IS129 100T-48S

Glutathione Peroxidase（GSH-PX）Assay Kit

Organism Species: *Pan-species* (General)

Instruction manual

FOR RESEARCH USE ONLY

NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

12th Edition

[INTENDED USE]

The kit is a colorimetric method for the in vitro quantitative measurement of Glutathione peroxidase (GSH-PX) activity in whole blood, tissues, mitochondria, cell membrane, serum, plasma etc..

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity(100T-48S/50T-24S)	Reagents	Quantity(100T-48S/50T-24S)
Reagent 1	1×2mL / 1×1mL	Reagent 4	1(Powder) / 1×25mL(Liquid)
Reagent 2 (Powder A)	1 / 1	Reagent 5(Powder)	4/2
Reagent 2 (Solution B)	1×50mL / 1×25mL	Reagent 6	4×3.07mg / 2×3.07mg
Reagent 3 (Powder)	1 / 1	Reagent 7	1×10mL / 1×5mL
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[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. A spectrophotometer capable of measuring absorbance at 412nm, glass cuvettes of 1cm light path.
2. Thermostatic water bath or air bath capable of controlling temperature at 37°C.
3. Desk centrifuge.
4. Transferpettor and tips.
5. Vortex mixer.
6. A source of pure water (preferably double distilled water and double distilled water).

[STORAGE OF THE KITS]

Stored at 4°C for 6 months.

[PRE-TEST ANNOUNCEMENTS]

Note 1: You only need to make 1~2 blank tubes and standard tubes for each batch.

Note 2: Different samples have different GSH-PX activities, so they also have different optimal sample concentrations. According to parabola proportion between inhibition percentage and enzyme activity, different samples have different sample volumes and different sample concentration, you need to determine an optimal sample volume and an optimal sample concentration before measure GPX activity in a new sample.

Optimal sample probing:

Do pre-test to determine optimal sample concentration: If you use this kit to do a new sample assay, then you need to make 3 sample tubes of different concentrations to do pre-test as follows:

Whole blood: Take 200μL 1:24, 1:49, 1:99 hemolysate (diluted by double distilled water) separately to do pre-test.

Tissue homogenate: Take 200μL 10%, 5%, 1% homolysate (different samples need different dilutions) separately to do pre-test.

Blood serum: Take 100μL undiluted, 1:1, 1:4, 1:9, 1:19 blood serum (diluted by physiological saline) to do pre-test.

Then calculate: Inhibition percentage=$(OD_{Nonenzyme}-OD_{Enzyme})/OD_{Nonenzyme})$ X 100%, result should be in range of **15%~55%**. Take the tube which inhibition percentage is between 45% to 50% as **optimal sample volume**.

Enzyme inhibition percentage and enzyme activity appear parabola proportion. If inhibition percentage is over 60% (curve appears flat in this part), then you need to dilute sample or decrease sample volume and try again. If inhibition percentage is lower than 20%, then you need to increase sample volume and try again.

In this way, it is great helpful for scientific result analysis and t-test; if inhibition ratio percentage is higher than 60% or lower than 10%, then there are no significant differences between different assay groups.

[REAGENT PREPARATION]

1. **Reagent 1 working solution preparation**: Take 0.1mL **stock solution(Reagent 1)**, add double distilled water until volume reaches 10mL, this operation equals to 100 times dilution. **Reagent 1 working solution** should be used soon after preparation, consider solution volume according to you need. Stored at 4ºC.
2. **Reagent 2 working solution preparation**: Take 170mL(100T-48S)/85mL(50T-24S) hot double distilled water(90-100ºC) to dissolve the **powder A(Reagent 2)** completely. Mix prepared solution A and solution B sufficiently. This mixture is supersaturated solution, so if there are crystals seed out after cooling and placing, then take supernatant for experiment. Stored at 4ºC or room temperature for 6 months.
3. **Reagent 3 working solution preparation**: Add 200mL(100T-48S)/100mL(50T-24S) double distilled water to dissolve **Reagent 3**, Stored in plastic bottle (given by our Institute) for 6 months.

Reagent 4 working solution preparation: For 100T-48S, the Reagent 4 is provided as powder form. Add 50mL double distilled water to dissolve **Reagent 4** (Powder). Stored at 4ºC away from light for 6 months. For 50T-24S, the Reagent 4 is provided as liquid form. It is the **Reagent 4 working solution preparation.** Stored at 4ºC away from light for 6 months.

