GPX4 Inhibitor Screening Assay Kit

Item No. 701880

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	96 wells Quantity/Size	Storage Temperature
701881	GPX4 Assay Buffer (10x)	1 vial/5 ml	-20°C
701882	GPX4 Enzyme (human, recombinant)	1 vial/275 µl	-80°C
703119	GPX NADPH	1 vial	-20°C
701883	GPX4 Glutathione	1 vial	-20°C
701884	GPX4 Glutathione Reductase	1 vial	-20°C
701885	GPX4 Cumene Hydroperoxide	1 vial/2.5 ml	-20°C
701886	GPX4 Inhibitor (ML-162)	1 vial/100 µl	-20°C
700020	Half Volume 96-Clear Plate	1 plate	RT
400012	96-Well Cover Sheet	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance at a wavelength of 340 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of ultrapure water is recommended. Pure water - glass-distilled or deionized - may not be acceptable *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Microcentrifuge tubes

INTRODUCTION

Background

Glutathione peroxidase 4 (GPX4) is a selenocysteine-containing glutathione peroxidase that reduces phospholipid hydroperoxides to phospholipid alcohols, thereby protecting cellular membranes from oxidative damage.[1,2] It is a monomeric protein consisting of a thioredoxin motif and a selenocysteine-glutamine-tryptophan catalytic triad. Three isoforms of GPX4, mitochondrial (mGPX4), also known as long-form GPX4, cytosolic (cGPX4), also known as short-form GPX4, and nuclear (nGPX4/snGPX4) are formed *via* alternative splicing and are ubiquitously expressed in mammals, with the highest mRNA levels found in testes.[2-4] Systemic knockout of *Gpx4* in mice is embryonic lethal but can be rescued by expression of the cGPX4 isoform.[5,6] *mGpx4*- or *nGpx4*-specific knockout mice are viable but display sperm structural abnormalities.[7,8] GPX4 is a key regulator of ferroptosis that inhibits ferroptotic cell death by preventing iron-dependent accumulation of toxic phospholipid hydroperoxide species.[2] The T allele of a SNP in GPX4, rs713041, is associated with an increased risk for a variety of cancers, as well as other diseases, while homozygous expression of the less common G allele in the rs3746165 SNP is associated with a reduced risk of lethal prostate cancer.[9,10] The role of GPX4 in cancer and ferroptotic cell death indicates the potential for pharmacological inhibition of GPX4 in the treatment of cancer.

About This Assay

Cayman's GPX4 Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human GPX4, a negative regulator of the ferroptosis pathway. The assay measures GPX4 indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPX4, is recycled to its reduced state by GR and NADPH:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPX4}} \text{R-O-H} + \text{GSSG} + \text{H}_2\text{O}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}). The rate of decrease in A_{340} is directly proportional to GPX4 activity. The GPX4 inhibitor ML-162 is included as a positive control.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted GPX4 Assay Buffer at a concentration 10X the desired final assay concentration (*e.g.*, for a 25 µM final assay concentration, a 250 µM stock should be made). This solution may contain up to 50% DMSO or up to 25% dimethyl formamide (DMF) or short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of DMSO in the assay will then be ≤5% and the final concentration of DMF or short-chain alcohols will be ≤2.5% (see 'Effects of Solvents' on page 18).

Reagent Preparation

NOTE: It is important that all reagents, except GPX4 Enzyme, are equilibrated to room temperature before proceeding with reagent preparation. The GPX4 enzyme should be stored on ice while the other reagents are allowed to reach room temperature.

1. **GPX4 Assay Buffer (10X)**

   Mix 2 ml of GPX4 Assay Buffer (10X) (Item No. 701881) with 18 ml of pure water to make 20 ml GPX4 Assay Buffer (1X). The GPX4 Assay Buffer (1X) should be discarded if not used within the same day. Once thawed, the GPX4 Assay Buffer (10X) may be stored at 4°C for at least one month.

