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Protocol

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Accurate and Precise Protocol to Estimate the Activity of Peroxiredoxin Enzyme

Thulfeqar Hamza[1,2], Mahmoud Hussein Hadwan*[2]

Abstract

Background: Accurate estimation of Prx activity poses many complications and interferences. The present protocol is free of interference and provides an effective alternative for the assessment of peroxide with high sensitivity. The assay can be used in clinical pathology laboratories since it is simple, rapid, and inexpensive. The systematic reagent consisted of AFS/ASA which acted as a sensitive probe for peroxide.

Methods: Prx activity was estimated by incubating samples in suitable concentrations of 1,4-dithio-DL-threitol (DTT) and hydrogen peroxide (H2O2) or t-Butyl hydroperoxide (t-BOOH), as the substrates. The enzymatic reaction was inhibited after incubation with a working reagent containing ammonium ferrous sulfate (AFS) and aminosalicylic acid (ASA).

Results: Residual peroxide reacted with the working solution to form a brown-colored ferriaminosalicylate (FAS) complex with a maximum absorbance (λmax) of 425 nm. This protocol used sodium azide (NaN3) to eliminate catalase interference and avoided using high concentrations of strong acid to inhibit the Prx reaction.

Conclusions: We concluded that the new protocol produced the same efficacy as the reference method since a strong correlation coefficient of comparison (r> 0.99) was found between both the FAS and ferrithiocyanate method.

Keywords: Amino Salicylic Acid, Ammonium Ferrous Sulfate, Dithiothreitol, Peroxiredoxin, T-Butyl Hydroperoxide.

Introduction

Peroxiredoxins (Prxs) are a family of antioxidant enzymes that are conserved within prokaryotic and eukaryotic organisms (1). The molecular size of these proteins ranges from 20 to 30 kilodaltons (kDa) which express different isoforms (2, 3). Mammalian cells express six Prxs, which are divided into three groups, depending on their structure and catalytic mechanisms (4).

Prxs alone comprise approximately 1% or more of cellular proteins in animal cells and functions to reduce 90% of mitochondrial peroxides and more than 99% of cytosolic peroxides (5). Physiologically, Prxs detoxify peroxides by coupling to enzymatic reactions that involve nicotinamide adenine dinucleotide phosphate (NADPH) (6).

Accurate estimation of Prx activity poses many complications. In animal cells, the dissociation of hydrogen peroxide (H_2O_2) can be performed by other peroxidase enzymes such as selenium dependent glutathione peroxidase and catalase, which makes it difficult to isolate and measure the activity of Prx *in vivo* with any specificity (7).

To overcome this obstacle, previous protocols have used the Ferrous Oxidation-Xylenol Orange (FOX) reagent to assess cumene hydroperoxide dissociation as a function of Prx activity (7). In parallel, cumene

1: Chemistry Dept., College of Science, University of Babylon, Iraq.
2: Pathological Analysis Department, Al-Mustaqbal University College, Hilla City, Babylon Governorate, Iraq.
***Corresponding author: Mahmoud Hussein Hadwan; Tel: +96 47811548657; E-mail: sci.mahmood.hadwan@uobabylon.edu.iq.**
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hydroperoxide, linoleic acid hydroperoxide and t-Butyl hydroperoxide (t-BOOH) are greatly reduced by Prxs compared to other peroxidases (8). In other protocols, Prx was assayed spectrophotometrically by linking their activities to the oxidation of NADPH through thioredoxin/thioredoxin reductase (7). In addition, this method could be used with other peroxide substrates.

The current assessment method demonstrates how to use spectrophotometric absorbance of organic (tert-butyl hydroperoxide) or inorganic peroxides (hydrogen peroxide) to quantify Prx activity. The activity of Prx was assessed by incubating samples with 1,4-dithio-DL-threitol dithiothreitol (DTT), and t-BOOH or H_2O_2, as the substrates. Following the Prx-substrate reaction, a working reagent that contained ammonium ferrous sulfate (AFS) and aminosalicylic acid (ASA) was used to inhibit the enzymatic reaction. Any undissociated peroxide molecule oxidized ferrous (Fe^{+2}) to ferric ion (Fe^{+3}). Fe^{3+} then complexed with ASA and produced a brown ferriaminosalicylate (FAS) complex that had a maximum absorbance (λ_{max}) of 425 nm. Our method produced a Prx reaction solution free of molecular interference.