4. **Reagent 5 working solution preparation**: Add 10mL double distilled water to dissolve a vial of **Reagent 5** (Powder, 100T-48S), can be stored at 4ºC away from light for 5 days.
5. **Standard solvent working solution preparation**: Mix **Reagent 7** (GSH standard solvent stock solution) with double distilled water at ratio of 1:9 (equals to 10 times dilution) to make working solution. Please use **standard solvent working solution** soon after preparation. Can be stored at 4ºC.
6. **1mmol/LGSH solution preparation**: GSH's molecular weight is 307. Before assay, Reconstitute the **Reagent**

6 (GSH standard powder, 3.07mg/vial) with GSH standard solvent working solution to 10mL. The solution is considered as **1mmol/L GSH solution**. Please use this solution soon after preparation.

7. **100μmol/LGSH solution preparation**: Mix 2mL 1mmol/LGSH solution and 18mL GSH solution working solution, adjust volume to 20mL. This solution is considered as 100μmol/L GSH standard solution. This solution is used to draw standard curve. If you don’t want to draw standard curve, then you can skip this step. (See in Appendix: GSH standard curve preparation).

8. **20μmol/L GSH standard solution**: Take 0.2mL 1mmol/L GSH solution, add GSH standard solvent working solution until mixture volume reaches to 10mL.This solution is considered as 20μmol/L GSH standard solution.

[SAMPLE PREPARATION]

1. Whole blood

① Take 20μL heparin anticoagulated whole blood, add distilled water until volume reaches to 1mL, 1:49 hemolysate is made. Dilute 10μL mouse/rat blood with distilled water to 1mL, 1:99 hemolysate is made.

② Mix sufficiently, place for 5 minutes until hemolysate in glass tube appears **completely transparent** towards light, then you can start assay.

③ GSH-PX activity in prepared hemolysate can maintain for only 45~60 minutes (extend to 120 minutes in cold weather. If you have not enough time to assay in the same day, then you can keep you samples in anticoagulated whole blood fridge(4～8ºC), enzyme activity keeps stable in 2~3 days.

Note 1: Please do preliminary experiment before formal experiment, details in **example of hemolysate optimal sample concentration and optimal sample volume probing**.

Note 2: Please make sure that erythrocytes are completely haemolyzed before measure blood GSH-PX activity (completely haemolyzed blood appears **transparent** towards light, **if it is not transparent enough, then you can freeze-thaw it once.** But erythrocytes of some rats and pigs can not be placed below 0ºC or they will be hard to haemolyze. For example, erythrocytes of diabetic rats and some normal rats are very hard to haemolyze after freezing. It is better to take 1-2 samples to do pretesting before formal experiment.place for 10 minutes and then you can start assay.

2. 10% tissue homogenate preparation

① Take tissue piece (0.2~1g), wash by ice-cold physiological saline. Remove blood, dry by filter paper, transfer in 5~10mL small flask.

② Use cylinder to measure volume of precooled homogenate medium (PH7.4，0.01mol/L sucrose，0.01mol/L Tris-HCl， 0.0001mol/L EDTA2Na solution) or physiological saline. Volume of homogenate medium or 0.86% physiological saline should be 9 times as tissue weight, transfer 2/3 total volume of homogenate medium or physiological saline into small flask. Cut tissue to small pieces by ophthalmology small scissor as quick as possible (please put small flask in ice water bath in hot weather).

③ Pour small tissue pieces to glass homogenate tube, then wash tissue residual by 1/3 volume of homogenate medium or 0.86% physiological saline, transfer all mixture into glass homogenater tube then start to make homogenate. Use left hand to hold homogenate tube to make sure its bottom in icewater bath, use right hand to insert bar in tube, grind by up-down rolling for several dozens times (6~8 minutes), grind sufficient to make sure tissue becomes homogenate. You can also use tissue triturator to grind at 10000～15000r/min to make 10% homogenate; incision tissue homogenizer can also be used (homogenate length: 10 seconds/time, interval is 30

seconds, do 3~4 times at 0～4°C) . Cardiac muscle needs longer homogenate time.

Note: Take optimal concentration to assay (according to pretesting result).

④ Centrifugate prepared 10% homogenate at 3000rpm (by common centrifuge or low temperature centrifuge) for 10~15 minutes. Remove sediment, keep supernatant.

⑤ Take supernatant for various kinds of assay according to your requirement.

3. **Mitochondrion preparation**

Take 5~10mL 10% tissue homogenate, centrifugate at 1000~2000rpm for 10 minutes (by common centrifuge or low temperature low speed centrifuge). Take supernatant, centrifugate at 8000~10000rpm (by low temperature high speed centrifuge) for 15 minutes, sediment is mitochondria.