2. **GPX4 Enzyme (human, recombinant)**

   This vial contains recombinant human GPX4 Enzyme (Item No. 701882). The enzyme should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 125 µl of GPX4 Enzyme with 875 µl GPX4 Assay Buffer (1X). This is enough enzyme to assay approximately 48 wells. It is recommended that the enzyme be diluted 5-10 minutes prior to performing the assay. The diluted enzyme is stable for 2 hours on ice. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

3. **GPX NADPH**

   This vial contains lyophilized NADPH (Item No. 703119). Reconstitute the contents of the vial with 4 ml GPX4 Assay Buffer (1X). The reconstituted NADPH is stable at room temperature for two hours or for two days when stored at 4°C.

4. **GPX4 Glutathione**

   This vial contains lyophilized Glutathione (Item No. 701883). Reconstitute the contents of the vial with 2.5 ml GPX4 Assay Buffer (1X). The reconstituted glutathione is stable at room temperature for two hours or for two days when stored at 4°C.

5. **GPX4 Glutathione Reductase**

   This vial contains lyophilized Glutathione Reductase (Item No. 701884). Reconstitute the contents of the vial with 2.5 ml GPX4 Assay Buffer (1X). The reconstituted glutathione reductase is stable at room temperature for two hours or for two days when stored at 4°C. *NOTE: Do not freeze the reconstituted reagent.*

6. **GPX4 Cumene Hydroperoxide**

   This vial contains 2.5 ml of a Cumene Hydroperoxide solution (Item No. 701885). Once thawed, vortex vigorously before use.

7. **GPX4 Inhibitor (ML-162)**

   This vial contains 100 µl of 0.5 mM GPX4 Inhibitor (ML-162) (Item No. 701886) in DMSO, which can be used as a positive control. Mix 20 µl GPX4 Inhibitor (ML-162) with 20 µl GPX4 Assay Buffer (1X). If all of the GPX4 Inhibitor (ML-162) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

ASSAY PROTOCOL

Plate Set Up

The 96-well plate included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control GPX4 Inhibitor (ML-162), be assayed in triplicate.

It is suggested that the contents of each well are recorded on the template sheet provided on page 21. A typical layout of samples to be measured in triplicate is shown in Figure 1, below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is important that all prepared reagents are equilibrated to room temperature.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- Measure absorbance at 340 nm using a plate reader.

Performing the Assay

1. **Background Wells**: add 40 µl GPX4 Assay Buffer (1X) and 10 µl of solvent to three wells. Use the same solvent concentration used for the unknown inhibitor and the positive control, GPX4 Inhibitor (ML-162).
2. **100% Initial Activity Wells**: add 20 µl of GPX4 Assay Buffer (1X), 20 µl of diluted GPX4 Enzyme, and 10 µl of solvent to three wells. Use the same solvent concentration used to dissolve the unknown inhibitor and the positive control, GPX4 Inhibitor (ML-162).
3. **Inhibitor/Positive Control Wells**: add 20 µl of GPX4 Assay Buffer (1X), 20 µl of diluted GPX4 Enzyme, and 10 µl of unknown inhibitor or the positive control GPX4 Inhibitor (ML-162) to three wells.
4. Mix the contents of the wells by pipetting, cover the plate with the 96-well Cover Sheet (Item No. 400012), and incubate at room temperature for 60 minutes.

NOTE: To observe greater than 90% inhibition of GPX4 by ML-162, a 60-minute pre-incubation is required. Other inhibitors may not require the entire pre-incubation to observe high levels of GPX4 inhibition. Thus, pre-incubation time may require optimization for new GPX4 inhibitors.

5. During GPX4 Enzyme and GPX4 Inhibitor (ML-162) pre-incubation, prepare mix containing glutathione and glutathione reductase. Mix 500 µl of reconstituted glutathione with 500 µl of reconstituted glutathione reductase. This will be enough for approximately 48 wells.
6. Remove the plate cover and add 20 µl of the glutathione/glutathione reductase mix to all wells being used.
7. Add 20 µl reconstituted NADPH to all wells being used and mix well by pipetting.
8. Add 10 µl cumene hydroperoxide to all wells being used and mix well by pipetting.
9. Remove the plate cover and measure absorbance at 340 nm using a plate reader every 30-60 seconds for 5 minutes.

