Human Peroxiredoxin 1 ELISA

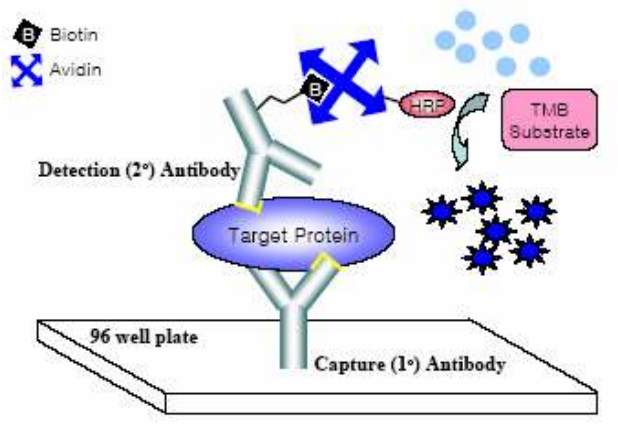
Cat. No.: RLF-EK0131R

1. Introduction

Organisms living under aerobic conditions have developed various anti-oxidative mechanisms to protect them from damage by reactive oxygen species (ROS). Peroxiredoxin (Prx) is a recently identified family of anti-oxidative proteins that includes six isoforms in mammals. They share a common reactive Cys residue in the N-terminal region, and are capable of serving as a peroxidase, involving thioredoxin and/or glutathione as the electron donor. Prx1-4 have an additional reactive Cys residue in the conserved C-terminal region, and show homology with >70% amino acid sequence similarity. Prx5 also contains an additional Cys in its C-terminal region that is less conserved. On the other hand, Prx6 has only the N-terminal Cys. These Prx family members are distributed in subcellular locations: Prx1, 2, and 6 in cytosol; Prx3 in mitochondria; Prx4 in ER and secretion; and Prx5 showing complicated distribution including peroxisome, mitochondria and cytosol, all of which are potential sites of ROS production. In addition to their role as a peroxidase, a body of evidence has accumulated to suggest that individual members also serve divergent functions that are associated with various biological processes such as the detoxification of oxidants, cell proliferation, cell differentiation and gene expression. These functions need not depend on peroxidase activity and; therefore, it seems likely that the divergence is due to unique molecular characteristics intrinsic to each member.

2. Principles of Method

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human Prx1. Samples are pippetted into these wells. Unbound Prx1 and other components of the sample are removed by washing, then biotin-conjugated monoclonal antibody specific to Prx1 is added. In order to quantitatively determine the amount of Prx1 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is then added to each microplate well. Next, a TMB-substrate solution is added to each well. Finally a sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. The absorbance (O.D. value) is directly proportional to the amount of captured Prx1.



Sandwich ELISA method

3. Intended Use

The AbFrontier human Peroxiredoxin 1 (human Prx1) ELISA kit is to be used for the in vitro quantitative determination of human Prx1 in human serum, human plasma, cell lysate or buffered solution. The assay will recognize both native and recombinant human Prx1.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. Storage and Stability

All components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. Chemical Hazard

- Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- All reagents containing Sodium Azide also contain Thimerosal as a preservative. Thimerosal contains Hg thus should be handled with great care.

6. Kit Contents

Contents	Number	Volume
96 Well Plate	1 (in aluminum foil bag with desiccant)	
Incubation Buffer	1	30 ml
Washing Buffer	1	(10X) 100 ml
Standard Protein	1 Glass vial (lyophilized)	
Standard/Sample Dilution Buffer	1	25 ml
Secondary Antibody	1	150 µl
AV-HRP	1	150 µl
Secondary Antibody/AV-HRP Dilution Buffer	1	25 ml
Substrate (TMB)	1	20 ml
Stop Solution	1	20 ml
Protocol booklet	1	
Plate sealers	3	

① 96 Well Plate

: Human Prx1 microtiter plate, one plate of 96 wells (16well strip x 6).

A plate using break-apart strips coated with a mouse monoclonal antibody specific to human Prx1.

② Standard Protein

: Lyophilized recombinant human Prx1.

③ Secondary Antibody

: Biotin labeled mouse anti human Prx1 antibody.

