitric oxide molecules and superoxide anion, generated by inflammation, causes nitration of guanosine. Since chemically modified nucleotides cause mutation during DNA replication, 8-Nitroguanine is thought to be a marker of DNA damage related to mutation and cancer.

Anti-Nitroguanosine antibodies have been developed jointly by Dr. Akaike at Kumamoto University and Dojindo Laboratories.

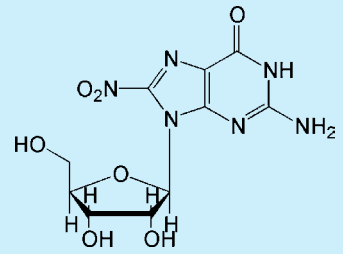


Fig. 1 Structure of 8-Nitroguanosine

Nitrated base of DNA and RNA detection

Anti-Nitroguanosine monoclonal antibody (Clone# NO_2G52)

Product Code: AB02

Because of its very high specificity, monoclonal antibody NO_2G52 recognizes 8-nitroguanine and 8-nitroguanosine, but it does not cross-react with normal nucleotide bases, 8-hydroxyguanine 8-hydroxydeoxy-guanosine, 3-nitrotyrosine, xanthine, or 2-nitroimidazole.

The specificity of NO_2G52 was determined by a competitive ELISA using an 8-nitroguanosine-BSA-coated plate. As shown in the figures below, NO_2G52 has very high affinity for 8-nitroguanine and 8-nitroguanosine, and it slightly cross-reacts with 8-bromoguanosine, 8-bromoguanine, and 8-chloroguanine.

Table 1 Reactivity of monoclonal antibody

strongly react(10 µmol/l)			
8-NO_2-guanosine	8-NO_2-guanine	8-NO_2-cGMP	8-NO_2-Xanthine
slightly react(>1 mmol/l)			
8-Br-guanosine	8-Br-guanine	8-Br-cGMP	8-Cl-guanine
no reaction			
guanosine	guanine	8-OH-guanine	
cytosine	xanthine	adenine	
adenosine	thymine	deoxythymidine	
uracil	uridine	3-NO_2-tyrosine	
2-NO_2-imidazole	8-OH-deoxyguanosine		

1. Specification

- ► Species: mouse(BALB/c)
- ► Clonality: monoclonal
- ► Isotype: IgG1
- ► Concentration: 1 mg/ml in PBS, contains 0.1% ProClin as a preservative

Type: IgG
Unit: 50 µg

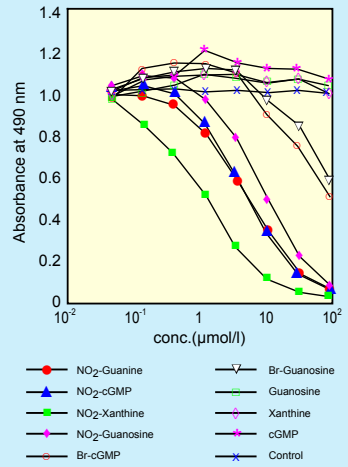


Fig. 2 Reactivity of Anti-Nitroguanosine monoclonal antibody$NO_2G52(IC_{50})$

Nitrated base of DNA and RNA detection

Anti-Nitroguanosine polyclonal antibody

Product Code: AB01

Anti-Nitroguanosine polyclonal antibody also recognizes 8-nitroguanine and 8-nitroguanosine, but it does not cross-react with normal guanosine, guanine, 8-hydroxyguanine, or 3-nitrotyrosine. Since this antibody was prepared using rabbits, it can be used for immuno-histostaining of rodent tissues such as mice or rats.

Table 2 Reactivity of polyclonal antibody

strongly react(10 µmol/l)		
8-NO_2-guanosine	8-NO_2-guanine	
no reaction		
guanosine	guanine	8-OH-guanine
3-NO_2-tyrosine		

Type: IgG
Unit: 50 µg

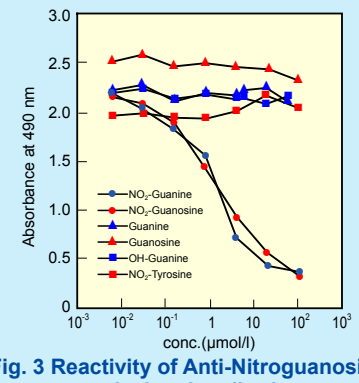


Fig. 3 Reactivity of Anti-Nitroguanosine polyclonal antibody

1. Example of Immunostaining(Fig. 4)

► Sample
Influenza virus-infected mouse lung

► Immunostaining

1. Fix the mouse lung with 2% periodate-lysine-paraformaldehyde.
2. Add anti-nitroguanosine antibody(10 µg/ml) to the lung sample.
3. Add alkaline phosphatase-conjugated secondary antibody.
4. Stain the sample with Vector red substrate kit I.

2. Notes

1. Freeze and thaw cycles can cause degradation of the antibody. After opening, store in the refrigerator.
2. If 8-Nitroguanosine staining was observed with polyclonal antibody, it is reccomended to confirm the experimental verification as follows,
   » no staining is observed by the competition with 8-Nitroguanine standard
   » no staining is observed by treating the sample with reducing agent, such as Sodium hydrosulfite
3. Monoclonal antibody can be used on human samples due to the high activity and selectivity.

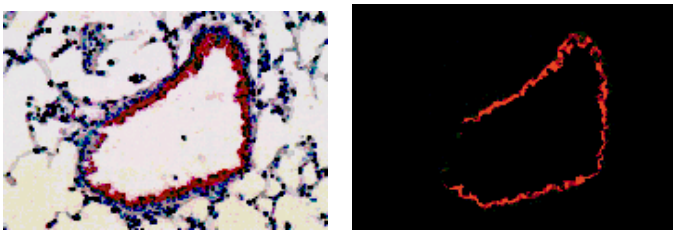


Fig. 4 Tissue staining with Anti-Nitroguanosine antibody

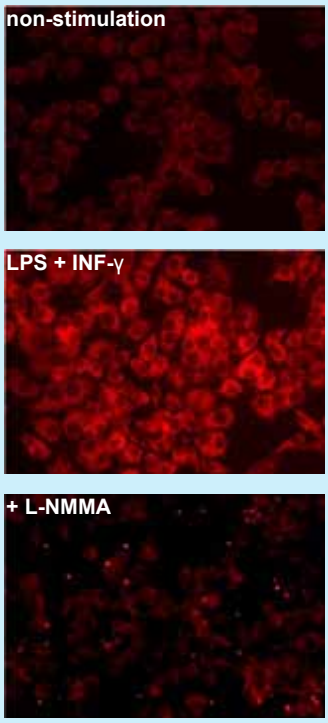


Fig. 5 Detection of guanine nitration on RAW264.7 cell

3. Specification

► Species: mouse(BALB/c)
► Clonality: monoclonal
► Isotype: IgG1
► Concentration: 1 mg/ml in PBS, contains 0.1% ProClin as a preservative

4. References

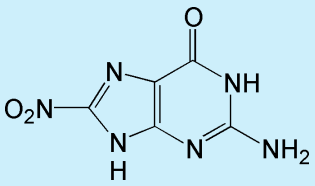
1. T. Akaike, *et al*., 8-nitroguanosine formation in viral pneumonia and its implication for pathogenesis, *PNAS*. 2003;**100**:685-690.
2. J. Yoshitake, *et al*., Nitric oxide as an endogenous mutagen for Sendai virus without antiviral activity. *J Virol*. 2004;**78**:8709-8719.
3. T. Sawa, *et al*., Protein S-guanylation by the biological signal 8-nitroguanosine 3',5'-cyclic monophosphate. *Nat Chem Biol*. 2007;**3**:727-735.
4. M. H. Zaki, *et al*., Cytoprotective function of heme oxygenase 1 induced by a nitrated cyclic nucleotide formed during murine salmonellosis. *J Immunol*. 2009;**182**:3746-3756.
5. Y. Terasaki, *et al*., Guanine nitration in idiopathic pulmonary fibrosis and its implication for carcinogenesis. *Am J Respir Crit Care Med*. 2006;**174**:665-673.
6. T. Sawa, *et al*., Analysis of urinary 8-nitroguanine, a marker of nitrative nucleic acid damage, by high-performance liquid chromatography-electrochemical detection coupled with immunoaffinity purification: association with cigarette smoking. *Free Radic Biol Med*. 2006;**40**:711-720.

Standard agent of 8-Nitroguanine detection

8-Nitroguanine(lyophilized)

Product Code: N455

8-Nitroguanine (lyophilized) is made by the lyophilization of its phosphate buffered saline solution, and is used in immunohistochemistry for absorption testing. Adding 0.4 ml of distilled water to the 8-Nitroguanine powder produces a 1.2 mmol/l of 8-Nitroguanine solution. 8-Nitroguanine/PBS solution is stable for one month at 4°C. If an antibody pre-treated with excessive 8-Nitroguanine shows negative staining, then the subsequent positive staining with this antibody will be specific for 8-nitroguanine or 8-nitroguanosine formed in DNA or RNA.



8-Nitroguanine(lyophilized)
$C_5H_4N_6O_3$ = 196.12
Unit: 100 µg

Detection of lipid peroxide by fluorescent microscopy and flowcytometry

Liperfluo

Product Code: L248

Liperfluo, a perylene derivative containing oligooxyethylene, is designed and exclusively developed by Dojindo for a detection of lipid peroxides and emits intense fluorescence by a lipid peroxide specific oxidation in organic solvents such as ethanol. Among fluorescent probes that detect Reactive Oxigen Species(ROS), Liperfluo is the only compound that can specifically detect lipid peroxides. Since the excitation and emission wavelengths of the oxidized Liperfluo are 524 nm and 535 nm, respectively, both a photo-damage against a sample and an auto-fluorescence from the sample can be minimized. The tetraethyleneglycol group linked to one end of diisoquinoline ring helps its solubility and dispersibility to aqueous buffer. Though Liperfluo oxidized form is almost nonfluorescent in an aqueous media, it emits fluorescence in lipophilic sites such as in cell membranes. Therefore it can easily be applied to lipid peroxide imaging by a fluorescence microscopy and a flow cytometric analysis for living cells.