Materials and Methods

Statistics

The statistical analyses were performed using Qi Macros for Microsoft Excel 2016 (QiMacros, Know Ware International, Denver, USA).

Chemicals

The chemicals including (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide (NaN_3), sodium hydroxide (NaOH), ammonium ferrous sulfate (AFS) and aminosalicylic acid (ASA) were purchased from Merck (Darmstadt, Germany). The chemicals including DTT, t-BOOH, HCl, and H_2O_2 were purchased from Fluka (Buchs, Switzerland). Potassium chloride and sodium chloride (BDH chemicals, England).

Instrument

A spectrophotometer (PG Instruments T80) was used for the assessment of Prx activity.

Reagents and solutions

1. Hydrochloric acid (HCl) solution (182 mM) was prepared by dissolving 1.5 ml HCl in 100 ml of distilled water (dH_2O).
2. To prepare 25 mM HEPES buffer (pH 7), 0.0393 g of diethylenetriaminepentaacetic acid was dissolved with 6 g of HEPES in 800 ml of dH_2O. To buffer the pH to 7, we used 1 M NaOH. The final volume was increased to 1000 ml through the addition of dH_2O. The pH of HEPES buffer was 7 for all future steps.
3. Hydrogen peroxide (320 µM) was prepared by dissolving suitable quantities of H_2O_2 in 25 mM HEPES buffer. The final concentration was standardized to the required concentration using a molar extinction coefficient of H_2O_2 at 240 nm (43.6 $M^{-1}cm^{-1}$).
4. To prepare 1 M t-BOOH (1 M), 130 µl of 70% t-BOOH (7.7 M) was mixed with 870 µl of 25 mM HEPES buffer.
5. To prepare 1mM t-BOOH, 100 µl of t-BOOH (1M) was mixed with 99.9 mL of 25 mM HEPES buffer.
6. To prepare 320 µM t-BOOH, 320 ml of t-BOOH (1mM) was mixed with 680 ml of 25 mM HEPES buffer.
7. To prepare 2.1 mM 1,4-dithio-DL-threitol, 0.0323 g DTT was dissolved in 100 ml of 25 mM HEPES buffer.
8. Sodium azide (320 µM) was prepared by diluting 10 ml of 10 mM NaN_3 in 312.5 ml of 25 mM HEPES buffer.
9. Working reagent (stop solution) was freshly prepared by mixing 50 ml of solution A with 50 ml of solution B. Solution A (2 mM AFS) consisted of 0.04 g AFS dissolved in 50 ml of 182 mM HCl solution. Solution B consisted of 0.2175 g ASA dissolved in 50 ml of 182 mM HCl solution.

Peroxiredoxin purification

Human Prx II was prepared in accordance with a protocol previously described (7).

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Tissue preparation

Male albino rats were purchased from the central animal house at the University of Babylon, College of Science, Iraq. Animal organs such as the kidney and liver were surgically excised. The tissues were washed with 0.9% (w/v) sodium chloride solution (BDH chemicals, England) to eliminate blood and other contaminants, and homogenized with cold 1.15% (w/v) potassium chloride solution (BDH chemicals, England). Finally, 25 mM HEPES buffer (1:500 dilution) was used to dilute the homogenous solution. The resulting tissue homogenate was used to evaluate Prx activity.

Procedures

Potassium thiocyanate method

We monitored Fe^{2+} oxidation in the presence of potassium thiocyanate with slight modification as described by Netto et al (10). Prx activity was evaluated by incubating 25 µl Prx enzyme sample in 25 mM HEPES buffer containing DTT for 10 min at 37 °C. Following incubation, t-BOOH or H_2O_2 (final concentration: 100 µM) was added to the reaction solution. The solution was then incubated for 10 min at 37 °C. After inhibiting the enzymatic reaction with 8% trichloroacetic acid, centrifugation (12,000×g for 5 min) was used to remove the precipitated protein. Then, 400 µl of 10 mM AFS and 200 µl of 2.5 M potassium thiocyanate (BDH chemicals, England) was added. Lastly, the absorbance was measured at 480 nm.