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute:
   a. Plot the absorbance values as a function of time and perform linear regression to obtain the slope (rate) of the linear portion of the curve (for example data, see Figure 2 on page 14). Use the absolute value in subsequent steps.

   OR

   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min.} = \frac{^{*}|\, A_{340}\,(\text{time 2}) - A_{340}\,(\text{time 1})\,|}{\text{time 2 (min.)} - \text{time 1 (min.)}}$$

**Use the absolute value.*

2. Determine the background-corrected rates by subtracting the average background rate from the 100% initial activity samples and inhibitor samples.
3. Determine the percent inhibition or percent initial activity for each inhibitor using one of the following equations:

$$\text{\% Inhibition} = \left[\frac{(\text{corrected 100\% initial activity} - \text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}}\right] \times 100$$

$$\text{\% Activity} = \left[\frac{(\text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}}\right] \times 100$$

4. Graph the percent inhibition or percent initial activity as a function of the inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition). Inhibition of recombinant human GPX4 by GPX4 Inhibitor (ML-162) is shown in Figure 3 (see page 16).

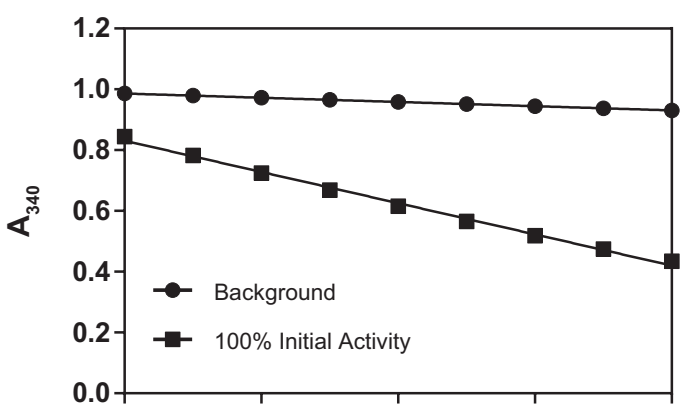


Figure 2. GPX4 Activity. The A_{340} of background and 100% initial activity wells plotted over time.

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[15]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{\left| \mu_{c+} - \mu_{c-} \right|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control or inhibitor sample
c-: Negative control or 100% initial activity

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's GPX4 Inhibitor Screening Assay Kit was determined to be 0.68.

Sample Data:

The data presented here is an example of data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

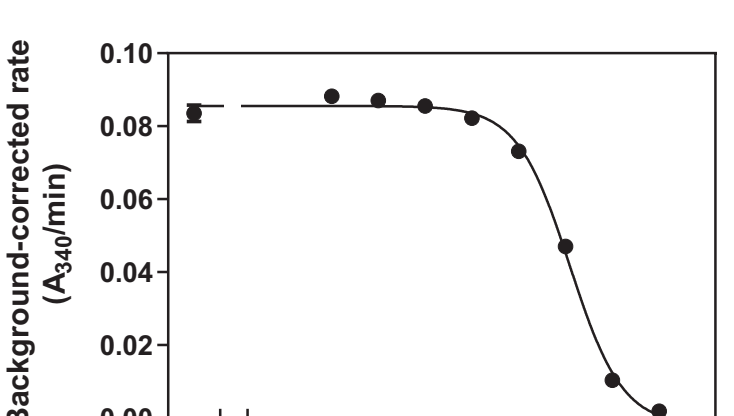


Figure 3. Inhibition of GPX4 by GPX4 Inhibitor (ML-162). Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity.