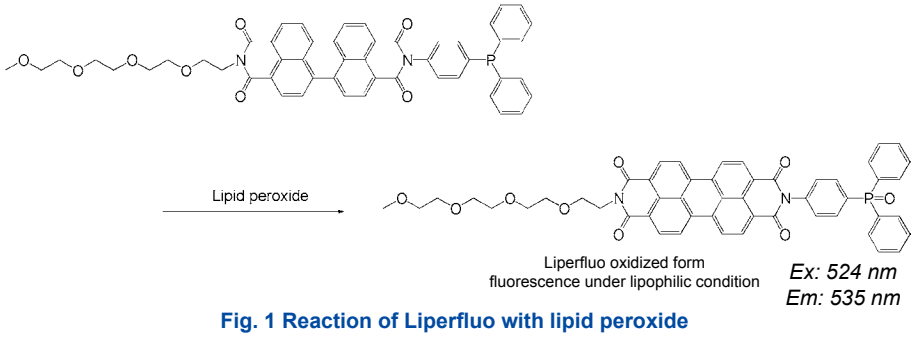


Fig. 1 Reaction of Liperfluo with lipid peroxide

1. General Protocol

1. Add 60 µl of DMSO to a vial containing Liperfluo(50 µg) and dissolve the product (concentration: 1 mmol/l).
   - If it is hard to dissolve it by pipetting, use vortex mixer, sonicator or warm the solution.
   - Cover the solution with an aluminum foil and use it within a day after the reconstition.
2. Add 10 µl of the Liperfluo solution to 1 ml of cell culture containing 1.0 x 10^5 cells.
   - Higher final concentration of DMSO may cause damage to cells.
   - Since the background fluorescence may be increased in culture medium, replacing the medium with an aqueous buffer such as PBS is recommended before the addition of Liperfluo solution.
3. Incubate the cell suspension at 37°C for 30 minutes.
4. Analyse the cells with a fluorescence microscope or a flow cytometer.
   - Although the Liperfluo oxidized form is almost non-fluorescent in an aqueous solution, wash the cells with PBS as necessary if the background fluorescence is high.

2. Live cell imaging of lipid peroxide (Fig. 4)

1. Innocurate SH-SY5Y cells(6.0 x 10^5 cells/well) to a 6-well plate.
2. Incubate the plate at 37 ºC for overnight.
3. Add Liperfluo, DMSO solution(final conc. 20 µM) and incubate at 37 ºC for 15 minutes.
4. Add either Cumene Hydroperoxide(final conc. 100 µM) or AIPH*(final conc. 6 mM).
5. Incubate at 37ºC for 2 hours.
6. Observe fluorescent by microscope**(Ex. 524 nm, Em. 535 nm).

* AIPH: 2,2'-azobis-[2-(2-imidazolin-2-yl)propane]dihydrochloride

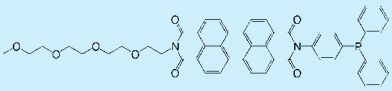
** Olympus IX-71 epifluorescent microscope, mirror unit: U-MNIBA3, exposure time: 10 sec, ISO: 800

3. References

1. K. Yamanaka, *et al*., A novel fluorescent probe with high sensitivity and selective detection of lipid hydroperoxides in cells, RSC Advances, 2012, **2**, 7894.
2. N. Soh, *et al*., Novel fluorescent probe for detecting hydroperoxides with strong emission in the visible range. *Bioorg Med Chem Lett*. 2006;**16**:2943-2946.
3. N. Soh, *et al*., Swallow-tailed perylene derivative: a new tool for fluorescent imaging of lipid hydroperoxides. *Org Biomol Chem*. 2007;**5**:3762-3768.

4. Specification

- ► Appearance: reddish black crystalline powder
- ► Purity: ≥90.0%(HPLC)



Liperfluo

N-(4-Diphenylphosphinophenyl)-N'-(3,6,9,12-tetraoxatridecyl)perylene-3,4,9,10-tetracarboxydiimide

$C_{51}H_{41}N_2O_8P$ = 840.85,

Unit: 50 µg x 5

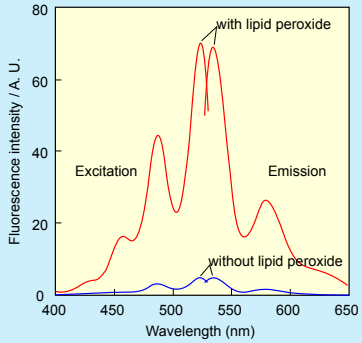


Fig. 2 Excitation and emission spectra of Liperfluo with or without lipid peroxide in ethanol.

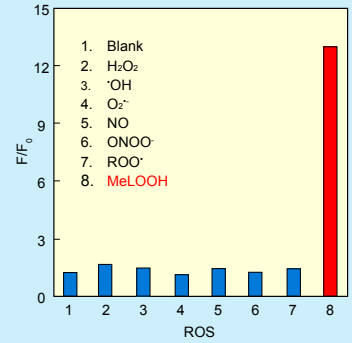


Fig. 3 Reaction selectivity of Liperfluo against the various reactive oxygen species.

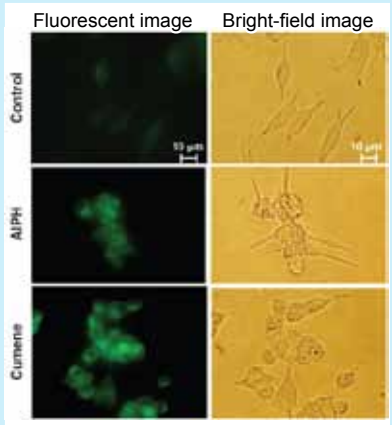


Fig. 4 Reaction selectivity of Liperfluo against the various reactive oxygen species.

Data was kindly provided from Dr. N. Noguchi, Doshisha University, System Life Science Laboratory.

Detection of lipid peroxide by HPLC

DPPP

Product Code: D350

Lipid peroxides are derived from unsaturated lipids, phospholipids, glycolipids and cholesterol esters. In food industry, lipid peroxides have been considered as one of a major cause of food deterioration. Meanwhile, there are many ongoing studies today that investigate the mechanism of lipid oxidation in human diseases, disorders, and aging. Hence, measuring amount of lipid peroxide in biological samples is significant, and an accurate method for detecting low level of lipid peroxides is eagerly anticipated. TBARS(Thiobarbituric acid reactive substances) assay is a well-established method and widely used for measuring lipid peroxidation. However, MDA(Malondialdehyde), one of a end-product generated in the lipid peroxidation, is not reflected in actual level of peroxidation because there are other source of MDA.
DPPP, Diphenyl-1-pyrenylphosphine, was developed by Dr. Meguro, *et al*. as a fluorescent probe for detecting lipid peroxide. It selectively reacts with hydroperoxides to generate DPPP oxide that emits fluorescence at 380 nm(ex: 352 nm), and makes it possible to quantify 0.1 to 7 nmol of hydroperoxide. In addition, the range of 1 to 2 pmol of lipid peroxide can be selectively detected with the combination of HPLC separation and the post-column reaction with DPPP.

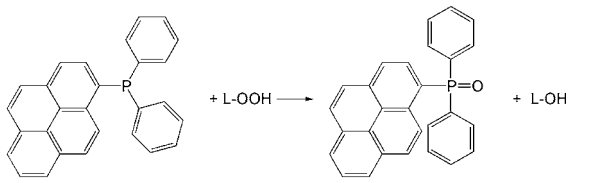
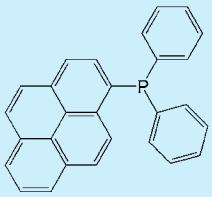


Fig. 1 Reaction scheme of DPPP with hydroperoxide



DPPP
Diphenyl-1-pyrenylphosphine
$C_{28}H_{19}P$ = 386.42
CAS No. [110954-36-4]
Unit: 10 mg

1. General Protocol

► HPLC detection of hydroperoxides in plasma sample
1. Prepare 10 mg/ml BHT(Butyl hydroxytoluene)/$CHCl_3$-Methanol(2:1) solution.
2. Dissolve a sample in 100 µl of the solution above.
3. Add 50 µl of DPPP solution(1 mg/10 ml in $CHCl_3$:Methanol=1:1) to the solution prepared in step 2.
4. Incubate the solution under dark condition for 60 minutes at 60°C.
5. Cool down the solution and measure the fluorescence intensity by HPLC.

► Determination of hydroperoxides on cell membrane(*in vivo*)
1. Disolve DPPP in DMSO and prepare 5 mM DPPP/DMSO solution.
2. Add the solution above to cell suspension(1 x 10^7 cells/ml) to the final concentration of 50 µM DPPP.
3. Incubate the cells at 37°C for 10 minutes.
4. Wash the cells twice with Hank's solution.
5. Stimulate the cells by adding H_2O_2 or Methyl linoleate hydroperoxide and measure flueorescence intensity.

2. References

1. K. Akasaka, *et al*., An Aromatic Phosphine Reagent for the HPLC-fluorescence Determination of Hydroperoxides -Determination of Phosphatidylcholine Hydroperoxides in Human Plasma. *Anal Lett*. 1988;**21**:965-975.
2. K. Akasaka, *et al*., Normal-phase High-performance Liquid Chromatograohy with a Fluorimetric Postcolumn Detection System for Lipid Hydroperoxides. *J Chromatogr A*. 1993;**628**:31-35.
3. Y. Okimoto, *et al*., A Novel Fluoresceint Probe Diphenyl-pyrenylphosphine to Follow Lipid Peroxidation in Cell Membranes. *FEBS Lett*. 2000;**474**:137-140.

3. Specification

► Appearance: slightly yellow powder
► Purity: ≥97.0%(HPLC)

4. Recent Publications

Title	Reference
Dysregulation of very long chain acyl-CoA dehydrogenase coupled with lipid peroxidation	Y. Kabuyama, *et al., Am J Physiol Cell Physiol*. 2010; **298**: C107 - C113.
Cell Death Caused by Selenium Deficiency and Protective Effect of Antioxidants	Y. Saito, *et al., J. Biol. Chem*. 2003; **278**: 39428 - 39434.
Induction of 1-cys peroxiredoxin expression by oxidative stress in lung epithelial cells	Han-Suk Kim , *et al., Am J Physiol Lung Cell Mol Physiol*. 2003; **285**: L363 - L369.
An Antisense Oligonucleotide to 1-cys Peroxiredoxin Causes Lipid Peroxidation and Apoptosis in Lung Epithelial Cells	Jhang Ho Pak, *et al., J. Biol. Chem*. 2002; **277**: 49927 - 49934.
1-Cys peroxiredoxin overexpression protects cells against phospholipid peroxidation-mediated membrane damage	Yefim Manevich, *et al., PNAS*. 2002; **99**: 11599 - 11604.

Detection of protein or DNA radical by WB, ELISA, and imaging

DMPO

Product Code: D048

Immuno-spin Trapping method was developed for detecting DNA and Protein radicals in biological analysis. ROS (Reactive Oxygen Species) produces modification of the structure and function of biomolecules that relate on the cause of variety diseases. To understand the mechanism of oxidative reactions, it is very important to analyze which molecules are involved in the oxidation process.
DMPO is the most popular spin-trapping reagent that traps radicals in protein and DNA samples. The DMPO-Protein or DMPO-DNA nitrone adducts are determined using a ELISA, Western Blotting, Mass Spectorometry, Imaging, and so on.
The purity of Dojindo's DMPO is higher than another commercialized DMPO. Since it does not contain impurities that might cause high background. Dojindo's DMPO does not require any pre-purification steps.

