TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Superoxide Dismutase Assay Kit Cuvette Format

Cat# 7502-100-K

Spectrophotometric assay for the analysis of Superoxide Dismutase in cell and tissue extracts.

Sufficient reagents for 100 tests

Superoxide Dismutase Assay Kit Cuvette Format

Cat# 7502-100-K

Table of Contents

		Page
I.	Introduction	1
II.	Precautions and Limitations	2
III.	Materials Supplied	2
IV.	Materials/Equipment Required but not Supplied	2
V.	Reagent Preparation	2
VI.	Preparation of Cell and Tissue Extracts	3
	Section A. Processing Samples	3
	Section B. Preparation of Extracts	4
	Section C. Differentiating SOD1, SOD2 and SOD3	4
VII.	SOD Assay Protocol	5
VIII.	Data Interpretation	5
IX.	References	7
X.	Troubleshooting	10
XI.	Related Products Available from Trevigen	10

© 2008 Trevigen, Inc. All rights reserved. Trevigen is a registered trademark and Ficoll-Hypaque™ is a trademark of Becton, Dickinson, and Company. Triton® is a registered trademark of Union Carbide Chemicals and Plastic Company

I. Introduction

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, ischemia reperfusion injury, athero-sclerosis and aging.[1-3] Superoxide Dismutases (SODs) catalyze the dismutation of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix (Fig. 1), and thus, SODs provide an important defense against the toxicity of superoxide radicals.[4]

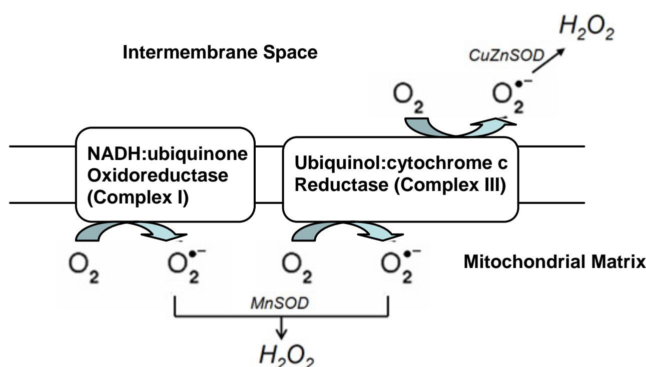


Figure 1. Hydrogen peroxide production by SODs.

In Trevigen's **Superoxide Dismutase Cuvette Assay,** superoxide radical ($O_2^{\bullet-}$) ions, generated from the conversion of xanthine to uric acid and H_2O_2 by xanthine oxidase (XOD), convert WST-1 to WST-1 formazan in a cuvette. WST-1 formazan absorbs light at 450 nm. SODs reduce superoxide ion concentrations and thereby lower the rate of WST-1 formazan formation.[5,6] The extent of reduction in the appearance of WST-1 formazan is a measure of SOD activity present in your experimental sample (Fig. 2).

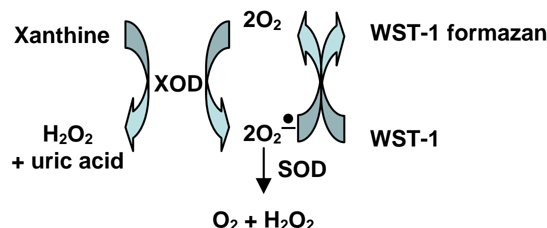


Figure 2. XOD and SOD antagonism in the generation of WST-1 formazan. Xanthine Oxidase (XOD) generates superoxide radical resulting in the reduction of WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion thereby reducing the rate of WST-1 formazan generation.

Trevigen's **Superoxide Dismutase Cuvette Assay** is free of interference by other catalytic activities, and is ideal for assaying SODs in mammalian tissue and cell lysates in a cuvette format. Unlike some other assay kits for SOD, this system is not greatly disturbed by trace metals. Sufficient reagents are provided for 100 tests. The assay is performed in as little as 10 minutes and relative SOD activity of the experimental sample is determined from percent inhibition of the rate of formation of WST-1 formazan. Furthermore, the assay is suitable for the assay of isozymes SOD1 (cytosolic Cu/Zn-SOD), SOD2 (mitochondrial Mn-SOD), and SOD3 (extracellular Cu/Zn-SOD).