4. **Serum**

Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 10 minutes at approximately 2,000rpm. Assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

5. **Plasma**

Collect plasma using heparin as an anticoagulant. Centrifuge samples for 10 minutes at 2,000rpm at 2-8°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

[ASSAY PROCEDURE]

1. Whole blood GSH-PX activity assay

(1) Enzymatic reaction: (Reagent 1 working solution is already prewarmed at 37°C).

	Non-enzyme tube	**Enzyme tube**
1mmol/L GSH (mL)	0.2	0.2
Hemolysate (mL)		0.2
Prewarm in 37°C water bath for 5 minutes.		
Reagent 1 working solution (mL)	0.1	0.1
React in 37°C water bath for 5 minutes accurately.		
Reagent 2 (mL)	2	2
Hemolysate (mL)	0.2	
Mix sufficiently, centrifugate at 3500~4000 rpm for 10 minutes, take 1mL supernatant for chromogenic reaction.		

(2）Chromogenic reaction:

	Blank tube	Standard tube	Non-enzyme tube	Enzyme tube
GSH standard solvent working solution (mL)	1			
20μmol/L GSH standard solution（mL）		1		
Supernatant（mL）			1	1
Reagent 3 working solution（mL）	1	1	1	1
Reagent 4 working solution（mL）	0.25	0.25	0.25	0.25
Reagent 5 working solution（mL）	0.05	0.05	0.05	0.05
Mix sufficiently, place at room temperature for 15 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 412nm (adjust zero by double distilled water).				

2. GSH-PX activity assay in tissues, mitochondria and cell membrane

(1) Enzymatic reaction (Reagent 1 working solution should be prewarmed in 37ºC water bath).

	Nonenzyme tube	Enzyme tube
1mmol/L GSH (mL)	0.2	0.2
Homogenate (mL)		0.2
Prewarm in 37ºC water bath for 5 minutes.		
Reagent 1 working solution (mL)	0.1	0.1
React in 37ºC water bath for 5 minutes accurately		
Reagent 2 working solution (mL)	2	2
Homogenate (mL)	0.2	
Mix sufficiently, centrifugate at 3500~4000rpm for 10 minutes, take 1mL supernatant for chromogenic reaction.		

(2) Chromogenic reaction:

	Blank tube	Standard tube	Nonenzyme tube	Enzyme tube
GSH standard solvent working solution (mL)	1			
20μmol/L GSH standard solution (mL)		1		
Supernatant (mL)			1	1
Reagent 3 working solution (mL)	1	1	1	1
Reagent 4 working solution (mL)	0.25	0.25	0.25	0.25
Reagent 5 working solution (mL)	0.05	0.05	0.05	0.05
Mix sufficiently, place at room temperature for 15 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 412nm (adjust zero by double distilled water).				

3. Blood serum (or plasma) GSH-PX activity assay

(1) Enzymatic reaction: (Reagent 1 working solution should be prewarmed at 37ºC water bath)

	Nonenzyme tube	Enzyme tube
1mmol/L GSH (mL)	0.2	0.2
Blood serum (or plasma) (mL)		0.1
Prewarm in 37ºC water bath for 5 minutes		
Reagent 1 working solution (mL)	0.1	0.1
React in 37ºC water bath for 5 minutes accurately.		
Reagent 2 working solution (mL)	2	2
Blood serum (or plasma) (mL)	0.1	
Mix sufficiently, centrifugate at 3500~4000rpm for 10 minutes, take 1mL supernatant for chromogenic reaction.		

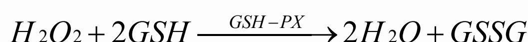
(2) Chromogenic reaction:

	Blank tube	Standard tube	Nonenzyme tube	Enzyme tube
GSH standard solvent working solution (mL)	1			
20μmol/L GSH standard solution (mL)		1		
Supernatant (mL)			1	1
Reagent 3 working solution (mL)	1	1	1	1
Reagent 4 working solution (mL)	0.25	0.25	0.25	0.25
Reagent 5 working solution (mL)	0.05	0.05	0.05	0.05
Mix sufficiently, place at room temperature for 15 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 412nm (adjust zero by double distilled water).				

[TEST PRINCIPLE]

Glutathione peroxidase (GSH-PX) is a widespread, important hydrogen peroxidase in organisms. It catalyzes reduced glutathione's reduction reaction with peroxide diagnostically leads to protection of cell structure and functions. GSH-PX's active site is Se-cysteine, selenium is necessary part of GSH-PX, 1 mol GSH-PX contains 4mol selenium. GSH-PX activity can be used as a biochemical indicator of Se level in vivo.

