Hydrogen Peroxide Ratiometric MaxSpec® Kit

Item No. 601460

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GENERAL INFORMATION

Materials Supplied

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

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 use of all associated reagents
2. Speed vac, vacuum filtration system, and filter plates. *NOTE: 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
5. LC-MS-grade formic acid
6. Absolute ethanol
7. HPLC-grade water
8. Catalase (for cell cultures), a minimum of 5,000 units (enough to make 100 ml of a 50 U/ml solution)

INTRODUCTION

About This Assay

Cayman's Hydrogen Peroxide Ratiometric MaxSpec® Kit enables the measurement of mitochondrial hydrogen peroxide (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 (MitoB-d_{15} and MitoP-d_{15}) to correct for extraction and detection variations.

- Certain tissues, such as heart and muscle tissue, will require several rounds of homogenization.
- Do not use tissue sections of greater 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 internal standard solution is consistent.

ASSAY PROTOCOL

Reagent Preparation

NOTE: Use well-rinsed or new glassware as trace detergents in glassware will decrease the performance of the mass spectrometer.

Mobile Phase A

Prepare a 0.1% formic acid solution in HPLC-grade water. After use, this solution should be stored in a cool, dry place and used within two weeks of preparation.

Mobile Phase B

Prepare a 0.1% formic acid solution in HPLC-grade acetonitrile. After use, this solution should be stored in a cool, dry place and used within two weeks of preparation.

Solution C

Prepare a solution of 95:5 acetonitrile:HPLC-grade water with 0.1% formic acid. After use, this solution should be stored in a cool, dry place and used within two weeks of preparation.

Solution D

Prepare a solution of 20:80 acetonitrile:HPLC-grade water with 0.1% formic acid. After use, this solution should be stored in a cool, dry place and used within two weeks of preparation.

MitoB and MitoP Calibrators Preparation

MitoB Calibrators

Dilute 5 mM MitoB MaxSpec® Standard (Item No. 601462) 1:5 in ethanol to make a 1 mM working stock solution (200 µl standard:800 µl ethanol) to be used in the preparation of calibrators. Calibrators can be stored at -20°C for up to two 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 (Item No. 601463) 1:10 in ethanol to make a 100 µM working stock solution (100 µl standard:900 µl ethanol) to be used in the preparation of calibrators. Calibrators can be stored at -20°C for up to two 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 (P4)
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, 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, however, it can 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. The protocol can be adjusted to better meet experimental demands.

1. Prepare a 1 µM MitoB working solution by diluting 10 µl of the 5 mM MitoB MaxSpec® Standard into 50 ml of cell culture media. Add catalase (50 U/ml) to 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 centrifuge at 120 × g for 5 minutes. Remove the 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 using a suspension cell line, centrifuge the cells (3 minutes at 1,000 × g) and use the supernatant.*
5. Flash-freeze the samples in liquid nitrogen and store at -80°C until sample preparation/extraction. 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 (Item No. 601461) to the samples and vortex for 30 seconds.
3. Add 500 µl of Solution C, vortex for 30 seconds, then centrifuge for 10 minutes at 16,000 × g in a benchtop centrifuge at room temperature. *NOTE: Replacing Solution C with 60:10 acetonitrile:HPLC-grade water with 0.1% formic acid may improve extraction efficiency for some tissue and urine samples.*
4. Transfer supernatant to a fresh tube and re-extract the pellet with an additional 500 µl of Solution C. Vortex, centrifuge, and combine supernatants. Completely dry samples under nitrogen or with a vacuum concentrator.
5. Reconstitute the samples in 100 µl of Solution D and transfer to an HPLC vial for LC-MS/MS analysis. HPLC samples may be stored at -80°C until analysis but should be sealed tightly to prevent evaporation of solvent.
6. Analyze these standards by LC-MS/MS as described in the **LC-MS/MS section** (see page 14). The response of MitoB relative to its deuterated internal standard 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 of 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. Extract these samples as described in the **Sample Preparation/Extraction** section and then 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 internal standard against concentration should be linear over the range 1–1,000 pmol, with an r^2 routinely >0.99. See **Plotting the Standard Curve** on page 13.

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 (pmol)	MitoP	Final Amount (pmol)
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, at a flow rate of 300 µl/min. Inject 5 µl of supernatant obtained from sample preparation. Elute MitoB, MitoP, and their internal standards at approximately 2.7-2.8 minutes using these conditions. Divert the flow from the mass spectrometer to waste between 0-2.5 minutes and 3-11 minutes of the sample elution to reduce the solvent entering the mass spectrometer.

Time (minutes)	Mobile Phase A	Mobile Phase 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: Ionize the eluted MitoB and MitoP by electrospray ionization in positive ion mode with nitrogen as the desolvation gas and argon as the collision gas. Use the following mass spectrometric parameters: source spray voltage, 3 kV; cone voltage, 62 V; ion source temperature, 150°C; collision energy, 52V; desolvation temperature, 500°C. Selectively monitor the MitoB and MitoP ions using multiple reaction monitoring in the same LC-MS/MS analysis. Use the MS/MS transitions described in Cochemé *et al.*[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 50:50 acetonitrile:HPLC-grade water (vol/vol) into the mass spectrometer at 5 µl/min. 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 50:50 acetonitrile:HPLC-grade water (vol/vol) into the mass spectrometer at 5 µl/min.