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the SOD Assay Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

Catalog Number	Component	Amount Provided	Storage Temperature
7501-500-01	SOD Standard	50 µl	-20°C
7501-500-02	10X SOD Buffer	20 ml	4°C
7501-500-03	Xanthine Oxidase (XOD)	3 ml	-20°C
7501-500-04	10X Xanthine Solution	2 ml	4°C
7501-500-05	20% Triton X-100	1 ml	4°C
7501-500-06	WST-1 Reagent	3 ml	4°C

IV. Materials/Equipment Required But Not Supplied

Reagents:

1. SOD Inhibitors or cells/tissue to be tested
2. 10X PBS (Trevigen # 4870-500-6)
3. Distilled water
4. Protease inhibitors (optional) such as phenylmethylsulfonylfluoride (PMSF)
5. Reagents to determine protein concentration
6. Ficoll-Hypaque™ (erythrocyte, lymphocyte and monocyte preparations)
7. Ethanol and Chloroform (erythrocyte preparation)

Disposables:

1. 1 - 200 µl and 100 - 1000 µl pipette tips
2. 0.5 and 1.5 ml microtubes
3. 15 ml conical (adherent and suspension cell preparation)
4. 50 ml conical (tissue preparation)

Equipment:

1. Micropipettes
2. Spectrophotometer and cuvettes for measuring absorbance at 450 nm
3. Centrifuge for processing samples

V. Reagent Preparation

1. **1X SOD Buffer**
   Dilute the 10X SOD Buffer to **1X (1:10)** with dH_2O. The **1X** SOD Buffer is used to prepare dilutions of SOD Standard, Cell Extraction Buffer and Master Mix.

2. **SOD Standard (Enzyme)**
   One unit of SOD reduces the rate of WST-1 formazan formation by 50%. The kit contains 50 µl of SOD Standard at a concentration of approximately 10 units/µl. The enzyme should be diluted appropriately with **1X** SOD Buffer just before use. **Note: Diluted enzyme should be used immediately and any remainder discarded. Store the stock SOD Standard at -20ºC.**

3. **1X Cell Extraction Buffer**
   Prepare sufficient amount of Cell Extraction Buffer. Preparation for 10 ml is as follows:

10X SOD Buffer (Cat# 7501-500-02)	1.0 ml
20% (v/v) Triton X-100 (Cat# 7501-500-05)	0.2 ml
dH_20	8.8 ml
200 mM PMSF (optional)	(50 µl)

4. **Master Mix**
   Prepare sufficient amount of Master Mix (887.5 µl per reaction):
   Preparation for 887.5 µl is as follows:

10X SOD Buffer (Cat# 7501-500-02)	88.75 µl
WST-1 Reagent (Cat# 7501-500-06)	25 µl
Xanthine Oxidase (Cat# 7501-500-03)	15 µl
dH_2O	759 µl

VI. Preparation of Cell and Tissue Extracts

Choose the appropriate protocol in Section A to Process Sample before proceeding to Section B: Please note that samples should be kept on ice to maintain enzyme activity.

Section A. Processing Samples

Suspension cells:

1. Centrifuge 2 to 6 x 10^6 suspension cells at 250 x g for 10 minutes at 4ºC. Discard the supernatant.
2. Suspend the cell pellet in 10 ml of ice-cold 1X PBS. Centrifuge at 250 x g for 10 minutes, discard supernatant, and place on ice.
3. Proceed to Section B. Preparation of Cytosolic Extracts

Adherent cells:

1. Wash 2 to 6 x 10^6 adherent cells with 1X PBS. Adherent cells may be harvested by gentle trypsinization.
2. Transfer to a 15 ml tube on ice. Centrifuge at 250 x g for 10 minutes at 4ºC and discard the supernatant.
3. Suspend the cell pellet in 10 ml of ice-cold 1X PBS. . Centrifuge at 250 x g for 10 minutes, discard supernatant, and place on ice.
4. Proceed to Section B. Preparation of Cytosolic Extracts

Erythrocytes:

1. Obtain blood in vacutainers containing heparin or EDTA. Dilute blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 X g for 30 min at 20°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Remove the remaining liquid from above the red blood cell/leukocyte pellet. Wash the pellet with 10 cell volumes of PBS.