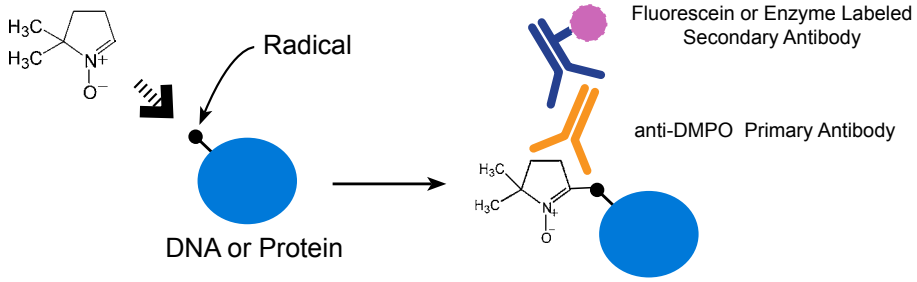


Fig. 1 Principle of Immuno-spin Trapping Method

1. Protocol Example : Radical DNA Detection

Referred Publication

Detection and imaging of the free radical DNA in cells--site-specific radical formation induced by Fenton chemistry and its repair in cellular DNA as seen by electron spin resonance, immuno-spin trapping and confocal microscopy. Bhattacharjee S, Chatterjee S, Jiang J, Sinha BK, Mason RP., *Nucleic Acids Res.* 2012, **40**, 5477-86

► Evaluation of radical DNA by ELISA

1. Treate RAW cells (1-3 x10^6 cells) with 100 µM $CuCl_2$,100 µM H_2O_2, and 100 mM DMPO and incubate for 12-15 hours at 5% CO_2 incubator.
2. Extract DNA from RAW cells and dilute DNA to 5 µg/ml in PBS.
3. Add 25µl of DNA solution and 25µl of Reacti-Bind DNA coating solution in each well of the plate and incubate for 2–4 hours at 37°C.
4. Wash the wells once with washing buffer (PBS containing 0.05% non-fat dry milk and 0.1% Tween-20).
5. Block with blocking buffer (PBS containing 3% non-fat dry milk) for 2 hours at 37°C.
6. Detect DMPO-DNA radical adduct with anti-DMPO antibody and HRP-conjugated secondary antibody.
7. After three washes, add the Immobilon chemiluminescence substrate each well and measure the intensity of luminesscense.

► Another application in this paper
Cell Imaging

2. Protocol Example : Radical Protein Detection

Referred Publication

Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. Chen CA, Lin CH, Druhan LJ, Wang TY, Chen YR, Zweier JL., *J Biol Chem.* 2011, **286**,

► Evaluation of radical protein by cell imaging

1. Prepare Bovine aortic endothelial cells (10^4 cells) in 35-mm dishes.
2. Add 10 µM Menadione and 50 mM DMPO and incubate cells.
3. Wash the cells with PBS and fix them with 3.7% paraformaldehyde for 10 minutes.
4. Permeabilize the cells with 0.25% Triton X-100 in TBST(Tris buffered saline with Tween) for 10 minutes and block the cells with 5% goat serum in TBST.
5. Visualize DMPO-protein radical adduct with anti-DMPO antibody and fluorescein labeled secondary antibody.
6. Analyze protein radicals by fluorescent microscopy.

► Other applications in this paper
Immunoblotting, Mass Spectrometry, Immunoprecipitation

3. Specification

► Appearance: colorless liquid
► Purity: ≥99.0%(GC)
► ESR spectrum: to pass test
► IR spectrum: authentic

DMPO
5,5-Dimethyl-1-pyrroline *N*-oxide
$C_6H_{11}NO$ = 113.16
CAS No. [3317-61-1]
Unit: 1 ml

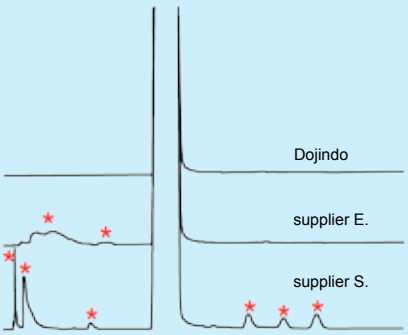


Fig. 2 Purity comparison in HPLC spectra
(*: impurities)

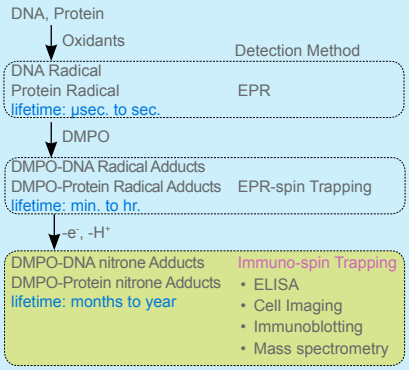


Fig. 3 Radical Detection Scheme

Detection of radical by EPR

DMPO

Product Code: D048

Because of potential cancer risks and their age-promoting effects, free radicals in living bodies have become a frequently studied subject. DMPO is the most frequently used spin-trapping reagent for the study of free radicals. It is suitable for trapping oxygen radicals, especially superoxides, and for producing adducts with characteristic EPR (ESR) patterns. However, most commercially available DMPO contains impurities that cause high backgrounds. Thus, DMPO requires further purification when running experiments on EPR. The quality of Dojindo's DMPO is well controlled and Dojindo's DMPO does not require any pre-purification process. There are no impurities to cause a background problem.

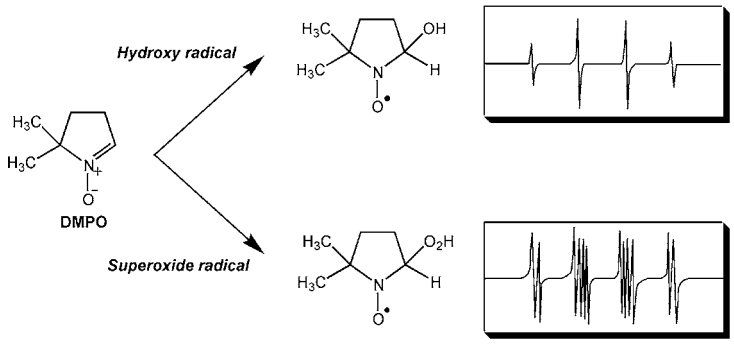
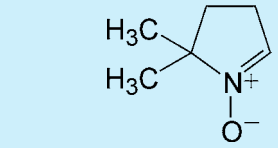


Fig. 1 ESR Spectera of DMPO Adducts



DMPO
5,5-Dimethyl-1-pyrroline *N*-oxide
$C_6H_{11}NO$ = 113.16
CAS No. [3317-61-1]
Unit: 1 ml

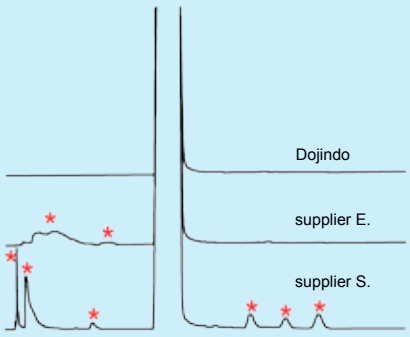


Fig. 2 Purity comparison in HPLC spectra
(*: impurities)

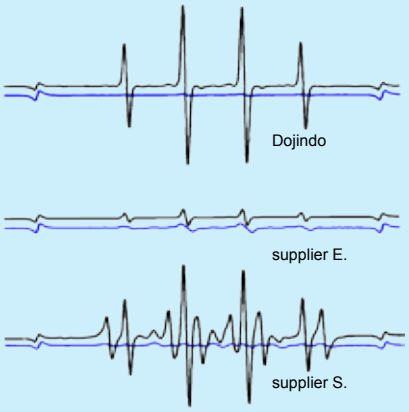


Fig. 3 Purity comparison of ESR spectra
(black: fenton reaction, blue: blank)

1. Protocol

► Evaluation of superoxide scavenging activities

1. Add 15 µl of DMPO and 50 µl of 5 mM hypoxanthine to 35 µl of 0.1 M Phosphate buffer(pH 7.8).
2. Add 50 µl of SOD standard or samples to be tested and voltex for 1-2 seconds.
3. Add 50 µl of 0.4 U/ml xanthine oxidase and voltex immediately.
4. Transfer the solution to ESR sample tube and measure ESR spectra after certain time of period, e.g. 1 minute.
5. Calculate relative intensity(DMPO-O_2^-/Mn^{2+}) from the peak height.

► C-, N-, and S-centered radicals Detection

1. Prepare a solution of 100 mM phosphate buffer (pH 7.4) containing 25 µM DTPA.
2. Make up a solution of the following peroxidase substrates: (A) 100 mM sodium formate (HCOONa); (B) 100 mM potassium cyanide (KCN); (C) 100 mM sodium azide (NaN_3); (D) 100 mM sodium sulfite (Na_2SO_3) in 100 mM phosphate buffer, pH 7.4.
3. Make up a solution of horseradish peroxidase with concentration of 4.0 mg/ml (~ 100 µM) and 1 mM solution of hydrogen peroxide (H_2O_2).
4. Make up a solution of DMPO with concentration of 1 M.
5. Prepare your reaction mixture to a total reaction volume of 200 µl and add 130 µl of buffer to an Eppendorf tube.
6. Add 20 µl DMPO of your 1 M DMPO solution, 20 µl of one of the substrates' stock solutions, 10 µl of 1 mM H_2O_2, and initiate the reaction with 20 µl HRP.
7. Vortex the tube, transfer the solution to a flat cell, and acquire the spectrum.
8. The final concentrations of the components are: 100 mM DMPO, 10 mM substrate (formate, cyanide, azide, sulfite), 50 µM H_2O_2, and 10 µM HRP.

*This protocol was kindly provided by Bruker Corporation.

2. Specification

Refer to previous page (Page 15)

3. Recent Publications

Title	Reference
Nonenzymatic displacement of chlorine and formation of free radicals upon the reaction of glutathione with PCB quinones	Yang Song, Brett A., and Garry R. Buettner, *et al., PNAS.* 2009; **106**: 9725 - 9730.
Manganese Superoxide Dismutase Modulates Hypoxia-Inducible Factor-1 Induction via Superoxide	Suwimol Kaewpila, and Larry W. Oberley, *et al., Cancer Res.* 2008; **68**: 2781 - 2788.
Hyperglycemia-Induced Reactive Oxygen Species Toxicity to Endothelial Cells Is Dependent on Paracrine Mediators	Julia V. Busik, and Maria B. Grant, *et al., Diabetes*, 2008; **57**: 1952 - 1965.
Overexpression of Extracellular Superoxide Dismutase Attenuates Heparanase Expression and Inhibits Breast Carcinoma Cell Growth and Invasion.	Melissa L.T., and Frederick E. Domann, *et al., Cancer Res.*, 2009; **69**: 6355 - 6363
Smoking Induces Bimodal DNA Damage in Mouse Lung	"Shunji Ueno and Kyosuke Temma, *Toxicol. Sci.* 2011; **120**: 322 - 330. "
Cardiac Myocyte–Specific Expression of Inducible Nitric Oxide Synthase Protects Against Ischemia/Reperfusion Injury by Preventing Mitochondrial Permeability Transition	Matthew B. and Aruni Bhatnagar, *et al., Circulation*. 2008; **118**: 1970 - 1978.

