Ab102500 – Hydrogen Peroxide Assay Kit (Colorimetric Fluorometric)

For the rapid, sensitive and accurate measurement of Hydrogen Peroxide in a variety of samples.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab102500
(use www.abcam.cn/ab102500 for China, or www.abcam.co.jp/ab102500 for Japan)

Storage and Stability: Store kit at -20°C in the dark immediately upon receipt. Kit can be stored for 1 year if components have not been reconstituted. Reconstituted components are stable for 2 months.
Aliquot components in working volumes before storing at the recommended temperature.

Materials Supplied:

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
H_2O_2 Assay Buffer	25 mL	-20°C	-20°C
OxiRed Probe (in DMSO)	200 µL	-20°C	4°C or -20°C. Protect from light.
HRP	1 vial	-20°C	4°C (up to 1 week) -20°C (up to 1 month)
H_2O_2 Standard	100 µL	-20°C	-20°C.

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:
- MilliQ water or other type of double distilled water (ddH2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (clear bottoms) for fluorometric assay
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using cells or tissue)

For deproteinization protocol
- Perchloric acid (PCA) 4M, ice cold
- Potassium hydroxide (KOH), 2M
- 10 kD Spin Columns (ab93349) – for fluid samples, if not performing PCA precipitation

Reagent Preparation:
- Briefly centrifuge small vials at low speed prior to opening.
- Aliquot reagents so that you have enough volume to perform the desired number of assays

H_2O_2 Assay Buffer: Ready to use as supplied. Equilibrate to Room temperature before use.
H_2O_2 Standard: Ready to use as supplied. Equilibrate to room temperature before use.
OxiRed Probe (in DMSO): Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before each use. Once the probe is thawed, solution is stable for 1 week at 4°C and 1 month at -20°C. Store protected from light.
HRP: Dissolve in 220 µL Assay Buffer. Keep on ice during the assay.

Standard Preparation
- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

Prepare a 10 mM H_2O_2 standard by diluting 10 µL of the provided H_2O_2 Standard (0.88 M solution) into 870 µL of ddH_2O. Prepare a 0.1 mM H_2O_2 Standard by diluting 10 µL 10mM H_2O_2 Standard into 990 µL dH2O.

For Colormetric Assay: Using the 0.1 mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.
For Fluorometric assay: Prepare a 10 µM H_2O_2 standard by diluting 100 µL of the 0.1 mM H_2O_2 Standard into 900 µL of ddH_2O. Using the 10 µM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

Δ Note: If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve. Each dilution has enough standard to set up duplicate readings (2 x 50 µL).

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount H_2O_2 (nmol/well)	
				Colorimetric	Fluorometric
1	0	150	50	0	0
2	30	120	50	1	0.1
3	60	90	50	2	0.2
4	90	60	50	3	0.3
5	120	30	50	4	0.4
6	150	0	50	5	0.5

Sample Preparation
- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the Deproteinization protocol before storing the samples. Alternatively, snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Avoid repeated freeze/thaw cycles. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Cell (adherent or suspension) samples:
1. Harvest the amount of cells necessary for each assay (initial recommendation: $2x10^6$ cells) & wash with cold PBS.
2. Resuspend cells in 500 µL (or ~4 volumes) of the Assay Buffer on ice.
3. Homogenize cells by using a Dounce homogenizer (10-50 passes) on ice.
4. Centrifuge, collect the supernatant, and transfer to a clean tube and put on ice.
5. Perform deproteinization protocol as described below

Tissue samples:
1. Harvest tissue necessary for each assay (initial recommendation: 10-100 mg) & wash in cold PBS.
2. Resuspend tissue in 500-1000 µL (or ~ 5 -6 volumes) of the assay buffer on ice.
3. Homogenize tissue with a Dounce homogenizer sitting on ice, with 10-15 passes.
4. Centrifuge sample for 2-5 minutes at 4°C at top speed using a microcentrifuge to remove any insoluble material.
5. Collect supernatant and transfer to a new tube and keep on ice.
6. Perform deproteinization protocol as described below

Plasma, Serum and Urine (and other biological fluids):

These fluids samples generally contain 0.8 – 6 µM H_2O_2.

1. Collect cell culture supernatant, serum, plasma, urine and other biological fluids.
2. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove particulate pellet and keep on ice.
3. Perform deproteinization protocol as described below. Alternatively, you can use 10kD Spin column (ab93349) to deproteinize biological fluids.

Deproteinization protocol:

1. Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.
2. Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. High protein concentration samples might need more PCA.
3. Incubate on ice for 5 minutes.
4. Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.
5. Precipitate excess PCA by adding ice-cold 2M KOH that equals 34% of the supernatant to your sample (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.
6. After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). If necessary, adjust pH with 0.1 M KOH or PCA.
7. Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.
8. Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

Sample Recovery:

The deproteinized samples will be diluted from the original concentration.
To calculate the dilution factor of your final sample, simply apply the following formula:

$$\%\ original\ concentration = \frac{initial\ sample\ volume}{initial\ sample\ volume + vol\ PCA + vol\ KOH} \times 100$$

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

Assay Procedure – Colorimetric & Fluorometric Assay

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

Set up reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 2-50 µL samples (adjust volume to 50 µL/well with Assay Buffer).

Reaction mix:

1. Prepare 50 µL of Reaction Mix for each reaction. Mix enough reagents for the number of assays (samples, standards, and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number samples + Standards +1

Component	Reaction Mix (µL)	
	Colorimetric	Fluorometric
Assay Buffer	46	48
OxiRed Probe*	2	1
HRP*	2	1

*For fluorometric readings, using 1 µL/well of the probe and HRP decreases the background readings, therefore increasing detection sensitivity.

2. Add 50 µL of the Reaction Mix into each well.
3. Incubate at room temperature for 10 min protected from light.
4. Measure output on a microplate reader

- Colorimetric assay: measure OD570 nm.
- Fluorometric assay: measure Ex/Em = 535/587 nm

Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (Standard #1) from all standards, controls and sample readings. This is the corrected absorbance.
3. Plot the corrected values for each standard as a function of the final concentration of H_2O_2.
4. Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
5. Extrapolate sample readings from the standard curve plotted using the following equation:

$$Sa = \left(\frac{Corrected\ absorbance - (y - intercept)}{Slope}\right)$$

Where:
Sa = sample amount from standard curve (pmol).
Sv = sample volume (µL).

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)