4. Determine the packed cell volume and add 10 cell volumes of cold dH_2O. Mix well and incubate on ice for 10-15 minutes to lyse the red blood cells. Lysis occurs when the opaque solution changes to a brilliant clear red solution, indicating the release of hemoglobin.
5. Precipitate the hemoglobin by adding 0.25 volumes of ethanol and 0.15 volumes of chloroform. Shake for 1 min and centrifuge at 10,000 x g for 10 minutes at 4˚C.
6. Recover the clear top layer and dialyze overnight at 4°C against 1X PBS or 50 mM Potassium Phosphate, pH 7.8.
7. Centrifuge the dialyzed erythrocyte extract to remove any precipitate that formed during the dialysis and place on ice.
8. Proceed to Section B. Preparation of Cytosolic Extracts

Lymphocytes and Monocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 X g for 30 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Dilute the blood mononuclear cells with 5 volumes of PBS and centrifuge at 400 x g for 10 minutes at 4ºC. Discard the supernatant
4. Suspend the cell pellet in 10 ml of ice-cold 1X PBS. Centrifuge at 250 x g for 10 minutes, discard supernatant, and place on ice.
5. Proceed to Section B. Preparation of Cytosolic Extracts.

Tissue:

1. Remove tissue and place in cold PBS in a 50 ml conical tube. Repeatedly wash the tissue with PBS to remove blood clots and other debris.
2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
3. Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 ml of cold 1X PBS.
4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
5. Transfer the PBS containing the single cell suspension to a 50 ml conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 minute to allow large aggregates of tissue to settle out of solution.
6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 ml conical centrifuge tube. Centrifuge at 400 x g for 10 minutes at 4ºC. Discard the supernatant. Suspend the cell pellet in 50 ml of ice-cold 1X PBS. . Centrifuge at 250 x g for 10 minutes, discard supernatant, and place on ice.
7. Proceed to Section B: Preparation of Cytosolic Extracts

Section B. Preparation of Cytosolic Extracts from Cells and Tissue

1. Measure the approximate volume of the cell pellets prepared above (except for erythrocytes) and suspend the cells in 5-10 volumes of cold 1X Cell Extraction Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4ºC to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. Occasionally, the pellet may float and can easily be removed with a pipet tip.
3. Determine the protein concentration of the cleared cell lysate.
4. If not assaying for SOD immediately, snap-freeze the cleared cell extract in 100 µl aliquots by immersing in liquid nitrogen and store at -80ºC. Avoid repeated freezing and thawing of the extract.
5. The detection of SOD in subcellular fractions is detailed in reference [10].

Section C. Differentiation between SOD1, SOD2, and SOD3

1. SOD2 can be inactivated by adding 400 µl or 800 µl of ice-cold chloroform/ethanol (37.5/62.5 (v/v)) to 250 µl of erythrocyte lysate or 500 µl of cell/tissue lysate, respectively, shaking for 30 sec, and then centrifuging at 2,500 x g for 10 min. Assay the upper aqueous phase for SOD1 immediately or freeze in aliquots at -80°C.
2. The addition of cyanide ion to a final concentration of 2 mM inhibits more than 90% of SOD1 activity. SOD2 is unaffected by cyanide.
3. SOD3 is isolated from the extracellular matrix of tissue. SOD3 has been found in serum and in cerebrospinal, ascitic, and synovial fluids. Ensure that all cells are removed from the extracellular fluid by centrifuging at 250 x g for 10 minutes at 4ºC. Assay the supernatant for SOD3 activity.