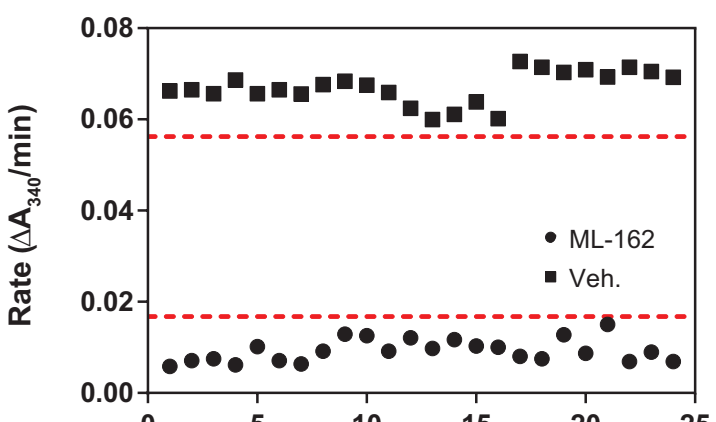


Figure 4. Typical Z′ data for the GPX4 Inhibitor Screening Assay Kit. Data are shown from 24 replicates each for vehicle control (Veh.) and 25 µM GPX4 inhibitor (ML-162) prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.68. The red lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents:

Samples may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (e.g., MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤5% for DMSO and ≤2.5% for DMF and short-chain alcohols.

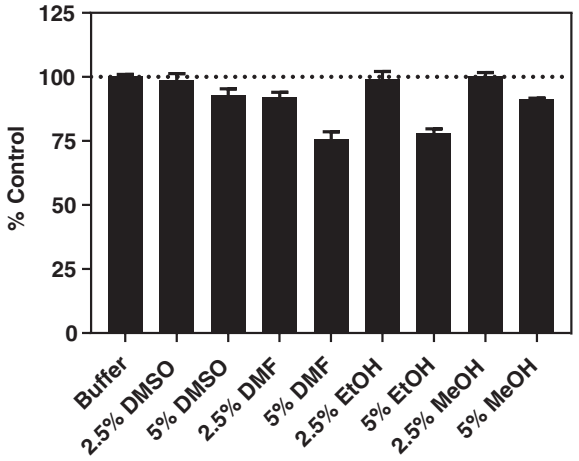


Figure 5. The effect of solvent on the readout of GPX4 activity. The data are shown as the mean ± standard deviation for quadruplicate reactions containing the indicated concentration of solvents.

Precision:

Intra-assay precision was determined by analyzing 8-24 measurements of the background, vehicle, and 25 µM GPX-4 inhibitor (ML-162) on the same day. The intra-assay coefficients of variation were 3.5, 2.9, and 5.3%, respectively. The intra-assay coefficient of variation for the IC_{50} values of eight inhibition curves performed on the same day was 9.1%.

Inter-assay precision was determined by analyzing inhibition with GPX4 Inhibitor (ML-162) in separate assays on different days. The inter-assay coefficient of variance for the IC_{50} values was 18.5%.