ASA method

Two protocols were used to assess Prx activity. Protocol 1 (Table 1) used H_2O_2 as the substrate, whereas protocol 2 (Table. 2) used t-BOOH as the substrate. NaN_3 was excluded from protocol 2 since catalase does not catalyze t-BOOH.

Table. 1. The steps involved in measuring Prx activity using H2O2 as the substrate.

Reagents	Test	Standard	Blank
HEPES buffer	525 µL	550 µL	1.050 µL
Sodium azide (NaN_3)	50 µL	50 µL	50 µL
Dithio-DL-threitol-1,4 *(DTT)	500 µL	500 µL	500 µL
Sample containing Prx enzyme (serum, RBC, and homogenous tissues)	25 µL	---	---
Incubate all test tubes for 10 min at 37 °C			
Reactions start with the addition of 500 µL of 320 µM t-BOOH, which yields an initial concentration of 100 µmol/L, followed by vigorous mixing.			
Mix all test tubes using a vortex, incubate at 37 °C for 10 min, after that add:			
Working solution	1000 µL	1000 µL	1000 µL
After 2 min, measure change in absorbance at 425 nm and against the reagent blank			

* The final concentrations for DTT and H_2O_2 were 100 µmol/L.

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Table. 2. The steps involved in measuring Prx activity using t-BOOH as the substrate.

Reagents	Test	Standard	Blank
HEPES buffer	575 µL	600 µL	1100 µL
Dithio-DL-threitol-1,4 *(DTT)	500 µL	500 µL	500 µL
Sample containing Prx enzyme (serum, RBC and homogenous tissues)	25 µL	---	---
Incubate all test tubes for 10 min at 37 °C			
Reactions start with the addition of 500 µL of 320 µM t-BOOH, which yields an initial concentration of 100 µmol/L, followed by vigorous mixing.			
Mix all test tubes using a vortex, incubate at 37 °C for 10 min, after that add:			
Working solution	1000 µL	1000 µL	1000 µL
After 2 min, measure change in absorbance at 425 nm and against the reagent blank			

Calculations

The following equation was applied to calculate Prx activity in each test tube. The first equation used to calculate unreacted peroxide, while the second used to calculate the activity.

The residual peroxide in test tube $= \frac{A.test}{A.STD} \times STD\ of\ Conc.$

Prx activity (µmol of peroxide utilized/min) =

$$\frac{Conc.of\ peroxide\ in\ STD - Conc.of\ peroxide\ in}{time\ (10\ min)} \times \frac{Total\ Volume}{Volume\ of\ Sample} \times Dilution\ Factor$$

Results

Prx containing samples were incubated with suitable concentrations of peroxide (H_2O_2 or t-BOOH) and DTT dissolved in 25 mM HEPES buffer. A working reagent containing AFS and ASA was used to stop the enzymatic reaction. Unreacted residual peroxide (H_2O_2 or t-BOOH) acted to oxidize Fe^{+2} to Fe^{+3} which then complexed with ASA to form a brown-colored aminosalicylate complex (Fig. 1) with a λ_{max} of 425 nm.

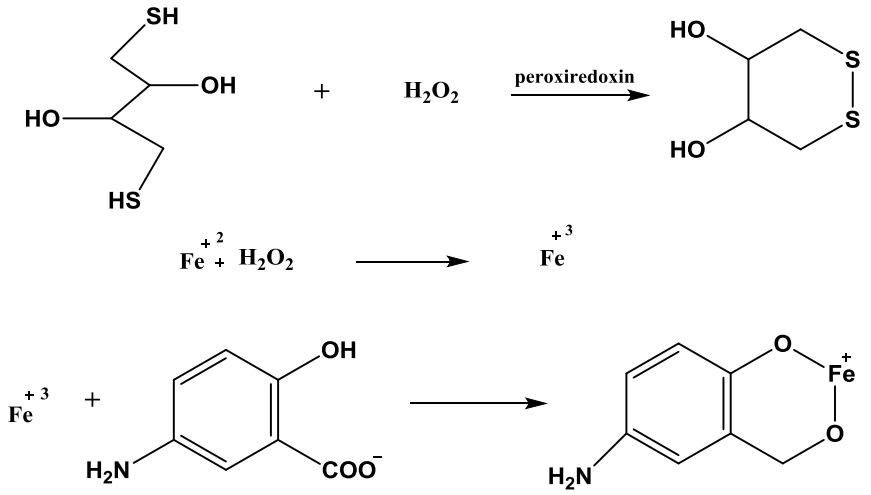

Fig. 1. Estimated Prx reaction. The final product included the production of a brown FAS complex.