VII. SOD Assay Protocol

A. Preparation of the Specrophotometer

1. Turn on your spectrophotometer and allow it to warm up for 30 minutes. Set the wavelength to 450 nm. If possible, set the cuvette chamber to 25ºC.

2. Blank the instrument against dH_2O.

B. SOD Standard Curve

Note: The SOD standard is used as a positive control to verify that the assay is working. It should not be used to calculate the units of SOD in your experimental sample.

1. The SOD standard (Cat# 7501-500-01) is provided at an approximate activity of 10 Units/µl. In 0.5 ml or 1.5 ml microtubes, prepare serial dilutions of the SOD standard with 1X SOD Buffer. Each level of SOD requires 100 µl per reaction. It is recommended that you make at least 150 µl of each serial dilution of SOD at the following concentrations: 5 units/100 µl (0.5 µl SOD Standard/100 µl), 2 units/100 µl (0.2 µl SOD Standard/100 µl), 1 unit/100 µl (0.1 µl SOD Standard/100 µl), 0.5 units/100 µl (0.05 µl SOD Standard/100 µl), and 0.25 units/100 µl (0.025 µl SOD Standard/100 µl).

2. Include a <u>100% Activity Control</u>: A reaction with 100 µl of 1X SOD Buffer (without SOD). This reaction provides the 100% activity reference point.

C. Biological Extracts

1. Make serial dilutions of your cell or tissue extracts with 1X SOD Buffer between 0.5 µg/100 µl to 100 µg/100 µl protein.

D. Setting up the Reactions

1. Add the following to a series of 1.5 ml tubes:

Component	Volume	Order of Addition
Master Mix	887.5 µl	1
Serial dilutions of your SOD standard, biological extract, or 1X SOD Buffer	100 µl	2

E. Initiation of Reactions

1. Initiate the reaction by adding 12.5 µl of 10X Xanthine solution (Cat# 7501-500-04). Cap the tube and mix well by inversion.

2. Immediately transfer the reaction to a cuvette. Place in the spectrophotometer and take absorbance readings at 450 nm every 0.5 minutes for 10 minutes at room temperature or 25ºC.

VIII. Data Interpretation

A. Determine rate of change in absorbance at 450 nm

1. Transfer the absorbance values at 450 nm (A450 nm) and the times points to an Excel or other suitable spread sheet program. Plot the A450 nm on the Y axis versus time in minutes on the X axis. Perform a linear regression on the curves, and display the linear equations. The change in absorbance at 450 nm as a function of time for the SOD standard is shown in **Figure 2**:

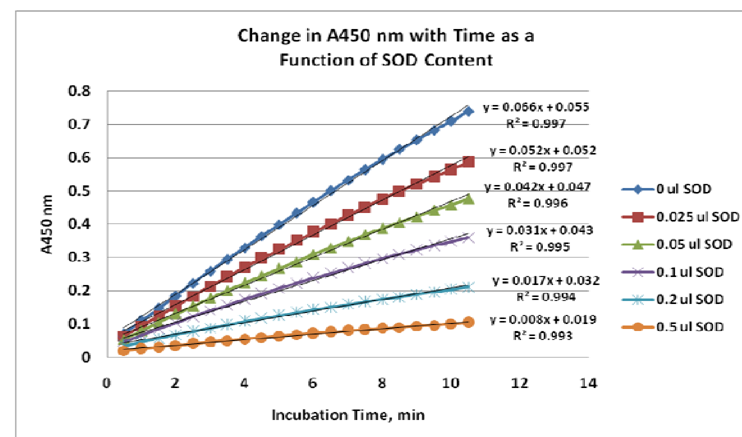


Figure 2: Change in absorbance at 450 nm with time for the SOD standard. A linear regression analysis is shown and used to determine the slopes of the curves.