Glutathione peroxidase (GSH-PX) can catalyze peroxide (H_2O_2) and reduced glutathione (GSH) to produce H_2O and oxidized glutathione (GSSG). GSH-PX activity can be represented by its enzymatic reaction rate. It is able to calculate enzyme activity by measuring GSH consumption in this enzymatic reaction.



$$H_2O_2 + 2GSH \xrightarrow{GSH-PX} 2H_2O + GSSG$$

GSH-PX activity is represented by catalyzed GSH reaction rate. These two substrates can undertake redox reaction without enzyme (non-enzymatic reaction), so GSH consumption caused by non-enzymatic reaction must be deducted when you calculate enzyme activity in conclusion.

GSH content assay: GSH reacts with dithio-dinitrobenzoic acid to produce 5-thio-dinitrobenzoic acid anions with relatively stable yellow color. It is able to calculate GSH content by measuring absorbance at 412nm.

[CALCULATION OF RESULTS]

1. **Whole blood**

a. Definition: Reacts at 37ºC for 5 minutes, 1μmol/L GSH concentration decreasing (already deduct effect of nonenzymatic reaction) in reaction system per 4μL whole blood is considered as 1 enzyme activity unit (U).

b. Formula::

$$\text{Whole blood GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times \text{Dilution times } (5^{*} \times \frac{1+X^{**}}{1+49^{***}})$$

Note: * According to operation table of enzymatic reaction, 0.5mL reaction solution is mixed with 2mL Reagent 2, this fact equals to 5 times dilution, so multiply with 5.

** X is whole blood dilution times. For example, if you dilute at ratio of 1:99, then X=99.

*** 1+49: When hemolysate is diluted at ratio of 1:49, taking 0.2mL hemolysate to assay equals to taking 4μL whole blood.

c. Example: hemolysate optimal sample concentration and optimal sample volume probing:

(1) Sample source：Fresh normal rat tail whole blood.

(2) Sample pretreatment:

Dilute rat whole blood with double distilled water at ratio of 1:24, 1:49, 1:59, 1:69, 1:79, 1:89, 1:99, 1:149, 1:199 separately in order to make a series of hemolysates of different concentrations. Take 0.2mL a series of hemolysates of different concentrations to assay according to whole blood assay operation table.

(3) Result:

OD_{Blank}		0.041	
$OD_{Standard}$		0.163	
Sample concentration	OD_{Enzyme}	$OD_{Non-enzyme}$	Inhibition percentage
1：24	0.079	0.451	82.48%
1：49	0.096	0.452	78.76%
1：59	0.107	0.450	76.22%
1：69	0.118	0.452	73.89%
1：79	0.130	0.450	71.11%
1：89	0.144	0.450	68.00%
1：99	0.163	0.449	63.70%
1：149	**0.231**	**0.450**	**48.67%**
1：199	0.298	0.449	33.63%

(4) Conclusion:

According to data above, optimal sample concentration of 1:149 can make inhibition ratio (inhibition

percentage=(($OD_{Nonenzyme}$-OD_{Enzyme})/$OD_{Nonenzyme}$) ×100%) in the range of 45%～55%. As result, it is better to take 0.2mL 1:149 diluted normal rat tail whole blood for GSH-PX formal experiment.

Example 1: Take 0.2mL 1:49 diluted hemolysate to assay. In results, $OD_{Nonenzyme}$ is 0.463, OD_{Enzyme} is 0.228, $OD_{Standard}$ is 0.165, OD_{Blank} is 0.041. Calculate as follows:

$$\text{Whole blood GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times 5 \times \frac{1+49}{1+49}$$

$$= \frac{0.463-0.228}{0.165-0.041} \times 20 \times 5 \times \frac{50}{50} = 189.52\ U$$

Example 2: Take 0.2mL 1:149 diluted rat hemolysate to assay, length of water bath is 5 minutes. In results, $OD_{Nonenzyme}$ is 0.451, OD_{Enzyme} is 0.205, $OD_{Standard}$ is 0.165, OD_{Blank} is 0.041. Calculate as follows:

$$\text{Whole blood GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times 5 \times \frac{1+149}{1+49}$$

$$= \frac{0.451-0.205}{0.165-0.041} \times 20 \times 5 \times \frac{150}{50} = 595.16\ U$$

2. **Tissues, mitochondria and cell membrane**

a. Definition: 1μmol/L GSH concentration decreasing (already deduct effect of nonenzymatic reaction) in reaction system per mg protein per minute is considered as 1 enzyme activity unit (U).