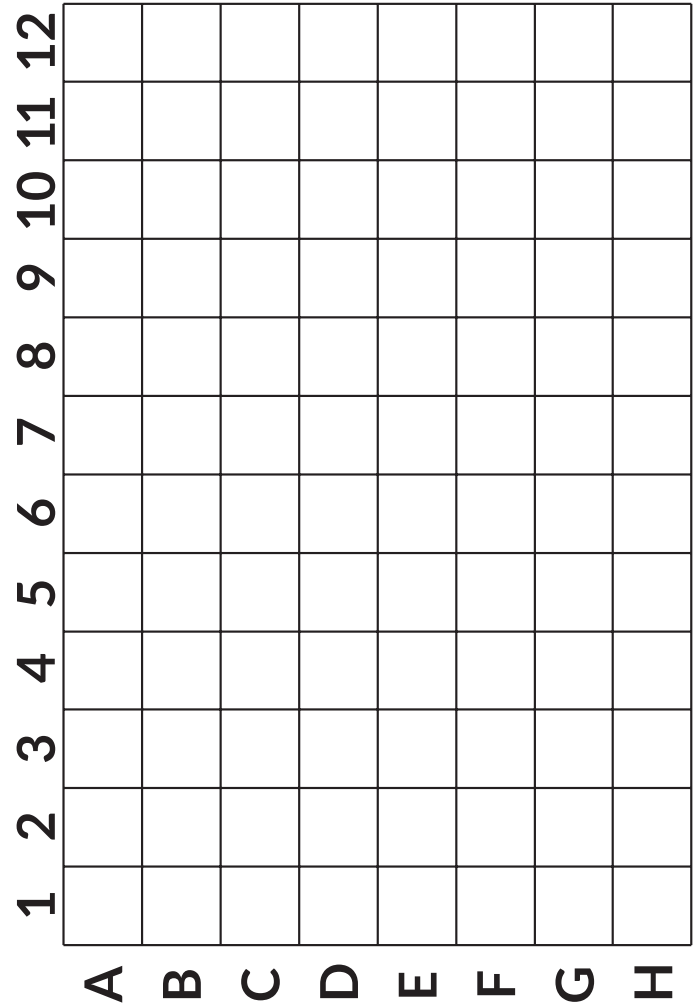
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
100% initial activity wells have a rate similar to background wells	Enzyme was not added to the well(s)	Make sure to add all of the components to the well(s)
No inhibition was seen with inhibitor	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Imai, H. and Nakagawa, Y. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radic. Biol. Med.* **34(2)**, 145-169 (2003).
2. Forcina, G.C. and Dixon, S.J. GPX4 at the crossroads of lipid homeostasis and ferroptosis. *Proteomics* **19(18)**, e1800311 (2019).
3. Maiorino, M., Scapin, M., Ursin, F., *et al.* Distinct promoters determine alternative transcription of *gpx-4* into phospholipid-hydroperoxide glutathione peroxidase variants. *J. Biol. Chem.* **278(36)**, 34286-34290 (2003).
4. Mannweiler, S., Amersdorfer, P., Trajanoski, S., *et al.* Heterogeneity of prostate-specific membrane antigen (PSMA) expression in prostate carcinoma with distant metastasis. *Pathol. Oncol. Res.* **15(2)**, 167-172 (2009).
5. Imai, H., Hirao, F., Sakamoto, T., *et al.* Early embryonic lethality caused by targeted disruption of the mouse PHGPx gene. *Biochem. Biophys. Res. Commun.* **305(2)**, 278-286 (2003).
6. Liang, H., Yoo, S.-E., Na, R., *et al.* Short form glutathione peroxidase 4 is the essential isoform required for survival and somatic mitochondrial functions. *J. Biol. Chem.* **284(45)**, 30836-30844 (2009).
7. Sullivan, A.F., McQuay, H.J., Bailey, D., *et al.* The spinal antinociceptive actions of morphine metabolites morphine-6-glucuronide and normorphine in the rat. *Brain Res.* **482(2)**, 219-224 (1989).
8. Andersson, M., Björkhem-Bergman, L., Ekström, L., *et al.* Detection of morphine-3-sulfate and morphine-6-sulfate in human urine and plasma, and formation in liver cytosol. *Pharmacol. Res. Perspect.* **2(6)**, e00071 (2014).
9. Barbosa, P., Abo El-Magd, N.F., Hesketh, J., *et al.* The role of rs713041 glutathione peroxidase 4 (*GPX4*) single nucleotide polymorphism on disease susceptibility in humans: A systematic review and meta-analysis. *Int. J. Mol. Sci.* **23(24)**, 15762 (2022).
10. Van Blarigan, E.L., Ma, J., Kenfield, S.A., *et al.* Plasma antioxidants, genetic variation in SOD2, CAT, GPX1, GPX4, and prostate cancer survival. *Cancer Epidemiol. Biomarkers Prev.* **23(6)**, 1037-1046 (2014).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.