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The FAS complex produced a single peak at 425 nm. The residual peroxide (H_2O_2 or t-BOOH) concentrations were directly proportional to the absorbance of the formed complex (Fig. 2).

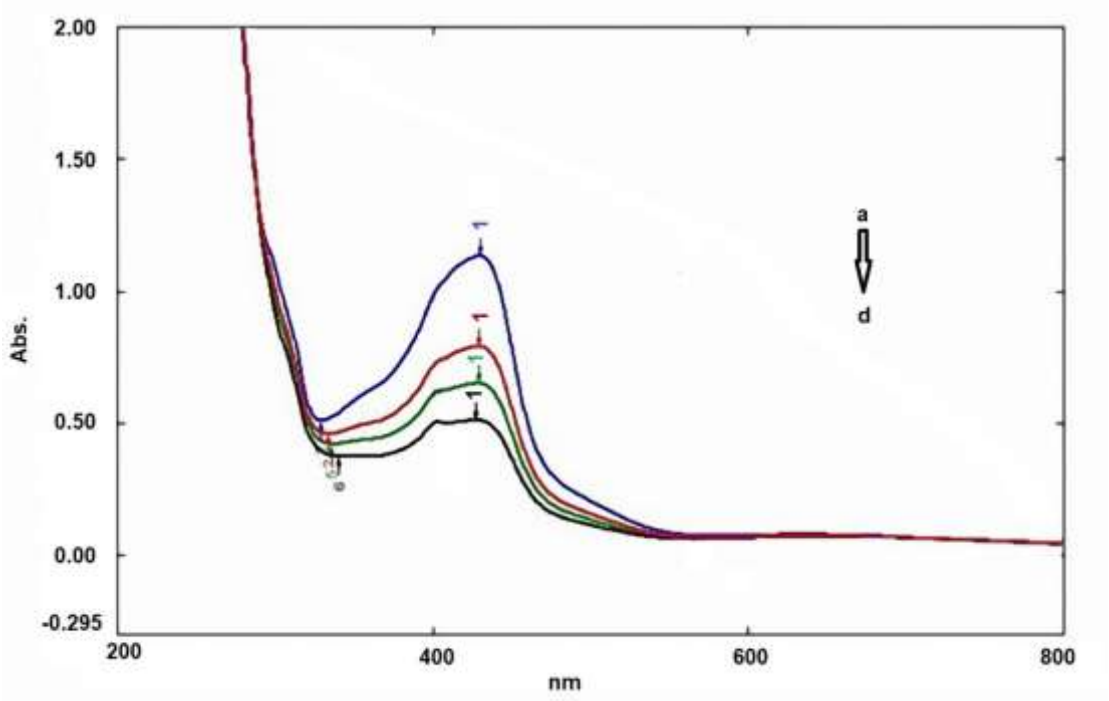

Fig. 2 A spectrophotometric spectrum of the FAS complex. The residual peroxide concentrations were directly proportional to the absorbance of the formed complex: (a) 200 µM H_2O_2; (b) 125 µM H_2O_2; (c) 100 µM H_2O_2; (d) 75 µM H_2O_2.

We compared the current FAS method to the ferrithiocyanate method to measure Prx activity in animal tissues (Table 3). The comparison produced compatible results.

Table 3. Prx activity was measured using the ferrithiocyanate and FAS protocol in male albino rat tissue.