B. Determine % inhibition of the rate of change in absorbance at 450 nm

1. The slope obtained in the absence of SOD (the 1X SOD Buffer control) should be maximal and is taken as the 100% value. All other slopes generated with SOD standards or biological extracts are compared to it. The % inhibition of the rate of increase in absorbance at 450 nm is calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Slope of 1X SOD Buffer Control} - \text{Slope of Sample}) \times 100}{\text{Slope of 1X SOD Buffer Control}}$$

C. Plot % inhibition versus volume SOD standard in the reaction

1. The SOD standard is provided with an activity of approximately 10 units/µl. Plot the % Inhibition versus the volume of SOD standard used in the reaction. A typical inhibition curve for the SOD standard is shown in **Figure 3**:

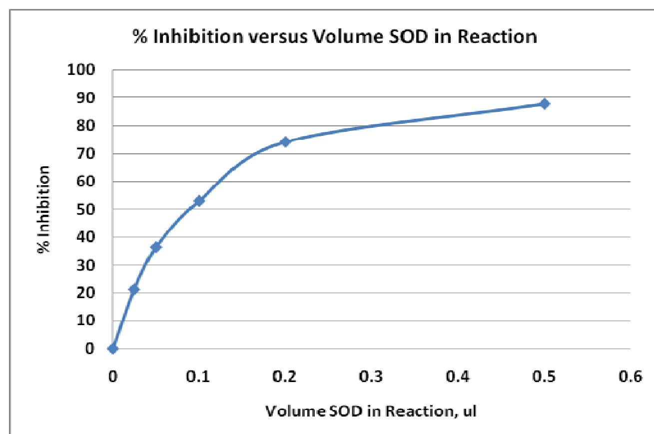


Figure 3. Inhibition curve for the SOD Standard.

D. Plot % Inhibition of your cell extract versus µg cell extract in the reaction

1. In a similar manner, plot % inhibition of your cell extract versus µg cell extract in the reaction. From the curve, determine the amount of protein in the cell extract which causes a 50% inhibition of the rate of increase in absorbance at 450 nm. This is shown in **Figure 4** for SOD activity in Jurkat cell extracts:

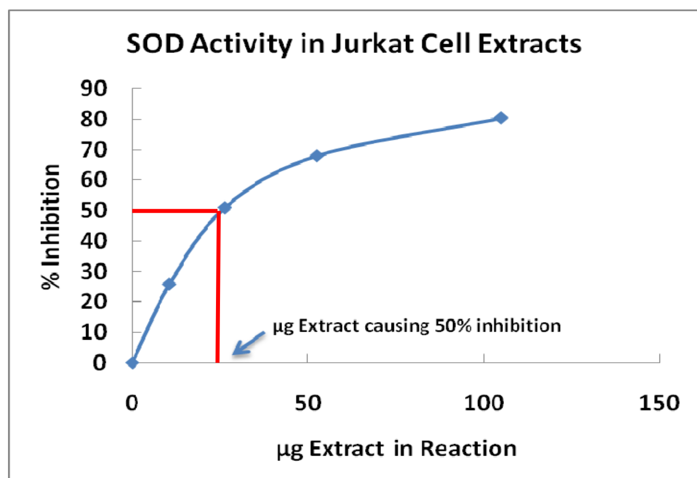


Figure 4. Inhibition curve for Jurkat cell extracts.

E. Calculation of SOD Activity in the standard and in cell/tissue extracts

1. Determine the amount of standard or cell extract protein that causes 50% inhibition. For example:

*I. Cell Extract (**Figure 4**):*

Amount of extract causing 50% inhibition	=	25.5 µg
SOD specific activity	=	1 unit/25.5 µg
	=	0.039 units/µg
Protein concentration of Jurkat cell extract	=	5.72 µg/µl
SOD concentration in extract	=	0.039 units/µg x 5.72 µg/µl
	=	0.224 units/µl