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, tune all standards to the transitions provided in Table 5 per the instructions recommended by the 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 approximately 9-11 minutes, with the peaks overlapping but with MitoB slightly before MitoP. Typical experimental chromatograms produced by LC-MS/MS analysis are shown in Figure 1, below.

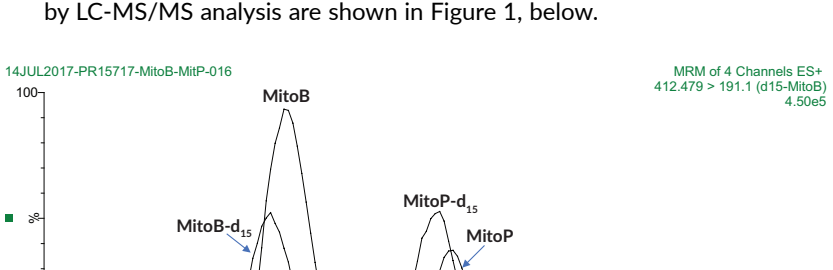

Figure 1. Extracted mass chromatogram of MitoB-d_{15}, MitoB, MitoP-d_{15}, and MitoP, obtained by LC-MS/MS analysis of HepG2 cells

3. Thaw the samples and standards in autosampler vials (if they were stored frozen), vortex briefly to redissolve, and centrifuge or filter to remove undissolved particulates, then keep at 4°C while awaiting injection by the autosampler. It is suggested to set up the vials in the following 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 mass spectrometer response for the samples and calibration curve by dividing the area for each compound by the area for its corresponding internal standards. Export the data into a spreadsheet program for further calculations. To produce the calibration curves, plot the mean mass spectrometer response against pmol of 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 injected MitoB stock or any that may occur during processing and storage. The means of multiple biological replicates should be determined with an appropriate measure of error.

Performance Characteristics

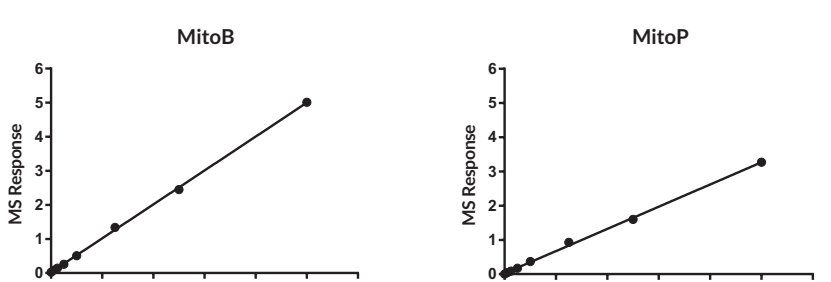

Figure 2. Standard curves for MitoB and MitoP

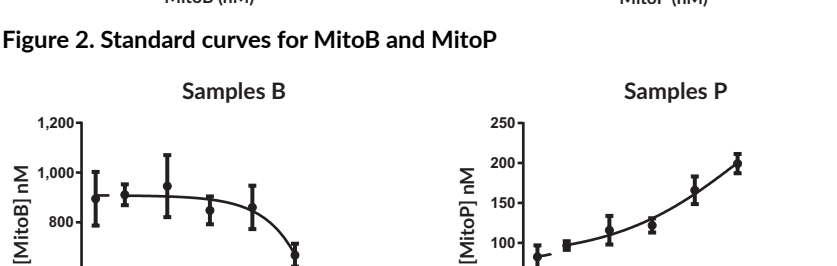

Figure 3. MitoB and MitoP concentrations in HepG2 cells treated with positive control menadione, followed by MitoB

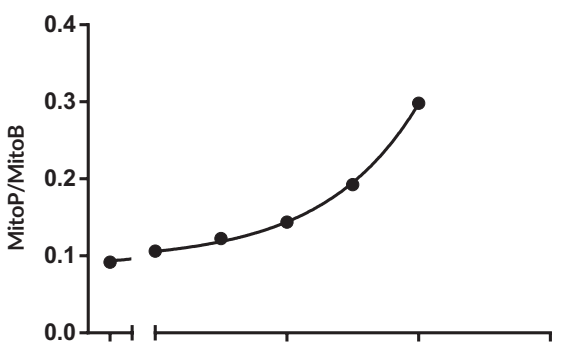

Figure 4. Ratio of MitoP to MitoB obtained from Figure 3. Data shows a concentration-dependent increase in the ratio of MitoP:MitoB in response to the positive control.

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 within the 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 internal standards detected in the system suitability run but not in samples	Error in the sample preparation/ extraction	Replace extraction solutions C and D
No MitoB or MitoP detected in the samples (but the calibration curve and the internal standards in the samples are detected)	Error in the fly injections	Check the injection solution for the correct concentration of MitoB (1 mM final concentration)
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).

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.

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