Samples	Type of substrate	Prx activity: (U l^{-1} protein) for serum and (U mg^{-1} protein) for tissues	
		FAS method [a]	Ferrithiocyanate method [a]
		Mean±SD	Mean±SD
Serum	H_2O_2	155±3	151±5
	t-BOOH	172±5	180±4
Erythrocytes	H_2O_2	31±1	33±2
	t-BOOH	19±0.5	18±1
Kidney	H_2O_2	12±0.7	11.5±1
	t-BOOH	9±0.5	9.0±0.7
Liver	H_2O_2	15±0.9	15.5±1
	t-BOOH	14±0.5	15±0.7

[a] mean of triplicate determinations

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The precision of the FAS protocol was assessed using four types of interfering biomolecules. The first contained 25 mM HEPES buffer; the second contained 5 mM of three types of monosaccharides: mannose, lactose and glucose which was dissolved in 25 mM HEPES buffer; the third contained 5 mM of three types of amino acids: aspartic acid, methionine, leucine, and histidine dissolved in 25 mM HEPES buffer; and the fourth contained 3% casein and 3% bovine serum albumin which was dissolved in 25 mM HEPES buffer. The enzymatic reaction consisted of 1 ml 1000 (U/l) Prx mixed with 9 ml aliquots of the solutions containing the interfering biomolecules. Total Prx activity was adjusted to 100 (U/L) using the thiocyanate method (9). The results in Table 4 demonstrates the correlation between relative percentage errors and interfering biological contaminants.

Table 4. Effect of interfering biomolecules on relative percentage errors during the measurement of Prx activity, using the FAS protocol.

	Added peroxiredoxin U/l	Found peroxiredoxin U/l	Relative error (%)
# Solution 1	100	100	0.00
# Solution 2	100	98	2.0
# Solution 3	100	103	3.0
# Solution 4	100	99	1.0

A Bland-Altman plot was applied to confirm sensitivity and linearity of the developed protocol (15). The sensitivity and linearity of the FAS protocol was evaluated for several Prx activities (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 U/ L). The linearity of the proposed assay was evaluated by comparing the results of the current method with the results obtained from the ferrithiocyanate method (9). Figure 3 showcased the results obtained from the Bland–Altman plot. The analysis elucidated the mean relative bias and the relative difference between the FAS and ferrithiocyanate method.

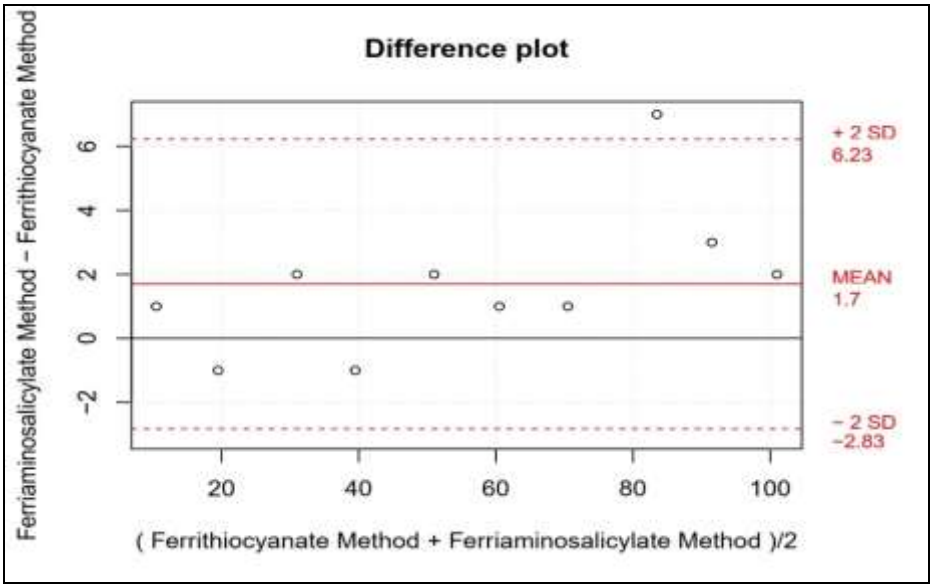

Fig. 3. Bland-Altman plot indicating the mean relative bias the relative difference between the ferrithiocyanate and FAS methods.

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Additionally, the Passing–Bablok analysis correlation for the FAS and ferrithiocyanate method showed good agreement (Fig. 4). Further, the Pearson correlation confirmed that there was a strong correlation (r> 0.98) between the different samples.

