Hydrogen Peroxide Ratiometric MaxSpec® Kit

Item No. 601460

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Concentration	Storage
601462	MitoB MaxSpec® Standard	1 ampule/1 ml	5 mM in Ethanol	-20°C
601463	MitoP MaxSpec® Standard	1 ampule/1 ml	1 mM in Ethanol	-20°C
601461	MitoB-d_{15}/MitoP-d_{15} Internal Standard Mixture	1 ampule/1 ml	10 µM MitoB-d_{15}, 5 µM MitoP-d_{15} in Ethanol	-20°C

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.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

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 on page 3 and used before the expiration date indicated on the outside of the box.

All reagents in the Hydrogen Peroxide Ratiometric MaxSpec® Kit may be used as provided. Reagents should be handled cold and resealed immediately after use to prevent solvent evaporation. Frozen reagents should be thawed completely prior to use to ensure uniform concentration. The entire contents of the Kit should be stored at -20°C when not in use.

Materials Needed But Not Supplied

1. A laboratory equipped for culture of mammalian cells and all associated reagents
2. Speed vac, vacuum filtration system and filter plates. *NOTE: That vacuum filtration is not necessary – alternatively, a 0.2 µm PVDF filter may be used for sample filtration.*
3. Mass spectrometry vials and lids (such as Phenomenex P/N AR0-9921-13C and AR0-8952-13B)
4. LC-MS grade acetonitrile (ACN)
5. LC-MS grade formic acid (FA)
6. Absolute grade ethanol
7. MilliQ deionized water
8. Catalase (for cell cultures), a minimum of 5,000 units (enough to make a 50 U/ml solution in 100 ml)

INTRODUCTION

About This Assay

Cayman's Hydrogen Peroxide Ratiometric MaxSpec® Kit enables the measurement of mitochondrial H_2O_2 in cell culture and animal models. MitoB (3-hydroxybenzyl)triphenylphosphonium bromide or MitoBoronic Acid) is taken up specifically by polarized mitochondria and accumulates in the matrix. The selective reaction of the arylboronic acid moiety of MitoB with H_2O_2 forms the phenol product, MitoP (MitoPhenol). Therefore, the increase in the MitoP/MitoB ratio over time indicates the level of mitochondrial H_2O_2. The ratio of MitoP/MitoB is measured in sample extracts by LC-MS/MS using deuterated internal standards (IS) (MitoB-d_{15} and MitoP-d_{15}) to correct for extraction and detection variations.

- Some tissues such as heart and muscle will require several rounds of homogenization
- Do not use tissue sections of more than ~100 mg as this will have an adverse effect on mass spectrometry detection
- Standards should be prepared in parallel with samples, and it is important that all samples are prepared using the same internal standard solution. Samples can be compared to a standard curve prepared on a separate day if the I.S. solution is consistent.
- Use well-rinsed or new glassware as trace detergents in glassware will decrease the performance of the mass spectrometer

ASSAY PROTOCOL

Reagent Preparation

Solution A

Prepare a solution of 99.9% Acetonitrile (ACN) and 0.1% Formic Acid (FA). Use well rinsed or new glassware as trace detergents in glassware will decrease the performance of the mass spectrometer. After use, this solution should be stored in a cool dry place and used within 2 weeks of preparation.

Solution B

Prepare a solution of 60% Acetonitrile and 0.1% Formic Acid in HPLC grade water. Use well rinsed or new glassware as trace detergents in glassware will decrease the performance of the mass spectrometer. After use, this should be stored in a cool dry place and used within 2 weeks of preparation.

Solution C

Prepare a solution of 20% Acetonitrile and 0.1% Formic Acid in HPLC grade water. Use well rinsed or new glassware as trace detergents in glassware will decrease the performance of the mass spectrometer. After use, this solution should be stored in a cool dry place and used within 2 weeks of preparation.

MitoB and MitoP Calibrators Preparation

MitoB Calibrators

Dilute 5 mM MitoB MaxSpec® Standard 1:5 in ethanol to make a 1 mM Working Stock (200 µl standard:800 µl ethanol) to be used in the preparation of calibrators. Calibrators can be stored at -20°C for up to 2 months.

