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Research
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Human Glutathione Peroxidase 1 ELISA

Cat. No.: RLF-EK0110R

1. Introduction

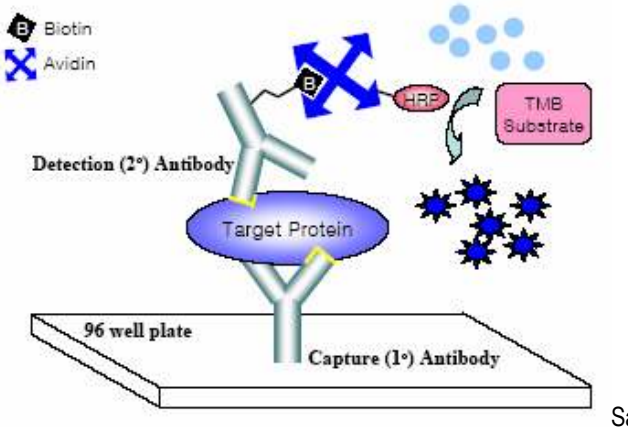
Glutathione peroxidases (Gpxs) are ubiquitously expressed proteins which catalyze the reduction of hydrogen peroxides and organic hydroperoxides by glutathione. There are several isoforms which differ in their primary structure and localization. The classical cytosolic /mitochondrial Gpx1 (cGpx) is a selenium-dependent enzyme, the first of the Gpx family to be discovered. Gpx2, also known as gastrointestinal Gpx (GI-Gpx), is an intracellular enzyme expressed only in the epithelium of the gastrointestinal tract. Extracellular plasma Gpx (pGpx or Gpx3) is mainly expressed in the kidney from where it is released into the blood circulation. Phospholipid hydroperoxidase Gpx4 (PH-Gpx) is expressed in most tissues and it can reduce many hydroperoxides including hydroperoxides integrated in membranes, hydroperoxy lipids in low density lipoprotein or thymine. All mammalian Gpx family members, except for the recently described Cys containing Gpx3 and epididymis-specific secretory Gpx (eGpx or Gpx5) isoforms, possess selenocysteine at the active site.

Because Gpx1 appears to have a major role in the prevention of oxidative stress, it may also be an important antiatherogenic enzyme. In mice, Gpx1 deficiency results in abnormal vascular and cardiac function and structure. From a clinical perspective, there is a report suggesting that low erythrocyte Gpx1 activity identifies patients with coronary artery disease who are at the high risk for cardiovascular events and that measurement of Gpx1 activity provides additional information on risk which might be useful in identifying patients who would benefit from preventive antioxidant treatment.

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 Gpx1. Samples are pipetted into these wells. Unbound Gpx1 and other components of the sample are removed by washing, then biotin-conjugated monoclonal antibody specific to Gpx1 is added. In order to quantitatively determine the amount of Gpx1 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 Gpx1.



Sandwich ELISA method

3. Intended Use

The AbFrontier human Glutathione Peroxidase-1 (human Gpx1) ELISA kit is to be used for the in vitro quantitative determination of human Gpx1 in human RBC or other cell lysate, serum, plasma or buffered solution. The assay will recognize both native and recombinant human Gpx1.

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 and 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	30ml
Wash Buffer	1	(10X) 100ml
Standard Protein	1 Glass vial (lyophilized)	
Standard/Sample Dilution Buffer	1	25ml
Secondary Antibody	1	(100X) 150ul
AV-HRP	1	(100X)150ul
Secondary Antibody/AV-HRP Dilution Buffer	1	25ml
Substrate (TMB)	1	20ml
Stop Solution	1	20ml
Protocol booklet	1	
Plate sealers	3	

① 96 Well Plate

: Human Gpx1 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 Gpx1.

② Standard Protein

: Lyophilized recombinant human Gpx1.

③ Secondary Antibody

: Biotin labeled mouse anti human Gpx1 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-chanel 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 Gpx1 standard

Reconstitute the human Gpx1 standard to 1μg/ml by adding 1ml of *Standard/Sample Dilution Buffer* into the standard protein glass vial containing lyophilized human Gpx1 protein. Swirl or mix gently, and allow to sit for 5 minutes to ensure complete reconstitution.