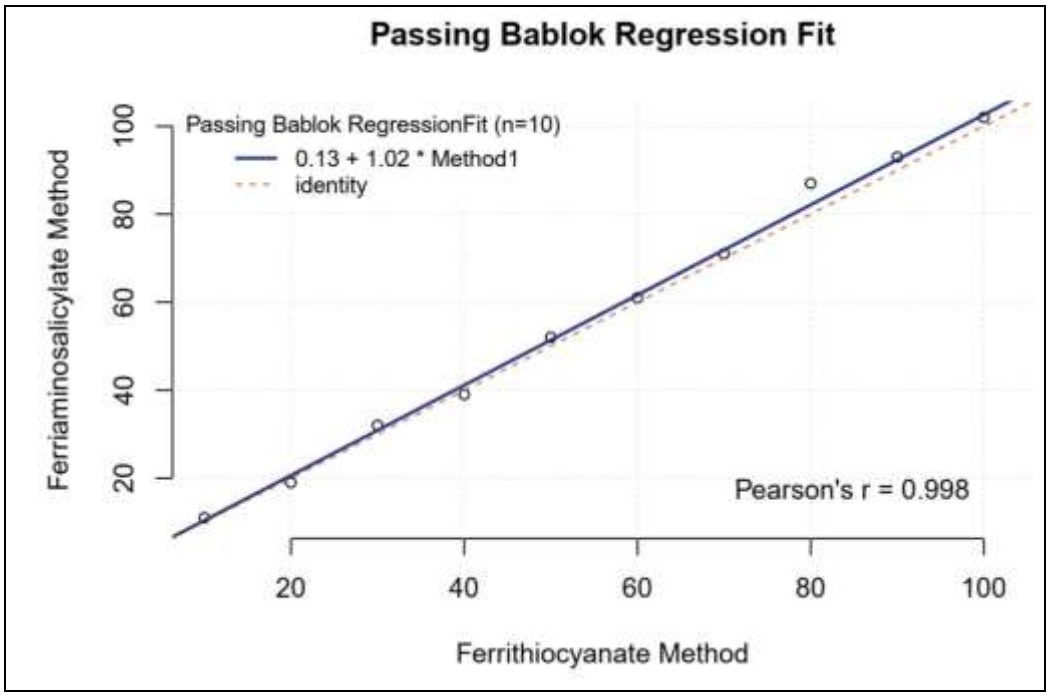

Fig. 4. The Passing–Bablok analysis correlation of the FAS and ferrithiocyanate methods.

Discussion

This assay was found to be suitable for Prx and can be used to estimate peroxide (H_2O_2 or t-BOOH) concentrations ranging from 1 to 1000 µM. The colored end product is stable and the absorbance at 425 nm remains constant for more than three hours.

Prx activity in liver homogenate was determined using the ASA method. Liver Prx activity was previously used to estimate oxidative stress in rats (10). Moreover, Prx protects against alcohol-induced oxidative injury in mice liver (11).

Previous studies demonstrated that the chronic consumption of ethanol in rats resulted in the hyper-oxidation of Prx I, but not other Prxs (11). Furthermore, the specific inactivation of Prx I was colocalized, in a large ratio, with cytochrome P450 2E1 (CYP2E1) in the endoplasmic reticulum membrane on the cytosolic side (12). The accumulation of the acidic inactive form of Prx III in rat livers decreased protease activity involved in the degradation of oxidized proteins (13). In addition, Prx II was identified based on its capacity to protect proteins from oxidative damage induced by reactive oxygen species, which are generated in the presence of DTT (14).

The current protocol was compatible with the previous method described by Khalifa and Hadwan (16). The two protocols used salicylic acid (SSA) derivatives to form colored complexes with unreacted residual peroxide. The disadvantages of the thiocyanate method included the high toxicity of thiocyanate and associated environmental risks. The substitution of thiocyanate with sulfosalicylic acid (SSA) is consistent with the rules and principles of greener chemistry (17, 18). According to the above results, the present protocol is free of interference and provides an effective alternative for the assessment of peroxide with high sensitivity. The results showed that this assay can be used in clinical pathology laboratories since it is simple, rapid, and inexpensive. The systematic reagent consisted of AFS/ASA which acted as a sensitive probe for peroxide.

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This study has been approved by the Ethics Committee of the University of Babylon, College of Science, Iraq.

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