Detection of superoxide radical by EPR

BMPO

Product Code: B568

Spin trapping analysis is one of the most reliable techniques for detecting and identifying short-lived free radicals. The EPR (ESR) spin trap reagent detects both superoxide and hydroxyl radicals produced by systems *in vitro* and *in vivo*. BMPO was developed as a spin trapping reagent that adducts superoxide and shows a much longer half-life ($t_{1/2}$=24 min) than other spin trap reagents. It gives us reproducible and steady results. Because BMPO is highly soluble in water, hydrophilic sample is applicable for analyzing the free radicals.

1. General Protocol

► Measuring hydroxy radical from Fenton reaction

1. Add 15 µl of BMPO solution, 75 µl of 1 mM H_2O_2 and 75 µl of 100 µM $FeSO_4$ to 50 µl of ddH_2O.
2. Transfer the solution to ESR sample tube and measure ESR spectra after certain time of period, e.g. 1 minute.
3. Calculate relative intensity from the peak height.

► Measuring superoxide radical from xanthine oxidase(XO) reaction

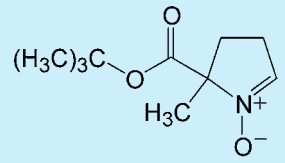
1. Dissolve 1 mg of BMPO with 1 ml of 50 mM Phosphate buffer(pH 7.4) (solution A).
2. Prepare 50 mM Phosphate buffer(pH 7.4) containing 1 mM DTPA and 0.4 mM Xanthine(solution B).
3. Prepare 50 mM Phosphate buffer(pH 7.4) containing 0.1 U/ml xanthine oxidase(solution C).
4. Mix 15 µl of solution A, 135 µl of solution B and 10 µl of solution C.
5. Transfer the solution to ESR sample tube and measure ESR spectra after certain time of period, e.g. 8 minutes.
6. Calculate relative intensity from the peak height.

2. References

1. H. Zhao, J. Joseph, H. Zhang, H. Karoui and B. Kalyanaraman, *Free Radic Biol Med*. 2001;**31**:599-606.
2. G. M. Rosen, P. Tsai, J. Weaver, S. Porasuphatana, L. J. Roman, A. A. Starkov, G. Fiskum and S. Pou, *J Biol Chem*. 2002;**277**:40275-40280.

3. Specification

► Appearance: white crystal or crystalline powder
► Purity: ≥99.0%(HPLC)



BMPO
5-*tert*-Butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide
$C_{10}H_{17}NO_3$ = 199.25
Unit: 50 mg

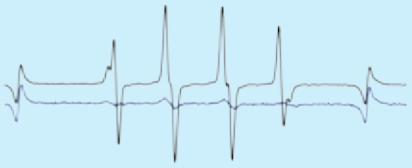


Fig. 1 ESR Spectra of hydroxy radical adduct
(black: fenton reaction, blue: blank)

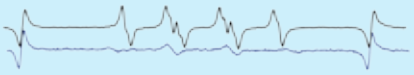


Fig. 2 ESR Spectra of superoxide radical adduct
(black: XO reaction, blue: blank)

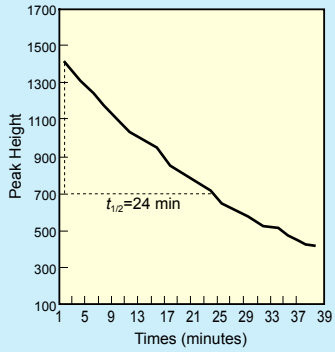


Fig. 3 Half-life of BMPO-superoxide radical adduct

NO Scavenger, NO detection by EPR

Carboxy-PTIO

Product Code: C348

Carboxy-PTIO is a stable, water-soluble organic radical that reacts with NO to form NO_2·. This reaction can be monitored by electron spin resonance (ESR). NO is an unstable molecule and has a complex reaction cascade for its metabolism in biological systems. Rapidly generated NO-related metabolites carry out various physiological activities. Commonly used NO scavengers such as hemoglobin trap NO; they also trap NOS inhibitors such as arginine derivatives. These NO scavengers also quench all other NO-related metabolites at the same time. In contrast, Carboxy-PTIO does not dramatically affect other NO-related product systems because it transforms NO to NO_2, which is a metabolite of NO. Thus, Carboxy-PTIO can be used to investigate the effects of NO separately from its downstream metabolites. Dr. Akaike and others showed that Carboxy-PTIO suppresses relaxation of the rat aorta ring, which is induced by acetylcholine, twice as effectively as NG-nitroarginine. Dr. Yoshida and others reported that downstream metabolites of NO, generated by treatment with Carboxy-PTIO, have an increased antiviral activity compared to NO alone. The NO metabolites play important roles in biological systems; therefore, they should be investigated separately from NO.

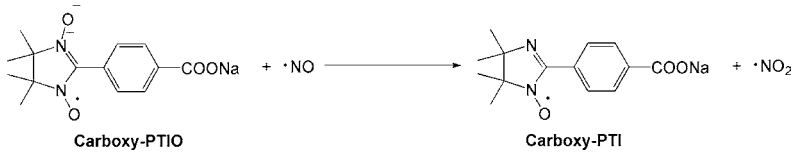


Fig. 1 Reaction of NO trapping

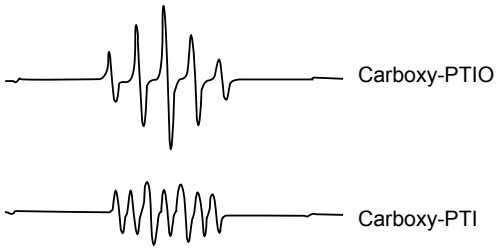
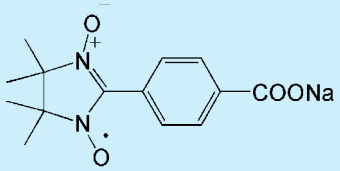


Fig. 2 ESR Spectra of Carboxy-PTIO and Carboxy-PTI



Carboxy-PTIO
2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, sodium salt
$C_{14}H_{16}N_2NaO_4$ = 299.28
CAS No. [148819-93-6]
Unit: 10 mg

2. References

1. E. F. Ullman, *et al*., Studies of Stable Free Radicals. X. Nitronyl Nitroxide Monoradicals and Biradicals as Possible Small Molecule Spin Labels. *J Am Chem Soc*. 1972;**94**:7049-7059.
2. Y. Miura, *et al*., Polymers Containing Stable Free Raficals, 5. Preparation of a Polymer Containing Imidazoline 3-Oxide 1-Oxyl Groups. *Macromol Chem Phys*. 1973;**172**:233-236.
3. K. Inoue, *et al*., Magnetic Properties of the Crystals of *p*-(1-Oxyl-3-Oxide-4, 4, 5, 5-Tetramethyl-2-Imidazolin-2-Yl)Benzoic acid and Its Alkali Metal Salts. *Chem Phys Lett*. 1993;**207**:551-555.
4. T. Akaike, *et al*., Antagonistic Action of Imidazolineoxyl N-Oxides Against Endothelium-Derived Relaxing Factor/NO Through a Radical Reaction. *Biochemistry*. 1993;**32**:827-832.
5. J. Joseph, *et al*., Trapping of Nitric Oxide by Nitronyl Nitroxides: an Electron Spin Resonance Investigation. *Biochem Biophys Res Commun*. 1993;**192**:926-934.
6. M. Yoshida, *et al*., Therapeutic Effects of Imidazolineoxyl N-Oxide Against Endotoxin Shock Through Its Direct Nitric Oxide-scavenging Activity. *Biochem Biophys Res Commun*. 1994;**202**:923-930.
7. T. Az-Ma, *et al*., Reaction Between Imidazolineoxil N-Oxide(Carboxy-PTIO) and Nitric Oxide Released from Cultured Endothelial Cells:Quantitative Measurement of Nitric Oxide by ESR Spectrometry. *Life Sci*. 1994;**54**:PL185-PL190.
8. H. Maeda, *et al*., Multiple Functions of Nitric Oxide in Pathophysiology and Microbiology: Analysis by a New Nitric Oxide Scavenger. *J Leukoc Biol*. 1994;**56**:588-592.

3. Specification

- Appearance: dark blue powder
- Purity: ≥97.0%(TLC)

4. Recent Publications

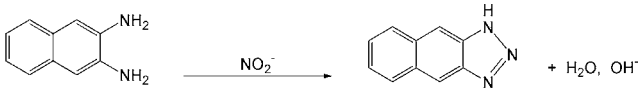
Title	Reference
Effect of Hypoxia on Susceptibility of RGC-5 Cells to Nitric Oxide	Takaki Sato and Tsunehiko Ikeda, *et al., Invest. Ophthalmol. Vis. Sci.* 2010; **51**: 2575 - 2586.
Upregulation of Nitric Oxide Production in Vascular Endothelial Cells by All-trans Retinoic Acid Through the Phosphoinositide 3-Kinase/Akt Pathway	Akira Uruno and Sadayoshi Ito, *et al., Circulation*. 2005; **112**: 727 - 736.
The Nitric Oxide–cGMP Pathway Controls the Directional Polarity of Growth Cone Guidance via Modulating Cytosolic Ca2+ Signals	Takuro Tojima and Hiroyuki Kamiguchi, *et al., J. Neurosci.* 2009; **29**: 7886 - 7897.
Nitric Oxide Transiently Converts Synaptic Inhibition to Excitation in Retinal Amacrine Cells	Brian Hoffpauir and Evanna Gleason, *et al., J Neurophysiol*. 2006; **95**: 2866 - 2877.

Detection of NO_2^- by fluorometric analysis

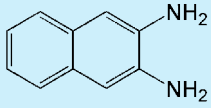
2,3-Diaminonaphthalene (for NO detection)

Product Code: D418

The Griess assay is a simple and popular method for detecting NO concentration. 2,3-Diaminonaphthalene (DAN) is a highly sensitive alternative to the Griess assay. The DAN method is 50-100 times more sensitive than the Griess assay: While the detection limit of the Griess assay is 1 mM, the limit of the DAN method is 10-50 nM. DAN reacts with NO_2^- in acidic conditions to produce fluorescent naphthalenetriazole. The wavelength of the emission maximum of naphthalenetriazole is 410 nm. However, detection at 450 nm is recommended to avoid fluorescent blanks and increase sensitivity. The fluorescent background of DAN is low for maximum sensitivity. The optimal reaction conditions of DAN with NO_2^- have been determined. The reaction should proceed at pH 2 at room temperature for 5 minutes, and the resulting fluorescence of naphthalenetriazole should be determined at a pH of 10 or more. DAN is a photosensitive reagent and sometimes becomes dark brown colored crystals. Since this brown product cannot be utilized for the fluorescent detection, recrystallization is necessary.