④ AV-HRP

: Avidin linked Horseradish Peroxidase (HRP, enzyme)

⑤ Substrate (Stabilized chromogen)

: Tetramethylbenzidine (TMB) solution

⑥ Stop Solution

: 1N solution of sulphuric acid (H_2SO_4).

⑦ Plate sealer

: Adhesive sheet.

- Do not mix or interchange reagents from different lots.

7. Materials Required But Not Provided

① Microtiter plate reader capable of measurement at or near 450nm.

② Calibrated, adjustable precision pipettes, preferably with disposable plastic tips (A manifold multi-channel pipette is desirable for large assays.)

③ Distilled or deionized water

④ Data analysis and graphing software

⑤ Vortex mixer

⑥ Polypropylene tubes for diluting and aliquoting standard

⑦ Absorbent paper towels

⑧ Calibrated beakers and graduated cylinders of various sizes

8. Reagent Preparation

1) Human Prx1 standard

Reconstitute the human Prx1 standard to 1 μg/ml by adding 1ml of *Standard/Sample Dilution Buffer* into the standard protein glass vial containing lyophilized human Prx1 protein. Swirl or mix gently, and allow to sit for 5 minutes to ensure complete reconstitution.

	Standard	Add	Into
#1	32.0ng/ml	32μl of the 1μg/ml standard	968μl of the *Standard/Sample Dilution Buffer*
#2	16.0ng/ml	500μl of tube #1	500μl of the *Standard/Sample Dilution Buffer*
#3	8.0ng/ml	500μl of tube #2	500μl of the *Standard/Sample Dilution Buffer*
#4	4.0ng/ml	500μl of tube #3	500μl of the *Standard/Sample Dilution Buffer*
#5	2.0ng/ml	500μl of tube #4	500μl of the *Standard/Sample Dilution Buffer*
#6	1.0ng/ml	500μl of tube #5	500μl of the *Standard/Sample Dilution Buffer*
#7	0.5ng/ml	500μl of tube #6	500μl of the *Standard/Sample Dilution Buffer*
#8	0ng/ml	1.0ml of the *Standard/Sample Dilution Buffer*	

2) Secondary Antibody

1. Equilibrate to room temperature, mix gently.

2. Mix 20μl *Secondary Antibody concentrated solution* (100X) + 2ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more if needed) Label as "Working Secondary antibody Solution".

3. Return the unused *Secondary Antibody concentrated solution* to the refrigerator.

3) AV-HRP

1. Equilibrate to room temperature, mix gently.

2. Mix 20µl *AV-HRP concentrated solution* (100X) + 2ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more as needed) Label as "Working AV-HRP Solution".

3. Return the unused *AV-HRP concentrated solution* to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.

2. Mix 1 volume *Wash buffer concentrate solution* (10X) + 9 volumes of deionized water. Label as "Working Washing Solution".

3. Store both the concentrated and the Working Washing Solution in the refrigerator.

*** Directions for washing**

1. Fill the wells with 300µl of "Working Washing Buffer".

Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.

If using an automated washer, the operating instructions for washing equipment should be carefully followed.

2. Incomplete washing or residual washing buffer in wells will adversely affect the assay and render false results.

3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

9. Assay Procedure

- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
- All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
- A standard curve must be run with each assay.
- If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
- Maintain a consistent order of sample and reagent additions from well to well. This ensures equal incubation times for all wells.

1) Determine the number of 16-well strips needed for assay. Insert these in the frame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).
2) Add 300µl of *Incubation buffer* to all wells and incubate the plate for 5 minutes at room temperature.
3) Thoroughly aspirate or decant the solution from the wells. Wash wells 2 times (See "Directions for washing").
4) For the standard curve, add 100µl of the standard to the appropriate microtiter wells. Add 100µl of the *Standard/Sample Dilution Buffer* to zero wells.
5) Serum requires at least 20 fold dilution in the *Standard/Sample Dilution Buffer.* And add 100 µl of diluted samples to each well.
6) Cover the plate with the plate cover and incubate for 2 hours at room temperature.
7) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
8) Pipette 100µl of "Working Secondary Antibody Solution" into each well.
9) Cover the plate with the plate cover and incubate for 1 hour at room temperature.
10) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
11) Add 100µl "Working AV-HRP Solution" to each well.
12) Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
13) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").
14) Pour enough Substrate you need into a tube or reagent boat. Add 100µl of *Substrate* to each well. The liquid in the wells should begin to turn blue.
15) Incubate the plate at room temperature. Avoid exposing plate to direct sunlight.