Tube	Concentration of MitoB Standard	Volume of Ethanol	Volume of MitoB (1 mM Working Stock)
B1	100 µM	900 µl	100 µl of 1 mM
B2	50 µM	500 µl	500 µl of 100 µM (B1)
B3	10 µM	900 µl	100 µl of 100 µM (B1)
B4	5 µM	900 µl	100 µl of 50 µM (B2)
B5	1 µM	900 µl	100 µl of 10 µM (B3)
B6	0 µM	100 µl	0 µl

Table 1. Volumes and concentrations of MitoB Calibrators

MitoP Calibrators

Dilute 1 mM MitoP MaxSpec® Standard 1:10 in ethanol to make a 100 µM Working Stock (100 µl Standard:900 µl ethanol) to be used in the preparation of calibrators. Calibrators can be stored at -20°C for up to 2 months.

Tube	Concentration of MitoP Standard	Volume of Ethanol	Volume of MitoP (100 µM Working Stock)
P1	25 µM	750 µl	250 µl of 100 µM
P2	10 µM	900 µl	100 µl of 100 µM
P3	5 µM	500 µl	500 µl of 10 µM (P2)
P4	1 µM	900 µl	100 µl of 10 µM (P2)
P5	0.5 µM	900 µl	100 µl of 5 µM (P3)
P6	0.1 µM	900 µl	100 µl of 1 µM (B4)
P7	0 µM	100 µl	0 µl

Table 2. Volumes and concentrations of MitoP Calibrators

Sample Preparation

Tissue Samples

MitoB and MitoP have been shown to work *in vivo*.[1] Follow this procedure for animal treatment and sample preparation and then follow the kit instructions to prepare curves and analyze samples.

Cell Samples

MitoB Treatment for Cell Culture Studies

MitoB was originally intended for use as an *in vivo* probe it can, however, be used in cell culture studies by sampling a portion of the cell culture media and measuring the concentration of H_2O_2.

NOTE: The following protocol is set up for 24 well cell culture plates, using a volume of 1 ml. Protocol can be adjusted to better meet experimental demands.

1. Make a 1 µM MitoB Working Solution by diluting 10 µl of 5 mM MitoB MaxSpec™ Standard into 50 ml of cell culture media. Also add catalase (50 U/ml) in the supernatant to control for peroxide changes outside the cell).
2. For adherent cells, carefully remove spent media by aspiration. For suspension cells, transfer the cell suspension to 1.5 ml microcentrifuge tubes and centrifugation at 120 × g for 5 minutes, remove supernatant by aspiration.
3. Replace cell culture media with MitoB Working Solution and incubate for 3-6 hours under standard conditions with an appropriate treatment (e.g., oxidative stress, antioxidant, etc.).
4. After 3-6 hours, transfer 0.5 ml of medium to a 1.5 ml microcentrifuge tube and quickly freeze the medium on dry ice or liquid nitrogen. *NOTE: If you are using a suspension cell line, you will need to centrifuge the cells (3 minutes at 1,000 × g) and use the supernatant.*
5. Flash-freeze the samples in liquid nitrogen and store them at -80°C until required. It is recommended that samples stored at -80°C be analyzed within 3-6 months.

Sample Preparation/Extraction:

1. Thaw the samples and transfer 200 µl of medium to a clean 1.5 ml microcentrifuge tube on ice.
2. Add 10 µl of MitoB-d_{15}/MitoP-d_{15} Internal Standard Mixture to samples and vortex for 30 seconds.
3. Add 50 µl of Solution A (99.9% ACN and 0.1% FA), and vortex for a further for 30 seconds, then centrifuge for 10 minutes at 16,000 × g in a benchtop centrifuge at room temperature.
4. Load the sample supernatant into a 1 ml syringe (by removing the plunger and pipetting from above) fitted onto a 0.22 µM PVDF filter pre-wetted with Solution A in HPLC-grade H_2O and filter slowly into a fresh microcentrifuge tube on ice. *NOTE: Samples must be filtered carefully to avoid excess pressure and rupture of the filter.*
5. Flash-freeze the samples in liquid nitrogen and store them at -80°C until required. It is recommended that samples stored at -80°C be analyzed within a 2 month timeframe.
6. Analyze these standards by LC-MS/MS as described in the LC/MS section below. The response of MitoB relative to its deuterated IS against concentration should be linear over the range 1–1,000 pmol, with an r^2 routinely >0.99.