IX. References

1. Kussmaul L, Hirst J. 2006. The mechanism of superoxide production by NADH: ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *PNAS* **103**: 7607-12.
2. Passos JF, Von Zglinicki T. 2006. Oxygen free radicals in cell senescence: are they signal transducers? *Free Radic Res* **40**:1277-83.
3. Lu T, Finkel T. 2008. Free radicals and senescence. *Exp Cell Res.* **314**:1918-22**.**
4. Szeto HH. 2006. Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. *AAPS J* **8**:E521-31.
5. Peskin AV, Winterbourn CC. 2000 A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clin Chim Acta* **293:**157–66.
6. Tan AS, Berridge MV. 2000 Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *J Immunol Methods* **238:**59–68.
7. Nebot C, Moutet M, Huet P, Xu J-Z, Yadan J-C, Chaudiere J 1993. Spectrophotometric assay of superoxide dismutase activity based on the activated auto-oxidation of a tetracyclic catechol. *Anal biochem* **214**:442-51.
8. Okado-Matsumoto A, Fridovich I. 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver. *J Biol Chem* **276**:38388-93.

X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No absorbance with SOD Buffer alone	Failure to add XOD or WST-1 reagents to the Master Mix	Add XOD or WST-1 reagents to the Master Mix
	Failure to initiate reaction with Xanthine	Add 12.5 µl Xanthine to initiate the reaction
No change in absorbance in wells with sample extracts	SOD concentration in samples too high	Add greater dilutions of sample to the reaction
No inhibition of WST-1 formazan formation in wells containing cell or tissue extracts	SOD activity in cells and tissues very low	Extend reaction to 20 minutes
		Modify the Master Mix to accommodate a sample volume of 200 µl. Maintain the final reaction volume at 1 ml

XI. Related Products from Trevigen

Oxidative Damage Kits

Catalog #	Description	Size
7510-100-K	Glutathione Reductase Assay	100 Reactions
7511-100-K	HT Glutathione Assay Kit	384 tests
7512-100-K	HT Glutathione Peroxidase Assay Kit	480 tests
7513-500-K	HT Glutathione Reductase Assay Kit	500 tests
7501-100-K	HT Superoxide Dismutase Assay Kit	480 tests

PARP Assay Kits:

Catalog #	Description	Size
4677-096-K	HT Universal Colorimetric PARP Assay w/ Histone Coated Strip Wells	96 samples
4676-096-K	Universal Chemiluminescent PARP Assay w/Histone Coated Strip Wells	96 samples
4690-096-K	HT F Homogeneous PARP Inhibition Assay Kit	96 tests
4667-250-01	Recombinant Human PARP Enzyme	250 µl
4668-100-1	Recombinant Human PARP (High Specific Activity)	1000 Units

DNA Damage Antibodies:

Catalog #	Description	Size
4410-PC-100	Fen-1	100 µl
4411-PC-100	γ-H2AX	100 µl
4350-MC-100	UVssDNA	100 µg
4431-MC-100	XPF	100 µg
Catalog #	Description	Size
4421-MC-100	XRCC1	100 µg
4354-MC-050	anti-8-oxo-dG	50 µl

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK 4040-100-FM	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4045-01K-FK 4045-01K-FM	Endonuclease III Kit	Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycytosine,5-hydroxy-uracil, methyl-tartonylurea, thymine ring saturated or fragmentation product	75 samples 100 samples
4130-100-FK 4130-100-FM	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4055-100-FK 4055-100-FM	T4-PDG Kit	Cis-syn isomers of cyclo-butane pyrimidine dimers	75 samples 100 samples
4065-100-FK 4065-100-FM	cv-PDG Kit	Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	75 samples 100 samples
4100-100-FK 4100-100-FM	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples 100 samples

CometAssay™ Kits:

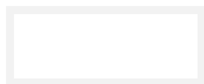
Catalog #	Description	Size
4250-050-K	CometAssay™ Kit	50 samples
4251-050-K	CometAssay™ Silver Kit	50 samples
4252-040-K	CometAssay™ Higher Throughput Kit	40 samples
4253-096-K	CometAssay™ Kit 96 Wells	96 samples
4254-200-K	CometAssay™ Silver Staining Kit	200 samples

Control Cells:

Catalog #	Description	Size
4256-010-CC	CometAssay™ Control Cells	10 assays

-notes-

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.



P6/16/08v1