- Do not cover the plate with aluminum foil (or other metal), or color may develop.

The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well.

- Keep the plate away from sun light because the *Substrate* is light sensitive,.
- Typically, reaction is stopped 5~10 minutes after treatment of Substrate, but this time can be adjusted as the user desires.

16) Add 100µl of *Stop Solution* to each well. The solution in the wells should change from blue to yellow.
17) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the *Stop Solution*.

18) Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve.

19) Read the human Prx1 concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the S*tandard/Sample Dilution Buffer*).

10. Characteristics

1) Typical result

The standard curve below is for illustration only and **should not be used** to calculate results in your assay.

A standard curve must be run with each assay.

Standard human Prx1 (ng/ml)	Optical Density (at 450nm)
0	0.070
0.5	0.077
1.0	0.093
2.0	0.136
4.0	0.236
8.0	0.480
16.0	0.974
32.0	1.985

Limitations

- Do not extrapolate the standard curve beyond the 32ng/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native human Prx1 in various matrices has not been investigated.

2) Sensitivity

The minimal detectable dose of human Prx3 was calculated to be 0.5ng/ml, by subtracting two standard deviations from the mean of 10 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

3) Specificity

The following substances have been were tested and found to have no cross-reactivity: human Prx2, Prx3, Prx4, Prx5, Prx6 and mouse Prx3 (mPrx3).

4) Precision

① Within-Run (Intra-Assay)

(n=10)

Mean (ng/ml)	SD (ng/ml)	CV (%)
4.29	0.38	8.91
7.92	0.76	9.63
16.75	1.06	6.34
32.32	3.09	9.57

② Between-Run (Inter-Assay)

(n=3)

Mean (ng/ml)	SD (ng/ml)	CV (%)
3.45	0.11	3.14
7.64	0.54	7.09
15.84	1.58	9.97
32.21	1.93	5.98

5) Recovery

Recovery upon addition is 93.9~100.0% (mean 97.0%)

Recovery upon dilution is 99.8~102.0% (mean 103.1%)

11. Troubleshooting

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase soaking time before aspiration/decanting step
	• Too much AV-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30°C before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing – unbound AV-HRP remaining	• Increase number of washes carefully
	• Too much AV-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual AV-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

12. References

1) Sue Goo Rhee et al. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. (2005) *Free Radic Biol Med.* **38**(12):1543-52
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BioVendor - Laboratorní medicína a.s.
CTPark Modrice
Evropska 873
664 42 Modrice
CZECH REPUBLIC
Phone: +420-549 124 185
Fax: +420-549 211 460
e-mail: info@biovendor.com
http://www.biovendor.com

European Union:

BioVendor GmbH
Im Neuenheimer Feld 583
D-69120 Heidelberg, GERMANY
Phone: +49-6221-433-9100
Fax: +49-6221-433-9111
e mail: roth@biovendor.com
e mail: abels@biovendor.com

China - Hong Kong Office:

BioVendor Laboratories Ltd.
Room 4008, Hong Kong Plaza, No.188
Connaught Road West
Hong Kong, CHINA
Phone: +852 28030523
Fax: +852 28030525
e-mail: lu@biovendor.com

USA, Canada and Mexico:

BioVendor, LLC
1463 Sand Hill Road, Suite 227
Candler, NC 28715, USA
Phone: +1-828-670-7807, +1-800-404-7807
Fax: +1-828-670-7809
e mail: infoUSA@biovendor.com

China – Mainland Office:

BioVendor Laboratories Ltd.
Room 2405, YiYa Tower, TianYu Garden, No.150
Lihe Zhong Road, Guang Zhou, CHINA
Phone: +86-20-87063029
Fax: +86-20-87063016
e-mail: jhsu@biovendor.com

Manufactured by

Abfrontier. Co. Ltd.
1Fl, Science Building C, Ewha University,
Daehyun-dong, Seodaemun-gu,
Seoul, Korea 120-750
http://www.abfrontier.com
e-mail : support@abfrontier.com