Preparation of Standard Curves for LC-MS/MS Analysis Cell Culture Samples:

1. Add 200 µl of control cell culture media to thirteen 1.5 ml microcentrifuge tubes labeled in accordance with the MitoB/P Calibrators prepared as described above.
2. Add 10 µl of the MitoB-d_{15}/MitoP-d_{15} Internal Standard Mixture to all tubes and vortex for 30 seconds.
3. Add 10 µl of each Calibrator (prepared as described) to the appropriately labeled tubes
4. Analyze these standards by LC-MS/MS as described in the LC-MS/MS section below. The response of MitoB or MitoP relative to its deuterated IS against concentration should be linear over the range 1–1,000 pmol, with an r^2 routinely >0.99. See Plotting the Standard Curve below.

Plotting the Standard Curve

Using the dilution schemes described on page 12, the final concentration of MitoB/P for each standard is as follows:

MitoB	Final Amount (pmols)	MitoP	Final Amount (pmols)
A	1,000	A	250
B	500	B	100
C	100	C	25
D	50	D	10
E	10	E	5
F	0	F	1
-	-	G	0

Table 3. Plotting the Standard Curve

LC-MS/MS

Analyze the samples by LC-MS/MS with multiple reaction monitoring in positive ion mode. A Waters Acquity UPLC with Xevo TQD mass spectrometer was used for the LC-MS/MS method development. These approaches can be easily adapted for any conventional LC-MS/MS system.

HPLC Setup: Fit the Acquity UPLC 1.7 µm BEH phenyl reverse phase column (2.1 x 100 mm) with a 1.7 µm BEH phenyl guard column (2.1 x 5 mm), and place in an Acquity UPLC system at ambient temperature. Set up a gradient elution method with Solutions A and B, delivered as outlined in the table below. The HPLC flow rate was 300 µl/min. 5 µl of supernatant obtained from sample preparation is injected. MitoB, MitoP, and their internal standards were eluted from 2.7-2.8 minutes using current conditions. The flow is diverted from mass spectrometer to waste at the time range from 0-2.5 minutes and 3-11 minutes during samples to reduce the solvent entering the mass spec.

Time (minutes)	%A	%B
0-0.5	90	10
1	70	30
3	30	70
4	30	70
5	5	95
7.5	5	95
8-11	90	10

Table 4. HPLC Mobile Phase Gradient

Mass Spectrometer Setup: The eluted MitoB and MitoP were ionized by electrospray ionization in positive ion mode with nitrogen as the desolvation gas and argon as the collision gas. The following mass spectrometric parameters are used: source spray voltage, 3 kV; cone voltage, 62 V; ion source temperature, 150°C; collision energy, 52V; desolvation temperature 500°C. The MitoB and MitoP ions are selectively monitored using multiple reaction monitoring in the same LC-MS/MS analysis. The MS/MS transitions used were the same as described in Nature Protocol.[2] For better sensitivity, the transitions may be optimized by directly infusing 1 µM stock solutions of MitoP-d_{15}, MitoP, MitoB-d_{15} or MitoB in 50% ACN/50% H_2O (vol/vol) into the mass spectrometer at 5 µl $min.^{-1}$.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)
MitoB	397.4	183.0
MitoB-d_{15}	412.5	191.1
MitoP	369.4	183.0
MitoP-d_{15}	384.5	191.1

Table 5. Precursor/Product Ion Parameters

1. Before running a full experiment be sure to tune all standards to the transitions provided in the previous table per the instructions recommended by your instrument manufacturer.
2. With the HPLC method set up and equilibrated, inject the MitoP/MitoB high calibration standard as a system suitability standard to ensure that the baseline of the LC-MS/MS trace is stable and that the LC-MS/MS system is responding appropriately to the compounds. MitoB and MitoP typically elute at about 9-11 minutes, with the peaks overlapping but with MitoB slightly before MitoP. Typical experimental chromatograms produced by LC-MS/MS analysis are shown n Figure 1, below.