2,3-Diaminonaphthalene *Naphthalenetriazole*



2,3-Diaminonaphthalene(for NO detection)
2,3-Diaminonaphthalene
$C_{10}H_{10}N_2$ = 158.20
CAS No. [771-97-1]
Unit: 10 mg

1. General Protocol

1. Dissolve 50 µg DAN in 1 ml 0.62 M HCl to prepare 0.31 mM DAN solution.[a)]
2. Mix 10 µl DAN solution with 100 µl $NaNO_2$ solution (0-10 mM) or sample solution.
3. Incubate the mixture at room temperature for 10-15 minutes.
4. Add 5 µl 2.8 M NaOH solution to the reaction solution.[b)]
5. Dilute 100 µl of this solution with 4 ml water, followed by fluorescent measurement with excitation wavelength at 365 nm and emission wavelength at 450 nm.
6. Prepare a calibration curve using this data where the X-axis is $NaNO_2$ concentration and the Y-axis is fluorescence intensity. Then use this calibration curve to determine the NO_2 concentration of the sample solution.
   a) Acidic conditions are required for a rapid reaction.
   b) Basic conditions (pH 10 or higher) are required for a high fluorescence signal.

2. References

1. W. R. Tracey, *et al.*, Comparison of spectrophotometric and biological assays for nitric oxide (NO) and endothelium-derived relaxing factor (EDRF): nonspecificity of the diazotization reaction for NO and failure to detect EDRF. *J Pharmacol Exp Ther.* 1990;**252**:922-928.
2. J. S. Pollock, *et al.*, Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *PNAS.* 1991;**88**:10480-10484.
3. S. Archer, Measurement of nitric oxide in biological models. *FASEB J.* 1993;**7**:349-360.
4. J. H. Wiersma, 2,3-Diaminonaphthalene as a Spectrophotometric and Fluorometric Reagent for the Determination of Nitrite Ion. *Anal Lett.* 1970;**3**:123-132.
5. C. R. Sawicki, Fluorimetric Determination of Nitrate. *Anal Lett.* 1971;**4**:761-775.
6. P. Damiani, *et al.*, Fluorometric determination of nitrite. *Talanta.* 1986;**8**:649-652.
7. T. P. Misko, *et al.*, A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem.* 1993;**214**:11-16.
8. G. L. Wheeler, et al., Rapid determination of trace amounts of selenium (IV), nitrite, and nitrate by high-pressure liquid chromatography using 2,3-diaminonaphthalene. *Microchem J.* 1974;**19**:390-405.

3. Specification

- ► Appearance: white or pale yellowish-brown powder
- ► Melting Point: 185°C to 200°C

4. Recent Publications

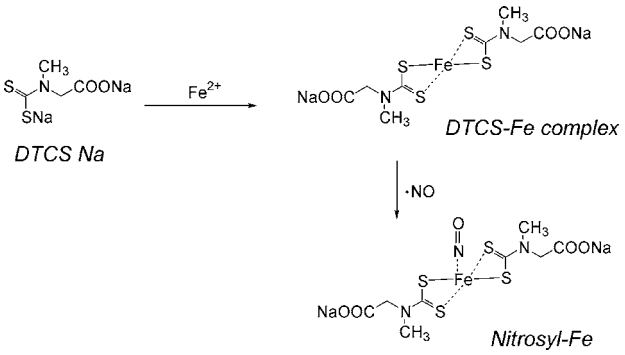
Title	Reference
Role of the Sphingosine Rheostat in the Regulation of Cnidarian-Dinoflagellate Symbioses	Olivier Detournay , *Biol. Bull.* 2011; **221**: 261 - 269.
TAK-242 (Resatorvid), a Small-Molecule Inhibitor of Toll-Like Receptor (TLR) 4 Signaling, Binds Selectively to TLR4 and Interferes with Interactions between TLR4 and Its Adaptor Molecules	N. Matsunaga, *et al., Mol. Pharmacol.* 2011; **79**: 34 - 41.
A Novel Cyclohexene Derivative, Ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242), Selectively Inhibits Toll-Like Receptor 4-Mediated Cytokine Production through Suppression of Intracellular Signaling	M li, *et al., Mol. Pharmacol.* 2006; **69**: 1288 - 1295.

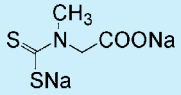
Detection of NO by EPR

DTCS Na

Product Code: D465

Diethyldithiocarbamate (DETC) is a good spin-trapping reagent for nitric oxide in vivo. However, DETC has not been widely utilized for NO detection in biological samples due to its poor water solubility. DTCS, an analog of DETC, forms a water-soluble iron(II) complex (Fe-DTCS). The Fe-DTCS complex then forms a complex with NO (NO-Fe-DTCS). Dr. Yoshimura successfully obtained two-dimensional ESR images of NO, induced by lipopolysaccharide in mouse peritoneum. DTCS sodium salt (DTCS Na) was used for this experiment because it is less toxic than ammonium salt (sodium salt LD_{50}: 1942 mg/kg; ammonium salt LD_{50}: 765 mg/kg). Since the Fe-DTCS complex is more stable than the other dithiocarbamate complexes in the air or in aqueous solutions, it could be a useful spintrapping reagent for biochemical research. The Fe-DTCS complex should be used immediately after preparation. An excessive amount of DTCS Na (usually 5 equivalents DTCS Na to $FeSO_4$) is required to make a more stable solution. Dithiocarbamates tend to decompose under physiological conditions to form toxic carbon disulfide.





DTCS Na
N-(Dithiocarboxy)sarcosine, disodium salt, dihydrate
$C_4H_5NNa_2O_2S_2 \cdot 2H_2O$ = 245.23
CAS No. [13442-87-0
Unit: 100 mg, 500 mg

1. General Protocol

► Preparation of Fe(II)-DTCS Complex

1. Dissolve 278 mg $FeSO_4$, $7H_2O$ (ferrous sulfate heptahydrate) with 20 ml water[a] to prepare 50 mM $FeSO_4$ solution.[b]
2. Dissolve 123 mg DTCS Na with 10 ml water[a] to prepare 50 mM DTCS solution.
3. Mix 1 ml DTCS Na solution with 8.8 ml buffer solution[a] (pH 7 or higher). Add 200 µl $FeSO_4$ solution just prior to use.[c]
   a) Purge any dissolved oxygen in the water or the buffer by nitrogen gas bubbling for at least 30 minutes prior to dissolving $FeSO_4$.
   b) The $FeSO_4$ solution can be stored at -20°C for at least 2 months.
   c) Fe(II)-DTCS complex is colorless. If the solution is brown, Fe(III)-DTCS may have formed by dissolved oxygen in the solution. However, the brown solution can still be used for NO trapping.

► Preparation of NO-Fe(II)-DTCS Complex

1. Under argon gas flow, add 200 µl of $FeSO_4$ solution to 9.4 ml buffer solution (pH 7 or higher). Then introduce NO into the solution by bubbling NO gas through a glass capillary for 15 minutes.
2. Add 400 ml of DTCS Na solution to the $FeSO_4$ solution, and continue to introduce NO by bubbling for another 5 minutes.
3. Remove excess NO with argon gas bubbling for 5 minutes, and store at -20°C. The NO-Fe(II)-DTCS solution can be stored at -20°C for at least 2 months in oxygen-free conditions.

2. References

1. T. Yoshimura, *et al*., In vivo EPR Detection and Imaging of Endogenous Nitric Oxide in Lipopolysaccharide-treated Mice. *Nat Biotechnol*. 1996;**14**:992-994.
2. B. Kalyanaraman, Detectionof Nitric Oxide by Electron Spin Resonance in Chemical, Photochemical, Cellular, Physiological, and Pathophysiological Systems. *Methods Enzymol*. 1996;**268**:168-187.
3. H. Yokoyama, *et al*., In vivo ESR-CT Imaging of the Liver in Mice Receiving Subcutenous Injection of Nitric Oxide-Bound Iron Complex. *Magn Reson Imaging*. 1997;**15**:249-253.

3. Specification

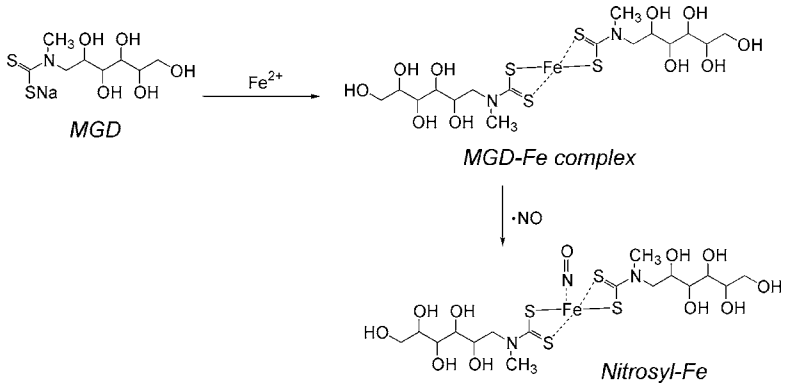
► Appearance: white or pale yellow powder

Detection of NO by EPR

MGD

Product Code: M323

MGD is a highly water-soluble dithiocarbamate-type chelator that generates with complexes many transitional method such as Fe and Cu. The diethyldithiocarbamate-Fe^{2+} complex has been used for NO detection by electron spin resonance (ESR). However, the poor solubility of this carbamate in an aqueous solution limits its application. Dr. Lai and others improved the technique using a water-soluble dithiocarbamate- Fe^{2+} complex, MGD-Fe^{2+}. They successfully detected in vivo NO of a nitroprusside-injected mouse and NO generated by an LPS injection using in vivo ESR. The MGD-Fe^{2+} complex is capable of NO detection under physiological conditions, and dissolved oxygen in the solution does not interfere with NO detection.



1. General Protocol

► Preparation of Fe(II)-MGD Complex

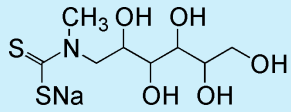
1. Dissolve 278 mg $FeSO_4$, $7H_2O$ (ferrous sulfate heptahydrate) with 20 ml water[a] to prepare 50 mM $FeSO_4$ solution.[b]
2. Dissolve 147 mg MGD with 10 ml water[a] to prepare 50 mM MGD solution.
3. Mix 1 ml MGD solution with 8.8 ml buffer solution[a] (pH 7 or higher) and then add 200 µl $FeSO_4$ solution prior to use.[c]
   a) Purge any dissolved oxygen in the water or buffer by nitrogen gas bubbling for at least 30 minutes prior to dissolving $FeSO_4$.
   b) The $FeSO_4$ solution can be stored at -20°C for at least 2 months.
   c) Fe(II)-DTCS is colorless. If the solution is brown, Fe(III)-DTCS may have formed because of dissolved oxygen in the solution. However, the brown solution can still be used for NO trapping.