Standard	Add	Into
100ng/ml	100μl of the 1μg/ml std.	900μl of the *Standard/Sample Dilution Buffer*
50ng/ml	50μl of the 1μg/ml std.	950μl of the *Standard/Sample Dilution Buffer*
25ng/ml	25μl of the 1μg/ml std.	975μl of the *Standard/Sample Dilution Buffer*
12.5ng/ml	12.5μl of the 1μg/ml std.	987.5μl of the *Standard/Sample Dilution Buffer*
6.25ng/ml	6.25μl of the 1μg/ml std.	993.75μl of the *Standard/Sample Dilution Buffer*
3.13ng/ml	3.13μl of the 1μg/ml std.	996.87μl of the *Standard/Sample Dilution Buffer*
1.56ng/ml	1.56μl of the 1μg/ml std.	998.44μl of the *Standard/Sample Dilution Buffer*
0ng/ml	1.0ml of the *Standard/Sample Dilution Buffer*	

2) Secondary Antibody

1. Equilibrate to room temperature, mix gently.

2. Mix 20 μl *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 20 μl *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) Wash 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 Wash 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 wash buffer in wells will adversly 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 u se (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 temperatur e.
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) Human serum, RBC lysate and cell lysate require at least 20 fold dilution in the *Standard/Sample Dilution Buffer.* And add 100µl 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 S*ubstrate* 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 the microtiter 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 adjust ed 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 Gpx1 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/SampleDilution 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 Gpx1 (ng/ml)	Optical Density (at 450nm)
0	0.095
1.56	0.126
3.13	0.159
6.25	0.212
12.5	0.363
25.00	0.681
50.00	1.295
100.00	2.440

Limitations

- Do not extrapolate the standard curve beyond the 100ng/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native human GPX1 in various matrices has not been investigated.

2) Sensitivity

The minimal detectable dose of human Gpx1 was calculated to be 1.56 ng/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

Rat Gpx1 and mouse Gpx1 had slight cross-reactivity. The following substances were tested and found to have no cross-reactivity: human Gpx3, Gpx4.

4) Precision

① Within-Run (Intra-Assay)

(n=7)

Mean (ng/ml)	SD (ng/ml)	CV (%)
2.87	0.12	4.2
4.81	0.17	3.5
11.64	0.38	3.3
25.82	0.94	3.6

② Between-Run (Inter-Assay)

(n= 8)

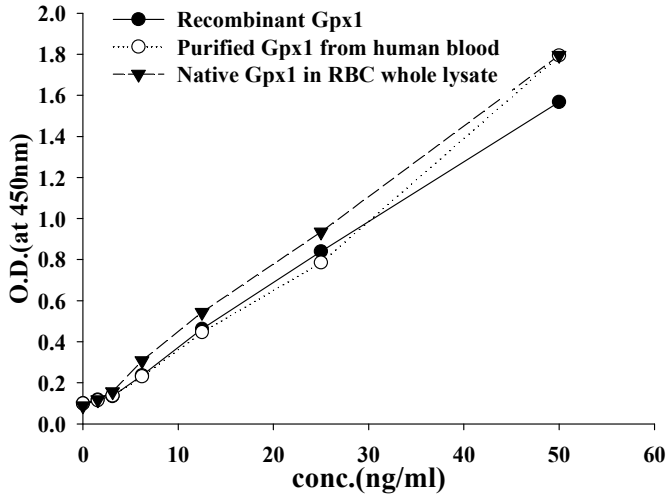
Mean (ng/ml)	SD (ng/ml)	CV (%)
3.02	0.22	6.8
5.29	0.16	3.0
11.82	0.96	8.2
25.53	1.99	7.9

5) Recovery

Recovery upon addition is 99.3~102.2% (mean 101.2%)

Recovery upon dilution is 97.85~103.44% (mean 100.7%)

6) Parallelism



Native human Gpx1 was serially diluted in *Standard/Sample dilution buffer*. The optical density of each dilution was plotted against the recombinant human Gpx1 standard curve.

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

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