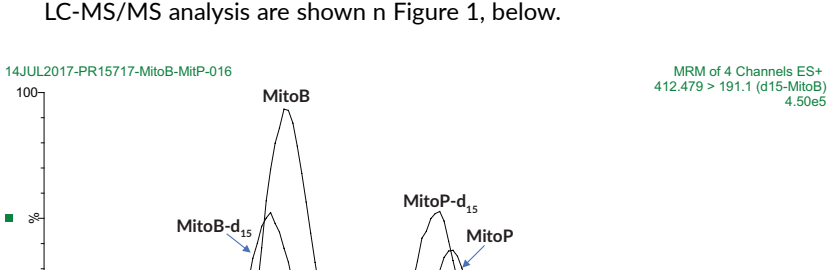

Figure 1. Extracted mass chromatogram of MitoB-d_{15}, MitoB, MitoP-d_{15}, MitoP, obtained by LC-MS/MS analysis of HepG2 cells.

3. Thaw the samples and standards in autosampler vials (if they were stored frozen) and keep them at 4°C while awaiting injection by the autosampler. Set up the vials in the following suggested order: solvent blank, 1 µM MitoB standard (Tube B5), 1 µM MitoP standard (Tube P4), solvent blank, calibration curve, and samples.
4. Determine peak areas for the MitoB, MitoP, and Internal Standards and calculate the MS response for the samples and calibration curve by dividing the area for each compound by the area for its corresponding internal standards. At this stage, export the data into Excel to facilitate further calculations. As each sample is injected twice, determine the mean MS response of the two injections. To produce the calibration curves, plot the mean MS response against pmol compound for MitoP and MitoB.
5. From the calibration curves determine the MitoP and MitoB concentrations in each sample and calculate the resulting MitoP/MitoB ratio. Correct the ratios for the t = 0 control, to correct for any background oxidation in the MitoB stock injected or any that occurs during processing and storage. The means of multiple biological replicates are determined along with an appropriate measure of error.

Performance Characteristics

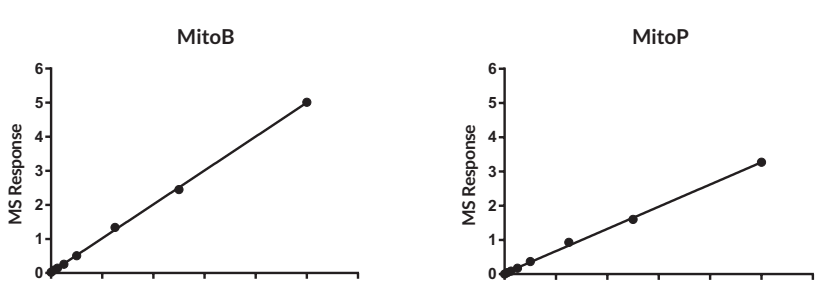

Figure 2. Standard Curves for MitoB and MitoP.

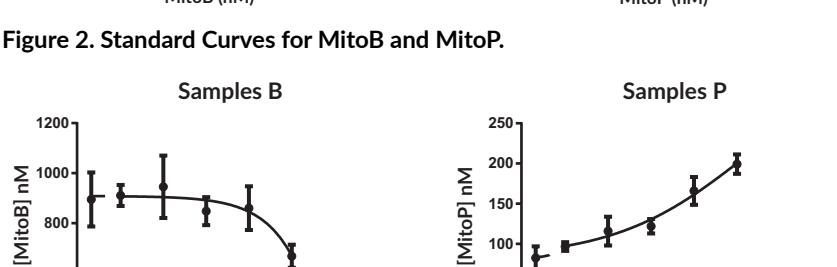

Figure 3. HepG2 cells treated with Menadione, followed by MitoB.