► Preparation of NO-Fe(II)-MGD Complex

1. Under argon gas flow, add 200 µl $FeSO_4$ solution to 9.4 ml buffer solution (pH 7 or higher). Then introduce NO into the solution by bubbling NO gas through a glass capillary for 15 minutes.
2. Add 400 µl MGD solution to the $FeSO_4$ solution and continue to introduce NO by bubbling for another 5 minutes.
3. Remove excess NO with argon gas bubbling for 5 minutes and store at -20°C. The NO-Fe(II)-MGD solution can be stored at -20°C for at least 2 months in oxygen-free conditions. Remove excess NO with argon gas bubbling for 5 minutes, and store at -20°C. The NO-Fe(II)-DTCS solution can be stored at -20°C for at least 2 months in oxygen-free conditions.

2. Specification

► Appearance: white crystalline powder
► Purity: ≥98.0%(HPLC)



MGD
N-(Dithiocarbamoyl)-*N*-methyl-D-gluca-mine, sodium salt
$C_8H_{16}NNa_2O_5S_2$ =293.34
CAS No. [94161-07-6(free acid)]
Unit: 500 mg

NO donor (Stable in acidic condition)

NOR Compounds

NORs are ideal NO donors with completely different chemical structures from the other NO donors. Although NORs do not have any ONO_2 or ONO moiety, they spontaneously release NO at a steady rate. Even though the NO release mechanism of NOR has not been completely determined, it is confirmed that the byproducts do not possess any significant bioactivities. NOR 3, isolated from Streptomyces genseosporeus, is reported to have strong vasodilatory effects on rat and rabbit aortas and dog coronary arteries. Its activity (ED_{50}=1 nM) is 300 times that of isosorbide dinitrate (ISDN). NOR 3 also increases the plasma cyclic GMP levels, whereas ISDN does not. NOR is a potent inhibitor of platelet aggregation and thrombus formation. NOR 3 (IC_{50}=0-7 mM) effectively inhibits 100% of ADP-initiated human platelet Aggregation, whereas ISDN inhibits only 32% of the total aggregation, even at 100 mM concentrations. NOR 3 has also been reported to have antianginal and cardioprotective effects in the ischemia/reperfusion system. In the rat methacholin-induced coronary vasospasm model, NOR 3 suppressed the elevation of the ST segment dose-dependently and significantly at 1 mg per kg. On the other hand, ISDN suppressed it significantly at 3.2 mg per kg. The difference in the NO release rate of NOR reagents was reflected even on the in vivo hypotensive effects. NOR may also be used orally in a 0.5% methylcellulose suspension. NOR is relatively stable in DMSO solution. NOR 1, which has the shortest half-life, is a promising reagent for making NO standard solutions for calibration. For the preparation of the standard solution, a precisely diluted NOR 1/DMSO solution is added to the buffer solutions.

Table 1 Half-life of NOR donors(pH 7.4)

NO Donors			
NOR 1	NOR 3	NOR 4	NOR 5
1.8 min	30 min	60 min	20 hrs

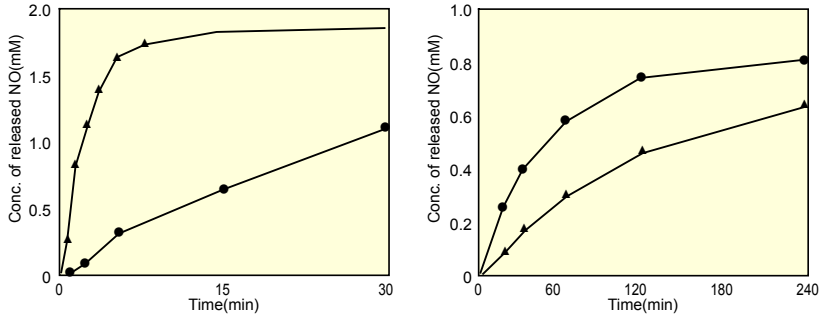


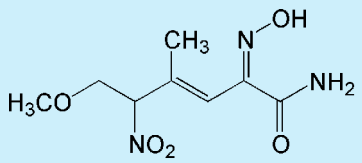
Fig. 1 Time course of releasing NO from NOR compounds

1. References

1. S. Shibata, *et al.*, Characteristics of the Vasorelaxing Action of (3E)-4-Ethyl-2-hydroxyimino-5-nitro-3-hexamide FK409, a New Vasodilator Isolated from Microbial Sources, in Isolated Rabbit Arteries. *J Cardiovasc Pharmacol.* 1991;**17**:508-518.
2. Y. Kita, *et al.*, Antianginal Effects of FK409, a New Spontaneous NO Releaser. *Br J Pharmacol.* 1994;**113**:1137-1140.
3. Y. Kita, *et al.*, Antiplatelet Activities of FK409, a New Spontaneous NO Releaser. *Br J Pharmacol.* 1994;**113**:385-388.
4. Y. Kita, *et al.*, Spontaneous Nitric Oxide Release Accounts for the Potent Pharmacological Actions of FK409. *Eur J Pharmacol.* 1994;**257**:123-130.
5. M. Hino, *et al.*, FK409, a Novel Vasodilator Isolated from the Acid-treated Fermentation Broth of *Streptomyces Griseosporeus* I. Taxonomy, Fermentation, Isolation, and Physico-chemical and Biological Characteristics. *J Antibiot.* 1989;**42**:1578-1583.
6. J. Decout, *et al.*, Decomposition of FK409, a New Vasodilator: Identification of Nitric Oxide as Metaborite. *Bioorg Med Chem Lett.* 1995;**5**:973-978.
7. S. Fukuyama, *et al.*, A New Nitric Oxide (NO) Releaser: Spontaneous NO Release from FK409. *Free Radic Res.* 1995;**23**:443-452.
8. Y. Kita, *et al.*, FR144420, a Novel, Slow, Nitric Oxide-releasing Agent. *Eur J Pharmacol.* 1995;**275**:125-130.
9. M. Kato, *et al.*, New Reagents for Controlled Release of Nitric Oxide. Structure-stability Relationships. *Bioorg Med Chem Lett.* 1996;**6**:33-38.
10. Y. Kita, *et al.*, FK409, a Novel Spontaneous NO Releaser: Comparative Pharmacological Studies with ISDN. *Cardiovasc Drug Rev.* 1996;**14**:148-165.
11. Y. Hirasawa, *et al.*, Antianginal Effects of FR144420, a Novel Nitric Oxide-releasing Agent. *Eur J Pharmacol.* 1996;**303**:55-59.
12. M. Sato, *et al.*, Nitric Oxide Raises Cytosolic Concentrations of Ca^{2+} in Cultured Nodose Ganglion Neurons from Rabbits. *Neurosci Lett.* 1996;**206**:69-72.
13. Y. Kita, *et al.*, Oral Biological Activities of Spontaneous Nitric Oxide Releaser are Accounted for by their Nitric Oxide-releaseing Rates and Oral Absorption Manners. *J Pharmacol Exp Ther.* 1996;**276**:421-425.

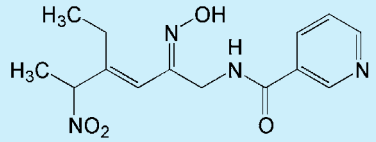
2. Specification

- Appearance: White or slightly yellow powder(NOR 1, NOR 4, NOR 5), White crystalline powder(NOR 3)
- Purity: ≥98.0%(HPLC)

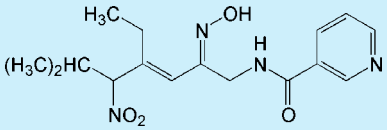


NOR 1 (Product code: N388)
(±)-(E)-4-Methyl-2-[(E)-hydroxyimino]-5- nitro-6-methoxy-3-hexenamide
$C_8H_{13}N_3O_5$ =231.21
CAS No. [163032-70-0]
Unit: 10 mg

NOR 3 (Product code: N390)
(±)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide
$C_8H_{13}N_3O_4$ =215.21
CAS No. [163180-49-2]
Unit: 10 mg



NOR 4 (Product code: N391)
(±)-*N*-[(E)-4-Ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridinecarboxamide
$C_{14}H_{18}N_4O_4$ =306.32
CAS No. [162626-99-5]
Unit: 10 mg



NOR 5 (Product code: N448)
(±)-*N*-[(E)-4-ethyl-3-[(Z)-hydroxyimino]-6-methyl-5-nitro-3-heptenyl]-3-pyridine-carboxamide
$C_{16}H_{22}N_4O_4$ = 334.37
Unit: 10 mg

NO donor (Stable in alkaline condition)

NOC Compounds

NOCs are stable NO-amine complexes that spontaneously release NO, without cofactors, under physiological conditions. The rate of NO release depends on the chemical structure of NOC. The mechanism of spontaneous NO generation by NOCs is very simple compared to other classical NO donors, such as nitroglycerin and nitropurusside, and the by-products do not interfere with cell activities. A single NOC molecule releases two NO molecules (as indicated in the reaction scheme); the release rate of the second NO molecule is very slow. NOCs can be used to add controlled amounts of pure NO to experimental systems at controlled rates with minimal side effects. The amount of NO released can be easily manipulated by altering the concentration and selection of NOC reagents. Dojindo offers four different NOCs (NOC 5, 7, 12, and 18) with different halflifes. Stock solutions of NOC prepared in alkaline solutions, such as aqueous NaOH, are relatively stable. However,the NOC stock solution should be used within one day because it degrades about 5% per day, even at -20ºC. The release of NO begins immediately after adding the stock solution to a sample solution.

1. General Protocol

1. Prepare 10 mM NOC stock solution using 0.1 M NaOH. Since the NOC stock solution is not stable, keep it on an ice bath and use it in one day.
2. Add an appropriate volume of the NOC stock solution to the sample solution in which NO is to be released. To maintain the pH of the sample solution, the volume of the NOC stock solution should not exceed 1/50 of the sample volume. The sample solution should have sufficient buffering action. NO will be released immediately after the addition of the NOC stock solution.

Table 1 Half-life of NOC donors

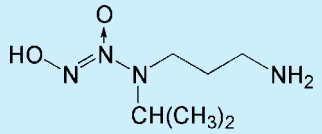
		NO Donors			
		NOC 5	NOC 7	NOC 12	NOC 18
pH	7.0	12 min	2.2 min	40 min	13 hrs
	7.2	20 min	3.8 min	1.2 hrs	18 hrs
	7.4	25 min	5 min	100 min	21 hrs
	7.6	42 min	8.2 min	3 hrs	34 hrs
	7.8	66 min	12.4 min	4.6 hrs	45 hrs

2. References

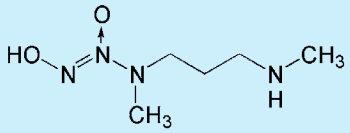
1. K. Hayashi, *et al.*, Action of Nitric Oxide as a Antioxidant Against Oxidation of Soybean Phosphatidylcholine Liposomal Membrane. *FEBS Lett.* 1995;**370**:37-40. (Noc 12)
2. S. Shibuta, *et al.*, Intracerebroventricular Administration of a Nitric Oxide-releasing Compound, NOC-18, Produces Thermal Hyperalgesia in Rats. *Neurosci Lett.* 1995;**187**:103-106. (NOC 18)
3. S. Shibuta, *et al.*, A new nitric oxide donor, NOC-18, exhibits a nociceptive effect in the rat formalin model. *J Neurol Sci.* 1996;**141**:1-5. (NOC 18)
4. N. Yamanaka, *et al.*, Nitric Oxide Released from Zwitterionic Polyamine/NO Adducts Inhibits Cu^{2+}-induced Low Density Lipoprotein Oxidation. *FEBS Lett.* 1996;**398**:53-56. (NOC 5, NOC 7)
5. D. Berendji, *et al.*, Nitric Oxide Mediates Intracytoplasmic and Intranuclear Zinc Release. *FEBS Lett.* 1997;**405**:37-41.
6. T. Ohnishi, *et al.*, The Effect of Cu^{2+} on Rat Pulmonary Arterial Rings. *Eur J Pharmacol.* 1997;**319**:49-55. (Noc 7)
7. Y. Adachi, *et al.*, Renal Effect of a Nitric Oxide Donor, NOC 7, in Anethetized Rabbits. *Eur J Pharmacol.* 1997;**324**:223-226. (Noc 7)
8. Y. Minamiyama, *et al.*, Effect of Thiol Status on Nitric Oxide Metabolism in the Circulation. *Arch Biochem Biophys.* 1997;**341**:186-192. (NOC 7)

3. Specification

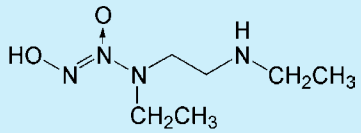
- ► Appearance: White powder
- ► Purity: ≥90.0%(HPLC)



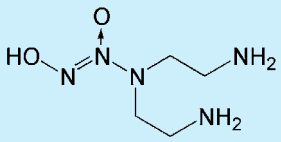
NOC 5 (Product code: N380)
1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene
$C_6H_{16}N_4O_2$ = 176.22
CAS No. [146724-82-5]
Unit: 10 mg, 50 mg



NOC 7 (Product code: N377)
1-Hydroxy-2-oxo-3-(*N*-methyl-3-amino-propyl)-3-methyl-1-triazene
$C_5H_{14}N_4O_2$ = 162.19
CAS No. [146724-84-7]
Unit: 10 mg, 50 mg



NOC 12 (Product code: N378)
1-Hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene
$C_6H_{16}N_4O_2$ = 176.22
CAS No. [146724-89-2]
Unit: 10 mg, 50 mg



NOC 18 (Product code: N379)
1-Hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-tria-zene
$C_4H_{13}N_5O_2$ = 163.18
CAS No. [146724-94-9]
Unit: 10 mg, 50 mg

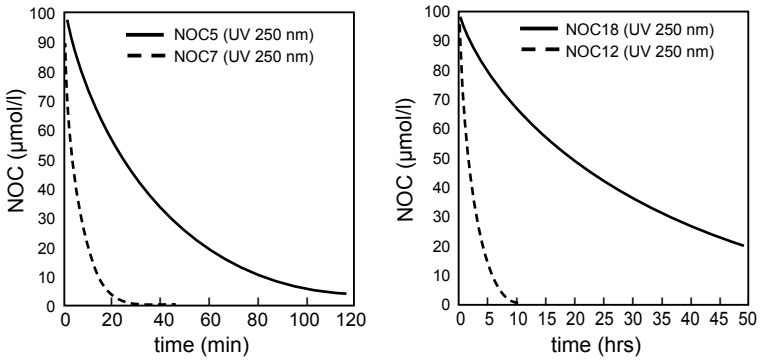


Fig. 1 Time course of NOC degradation
(100 µM NOCs in 100 mM PBS, pH7.0, 37°C)

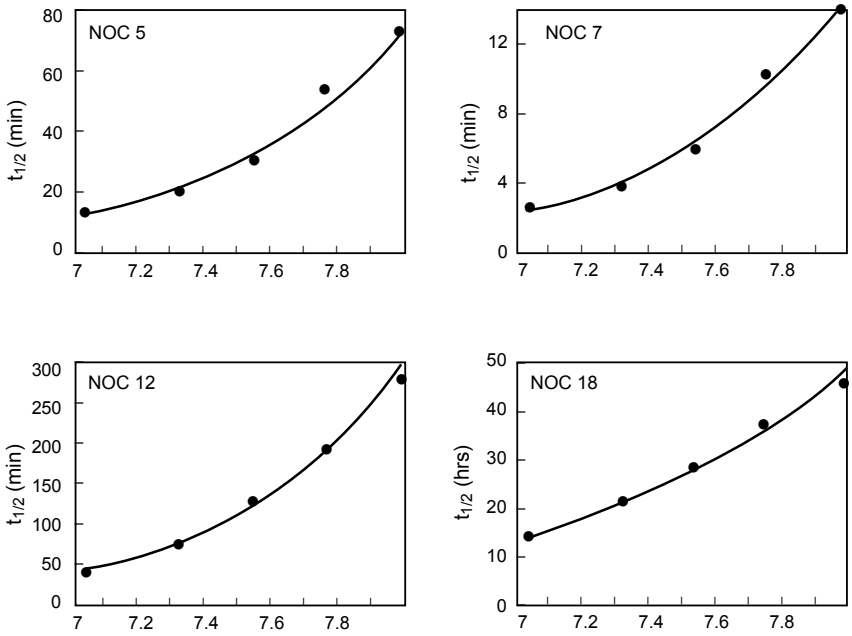


Fig. 2 Half life of NOC compounds in 100mM PBS(37°C)
under the various pH conditions

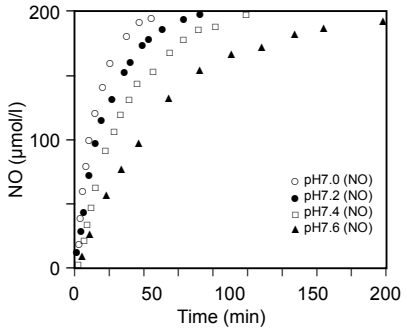


Fig. 3 pH dependency of releasing NO from NOC 5
(0.1 mM NOC 5, in 100 mM PBS at 37°C)

NO donor from nitrosothiol compound

S-Nitrosoglutathione

Product Code: N415

Nitrosothiol compounds release NO and become disulfides under specific physiological conditions. While most of the S-nitrosothiol compounds are unstable, S-Nitrosoglutathione is exceptionally stable. Furthermore, S-Nitrosoglutathione is water-soluble. Although S-nitrosothiol is a good NO donor with no nitrate tolerance, there is evidence that S-nitrosothiol itself has NO-like activity during guanylate cyclase activation. Another important reaction of nitrosothiol is NO transfer to other thiol compounds. Since it depends on the pKa of thiols, this transfer reaction proceeds at physiological pH levels.
Previous research found that the vasorelaxant properties of endothelium-derived relaxation factor (EDRF) are more similar to *S*-nitrosocysteine than NO; however, this does not seem to be the current majority view. Though nitrosothiol is one of the most important factors for the study of the NO pathway, only a few nitrosothiols, such as SNAP and *S*-nitrosoglutathione (GSNO), are stable enough for use as NO donors. Unfortunately, SNAP is insoluble in water. Thus, GSNO and *S*-nitrosocysteine (SNC) are the only commercially available water-soluble nitrosothiols. Nitrosothiols release nitric oxide, and form disulfides as shown below.

$$2RSNO \rightarrow RSSR + 2NO$$

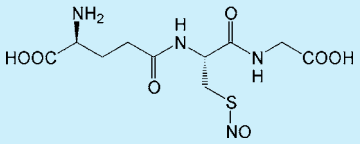
This reaction is accelerated by light and heat. If GSNO is incubated at 37°C without light, NO will not be released spontaneously. Metal ions, such as Cu(II), Cu(I), and Hg(II), also accelerate the reaction. Thus, masking reagents such as EDTA prevent the releasing reaction. Another important characteristic of nitrosothiols is their ability to carry out nitrosation. This reaction is faster than the decomposition of RSNO itself, and proceeds readily at physiological pH levels. The reaction rate depends on the pKa of the thiol. The vasorelaxant activities of nitrosothiols in rat aortic rings have been reported as follows:

SNAP > GSNO = SNAC (*S*-Nitroso-N-acetylcysteine)
> CoAsNO (*S*-Nitroso-coenzyme A) > CYCNO (*S*-Nitrosocysteine)

The inhibitory potencies of nitrosothiols for the platelet aggregation have been reported as follows:

GSNO > NO > SNAP > SIN-1

Denitrosation of *S*-nitrosothiol is not spontaneous, and it needs to be catalyzed on the surface of external vascular membranes. *S*-nitroso-L-cysteine raises the intracellular calcium level of a PC12 cell by modifying the thiol group of a caffeine-sensitive moiety of the calcium-induced calcium release (CICR) channel. GSNO has been shown to reduce the blood pressure of anesthetized dogs (0.2 mg/kg) and monkeys (10 mg/kg) through the inhibition of the platelet aggregation.



S-Nitrosoglutathione
N-(*N*-L-γ-Glutamyl-*S*-nitroso-L-cysteinyl)glycine
$C_{10}H_{16}N_4O_7S$ = 336.32
CAS No. [57564-91-7]
Unit: 25 mg, 100 mg