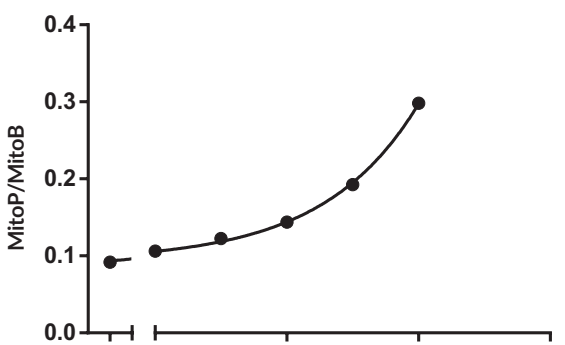

Figure 4. Ratio of MitoP to MitoB obtained from Figure 3. Data shows a concentration dependent increase in the ratio of MitoP:MitoB in response to positive control.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No MitoB or MitoP detected in the system suitability standard	A. Fault with the LC-MS/MS system B. Incorrect method settings C. Standards made incorrectly	A. Check for hardware issues such as gas supply, column, and LC fluidic connections. Reset the system if necessary and rerun the samples B. Check software settings (monitored transistions) and all HPLC and mass spectrometer method settings. Incorrect gradient settings, improper flow rate, incorrect tune file, and many other method related settings can result in the inability to detect the desired peaks. C. Prepare fresh standards and recheck mass spectrometer tuning.
MitoB, MitoP, or ISs detected in the system suitability run but not in samples	Error in the sample preparation/ extraction	Replace extraction solutions B and C
No MitoB or MitoP detected in the samples (but the calibration curve and the ISs in the samples are detected)	Error in the fly injections	Check the injection solution for the correct concentration of MitoB (1 mM Final).
Rising pressure in the HPLC	Insoluble debris that has bypassed the filtration and centrifugation clean-up steps	This may occur due to precipitation in the auto sampler vials during storage. If so, re-centrifuge and/ or re-filter the samples as described in protocol. It may also be necessary to replace the guard column and prepare fresh mobile phase solutions
Change in HPLC peak shape or retention time	Deterioration of the LC column or mobile phase not equilibrated.	Replace the guard column and possibly the HPLC column. If this occurs during a sample run, then any changes to the analysis of the calibration curves and standards run at different times during the analysis can be used to account for any drift in mass spectrometer response. Retention time drift is typically a result of an unequilibrated column. Wash column with 10-20 column volumes of mobile phase and check retention time reproducibility with repeat standard injections.

References

1. Cochemé, H.M., Quin, C., McQuaker, S.J., *et al.* Measurement of H_2O_2 within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. *Cell Metabolism* **13(3)**, 340-350 (2011).
2. Cochemé, H.M., Logan, A., Prime, T.A, *et al.* Using the mitochondria-targeted ratiometric mass spectrometry probe MitoB to measure H_2O_2 in living Drosophila. *Nature Protocols* **7(5)**, 946-958 (2012).

Additional Reading

1. Latorre-Pellicer, A., Moreno-Loshuertos, R., Lechuga-Vieco, A.V., *et al*. Mitochondrial and nuclear DNA matching shapes metabolism and healthy ageing. *Nature* **535(7613)**, 561-565 (2016).
2. Chouchani, E.T., Pell, V.R., Gaude, E., *et al*. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **515(7527)**, 431-435 (2014).
3. Logan, A., Shabalina, I.G., Prime, T.A., *et al*. *In vivo* levels of mitochondrial hydrogen peroxide increase with age in mtDNA mutator mice. *Aging Cell* **13(4)**, 765-768 (2014).
4. Salin, K., Auer, S.K., Villasevil, E.M., *et al*. Using the mitoB method to assess levels of reactive oxygen species in ecological studies of oxidative stress. *Sci. Rep.* **7(41228)**, (2017).
5. Rosen, G.M., Pou, S., Ramos, C.L., *et al.* Free radicals and phagocytic cells. *FASEB Journal* **9**, 200-209 (1995).

NOTES

Warranty and Limitation of Remedy

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