1. References

1. A. Gibson, *et al*., An Investigation of Some S-Nitrosothiols, and of Hydroxy-arginine, on the Mouse Anococcygeus. *Br J Pharmacol*. 1992;**107**:715-721.
2. M. W. Radomski, *et al*., S-Nitroso-glutathione Inhibits Platelet Activation in Vitro and in Vivo. *Br J Pharmacol*. 1992;**107**:745-749.
3. R. M. Clancy, *et al*., Novel Synthesis of S-Nitrosoglutathione and Degradation by Human Neutrophils. *Anal Biochem*. 1992;**204**:365-371.
4. J. W. Park, *et al*., Transnitrosation as a Predominant Mechanism in the Hypotensive Effect of S-Nitrosoglutathione. *Biochem Mol Biol Int*. 1993;**30**:885-891.
5. D. Barrachina, *et al*., Nitric Oxide Donors Preferencially Inhibit Neuronally Mediated Rat Gastric Acid Secretion. *Eur J Pharmacol*. 1994;**262**:181-183.
6. E. A. Konorev, *et al*., S-Nitrosoglutathione Improves Functional Recovery in the Isolated Rat Heart After Cardioplegic Ischemic Arrest-evidence for a Cardioprotective Effect of Nitric Oxide. *J Pharmacol Exp Ther*. 1995;**274**:200-2006.
7. S. C. Askew, *et al*., Catalysis by Cu^{2+} of Nitric Oxide Release from S-Nitrosothiols (RSNO). *J Chem Soc Perkin Trans 2*. 1995;741-745.
8. D. J. Banett, *et al*., NO-group Transfer(Transnitrosation) between S-Nitrosothiols and Thiols. Part 2. *J Chem Soc Perkin Trans 2*. 1995;1279-1282.
9. J. G. De Man, *et al*., Effect of Cu^{2+} on Relaxations to the Nitrergic Neurotransmitter, NO and S-Nitrosothiols in the Rat Gastric Fundus. *Br J Pharmacol*. 1996;**119**:990-996.
10. A. P. Dicks, *et al*., Generation of Nitric Oxide from S-Nitrosothiols Using Protein-bound Cu^{2+} Sources. *Chem Biol*. 1996;**3**:655-659.
11. J. A. Cook, *et al*., Convenient Colorimetric and Fluorometric Assays for S-Nitrosothiols. *Anal Biochem*. 1996;**238**:150-158.
12. S. X. Liu, *et al*., Nitric Oxide Donors: Effects of S-Nitrosoglutathione and 4-Phenyl-3-furoxancarbonitrile on Ocular Blood Flow and Retinal Function Recovery. *J Ocul Pharmacol Ther*. 1997;**13**:105-114.
13. C. Alpert, *et al*., Detection of S-Nitrosothiols and Other Nitric Oxide Derivatives by Photolysis-chemiluminescence Spectrometry. *Anal Biochem*. 1997;**245**:1-7.
14. T. Akaike, *et al*., Nanomolar Quantification and Identification of Various Nitrosothiols by High Performance Liquid Chromatography Coupled with Flow Reactors of Metals and Griess Reagent. *J Biochem*. 1997;**122**:459-466.

2. Specification

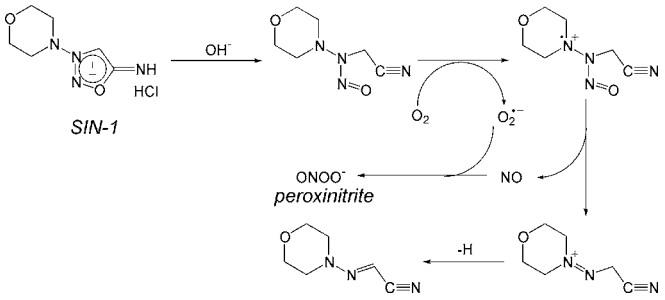
- Appearance: Pink powder
- Purity: ≥90.0%(HPLC)

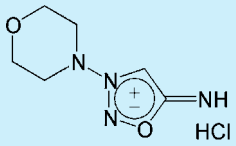
ONOO$^-$(peroxynitrite) donor

SIN-1

Product Code: S264

SIN-1, a metabolite of the vasodilator molsidomine, is utilized to separately estimate the effectiveness of NO and peroxynitrite with other NO donors. SIN-1 spontaneously decomposes in the presence of molecular oxygen to generate NO and superoxide. Both products bind very rapidly to form peroxynitrite (rate constant k: $3.7x10^{-7}$ $M^{-1}s^{-1}$). Therefore, SIN-1 is a useful compound that generates peroxynitrite in an efficient manner. Peroxynitrite is a very strong oxidant that generates hydroxyl and nitrosyldioxyl radicals under physiological conditions. Peroxynitrite also decomposes to generate nitrate ion quickly in acidic conditions and slowly in basic conditions. Those species have a different bioactivity from NO.





SIN-1
3-(4-Morpholinyl)sydnonimine, hydrochloride
$C_6H_{11}ClN_4O_2$ = 206.63
CAS No. [16142-27-1]
Unit: 25 mg

1. References

1. M. Feelisch, *et al*., On the Mechanism of NO Release from Sydnonimines. *J Cardiovasc Pharmacol*. 1989;**14**:S13-S22.
2. M. Feelisch, The Biochemical Pathways of Nitric Oxide Formation from Nitrovasodilators: Appropriate Choice of Exogenous NO Donors and Aspects of Preparation and Handling of Aqueous NO Solutions. *J Cardiovasc Pharmacol*. 1991;**17**:S25-S33.
3. N. Hogg, *et al*., Production of Hydroxyl Radicals from the Simultaneous Generation of Superoxide and Nitric Oxide. *Biochem J*. 1992;**281**:419-424.
4. M. E. Murphy, *et al*., Nitric Oxide Hyperpolarizes Rabbit Mesenteric Arteries via ATP-sensitive Potassium Channels. *J Physiol*. 1995;**486**:47-58.
5. H. Kankaanranta, *et al*., 3-Morpholino-sydnonimine-induced Suppression of Human Neutrophil Degranulation in Not Mediated by Cyclic GMP, Nitric Oxide or Peroxynitrite: Inhibition of the Increase in Intracellular Free Calcium Concentration by N-Morpholinoiminoacetoni. *Mol Pharmacol*. 1997;**51**:882-888.
6. S. Yamamoto, *et al*., Subarachnoid Hemorrhage Impairs Cerebral Blood Flow Response to Nitric Oxide but Not to Cyclic GMP in Large Cerebral Arteries. *Brain Res*. 1997;**757**:1-9.
7. S. Pfeiffer, *et al*., Interference of carboxy-PTIO with Nitric-oxide and Peroxynitrite-mediated Reactions. *Free Radic Biol Med*. 1997;**22**:787-794.
8. M. B. Herrero, *et al*., Tyrosine Nitration in Human Spermatozoa: A Physiological Function of Peroxynitrite, the Reaction Product of Nitric Oxide and Superoxide. *Mol Hum Reprod*. 2001;**7**:913-921.
9. P. D. Lu, *et al*., Cytoprotection by Pre-emptive Conditional Phosphorylation of Translation Initiation Factor 2. *EMBO J*. 2004;**23**:169-179.

2. Specification

- Appearance: White needles or slightly yellowish-white crystalline powder
- Purity: pass test(TLC)
- Melting Point: 180°C to 190°C

Detection of 3-DG by HPLC (Fluorometric)

3-Deoxyglucosone Detection Reagents

Product Code: D536

Advanced glycation end-products (AGEs) have been studied as one of the causes of diabetic complications. Several compounds have been identified as AGEs, including pyralline, pentosidine, imidazolone, and pyropyridine. Glyoxal and methylglyoxal are reactive dicarbonyl compounds generated by glucose self-oxidation that are known to be AGE precursors. Another dicarbonyl compound, 3-Deoxyglucosone (3-DG), is also known to be one of the AGE precursors. 3-DG is derived from the Amadori rearrangement products of proteins and sugars in early stages of the Maillard reaction. 3-DG is also derived from fructose, which is present in high levels in diabetic patients, by a selfcondensation reaction. Fructose-3-phosphate has been found to enhance cross-linking reactions of lens proteins in a diabetic rat model. Therefore, 3-DG derived from fructose-3-phosphate has been studied as a possible cause of cataracts.
There are two methods for determining 3-DG levels: HPLC and mass spectrometry (MS). However, there is some discrepancy between the HPLC and MS methods when measuring 3-DG levels in vivo. HPLC analysis is based on a fluorescent compound, 2-(2,3,4-trihydroxybutyl)-benzo[g]quinoxaline, generated by a coupling reaction between 3-DG and 2,3-diaminonaphthalene. Analogs of 2,3-diaminonaphthalene, such as 1,2-diamino-4,5-dimethoxy-benzene and 1,2-diamino-4,5-methylenedioxybenzene, can also be used.

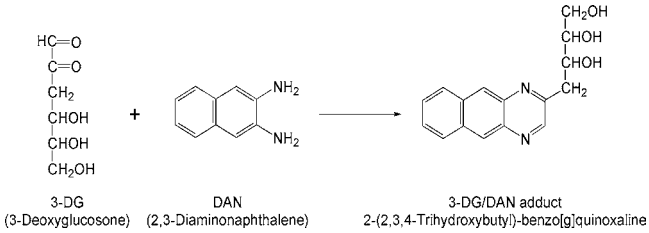


Fig. 1 Principal of 3-DG detection

2. General Protocol

► HPLC Method: Human Serum

1. Add 60% perchloric acid solution to 1 ml human serum and spin at 3,000 xg for 20 minutes at 4°C.
2. Dilute the supernatant with bicarbonate buffer, then add 0.1 ml of 2,3-Diaminonaphthalene / methanol solution and 25 µl of 1 ppm 3,4-hexanedione as an internal standard.
3. Incubate the mixture at 4°C overnight.
4. Extract the mixture with 4 ml ethyl acetate, and add 4 ml methanol to the extract.
5. Analyze the mixture with reverse-phase HPLC at 267 nm excitation and 503 nm emission for fluorescent detection or at 268 nm for UV detection. Data correlates well with HbA1c level.
   * Normal serum 3-DG level: 12.8±5.2 ng/ml
   * Serum 3-DG level of diabetic patient: 31.8±11.3 ng/ml

3. References

1. K. J. Knecht, *et al*., Detection of 3-Deoxyfructose and 3-Deoxyglucosone in Human Urine nd Plasma: Evidence for Intermediate Stages of the Maillard Reaction in Vivo. *Arch Biochem Biophys*. 1992;**294**:130-137.
2. T. Niwa, *et al*., Presence of 3-Deoxyglucosone, a Potent Protein Crosslinking Intermediate of Maillard Reaction, in Diabetic Serum. *Biochem Biophys Res Commun*. 1993;**196**:837-843.
3. H. Yamada, *et al*., Increase in 3-deoxyglucosone levels in diabetic rat plasma. Specific in vivo determination of intermediate in advanced Maillard reaction. *J Biol Chem*. 1994;**269**:20275-20280.

Contents of the Kit

DAN [2,3-Diaminonaphthalene] 10 mg x 1

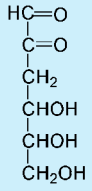
3-DG/DAN adduct [2-(2,3,4-Trihydroxybutyl)benzo[g]quinoxaline] 1 mg x 1

Standard substrate for AGE precursor

3-Deoxyglucosone

Product Code: D535

3-DG can be utilized for AGE production or as a standard for 3-DG level detection in plasma or serum samples.



3-Deoxyglucosone
3-Deoxy-D-erythro-hexos-2-ulose
$C_6H_{10}O_5$ = 162.14
CAS No. [4084-27-9]
Unit: 1 mg

1. Specification

► Appearance: white or white pale yellow solid
► Purity: ≥99.0%(HPLC)

2. Recent Publications

Title	Reference
Glutathione depletion as a mechanism of 3,4-dideoxyglucosone-3-ene-induced cytotoxicity in human peritoneal mesothelial cells: role in biocompatibility of peritoneal dialysis fluids	T. Yamamoto, *et al., Dial. Transplant.* 2009; **24**: 1436 - 1442.
A novel class of advanced glycation inhibitors ameliorates renal and cardiovascular damage in experimental rat models	Y. Izuhara, *et al, Nephrol. Dial. Transplant.* 2008; **23**: 497 - 509.