Note: Tissue protein assay: According to experimental methodology, use biuret method, Coomassie brilliant blue method, ultraviolet method or salicylsulfonic acid method to measure tissue protein content in milligrams.

b. Formula:

$$\text{Tissue GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times \text{Dilution times } (5^*) \div \text{Reaction time length} \div (\text{Sample volume} \times \text{Protein content in sample})$$

Note: * According to operation table of enzymatic reaction, 0.5mL reaction solution is mixed with 2mL Reagent 2, this fact equals to 5 times dilution, so multiply with 5.

c . Example: tissue homogenate optimal sample concentration and optimal sample volume probing:

(1) **Sample source**： Use normal mouse liver tissue to make 10% liver homogenat supernatant, dilute with physiological saline to 1% for assay.

(2) Sample pretreatment:

Dilute 1% liver homogenate with physiological saline to 1.0%, 0.5%, 0.4%, 0.3%, 0.25%, 0.1%, 0.05% separately in order to make a series of homogenates of different concentrations. Take 0.2mL a series of homogenates of

different concentrations to assay according to tissue assay operation table.

(3) Result:

OD_{Blank}		0.041	
$OD_{Standard}$		0.163	
Sample concentration	OD_{Enzyme}	$OD_{Nonenzyme}$	Inhibition percentage
0.05%	0.411	0.463	11.23%
0.10%	0.356	0.464	23.28%
0.20%	0.283	0.462	38.74%
0.25%	**0.244**	**0.461**	**47.07%**
0.30%	0.213	0.462	53.89%
0.40%	0.175	0.461	62.04%
0.50%	0.145	0.46	68.47%
1.00%	0.085	0.46	81.52%

(4) Conclusion:

According to data above, optimal sampling concentration of 0.25%~0.3% can make inhibition ratio (inhibition percentage=(($OD_{Nonenzyme}$-OD_{Enzyme})/$OD_{Nonenzyme}$) ×100%) in the range of 45%～55%. As result, it is better to take 0.2mL 0.25% normal mouse liver tissue homogenate for GSH-PX formal assay.

Example 1: Take 0.2mL 0.25% mouse liver tissue homogenate to assay. In results, $OD_{Nonenzyme}$ is 0.480, OD_{Enzyme} is 0.172, $OD_{Standard}$ is 0.163, OD_{Blank} is 0.041, protein content in 1% homogenate is 0.910mg/mL, standard solution concentration is 20μmol/L . Calculate as follows:

$$\text{Liver tissue GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration (20}\mu\text{mol/L)} \times \text{Dilution times}$$

$$\div \text{Reaction time length} \div (\text{Sample volume} \times \text{Protein content in sample})$$

$$= \frac{0.480-0.172}{0.163-0.041} \times 20 \times 5 \div 5 \div (0.910 \div 4^{*} \times 0.2)$$

$$= 1109.71\text{U}$$

Note: * 0.25% mouse liver homogenate is used to measure GSH-PX activity, 1% mouse liver homogenate is used to measure protein content, so in calculation, it should be divided by 4.

Example 2: Take 0.2mL 0.5% crucian liver tissue homogenate to assay. In results, $OD_{Nonenzyme}$ is 0.454, OD_{Enzyme} is 0.264, $OD_{Standard}$ is 0.163, OD_{Blank} is 0.041, protein content in 1% homogenate is 0.8367mg/mL, standard solution concentration is 20μmol/L . Calculate as follows:

$$\text{Liver tissue GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration (20}\mu\text{mol/L)} \times \text{Dilution times}$$

$$\div \text{Reaction time length} \div (\text{Sample volume} \times \text{Protein content in sample})$$

$$= \frac{0.454-0.264}{0.163-0.041} \times 20 \times 5 \div 5 \div (0.8367 \div 2^{*} \times 0.2)$$

$$= 372.266\text{ U}$$

Note: * 0.5% crucian liver homogenate is used to measure GSH-PX activity, 1% crucian liver homogenate is used to measure protein content, so in calculation, it should be divided by 2.

Example 3: Take 0.2mL 10% rat testis tissue homogenate to assay. In results, $OD_{Nonenzyme}$ is 0.468, OD_{Enzyme} is 0.258, $OD_{Standard}$ is 0.163, OD_{Blank} is 0.041, protein content in 10% homogenate is 6.941mg/mL, standard solution concentration is 20μmol/L . Calculate as follows:

$$\text{Testis tissue GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times \text{Dilution times} \div \text{Reaction time length} \div (\text{Sample volume} \times \text{Protein content in sample})$$

$$= \frac{0.468-0.258}{0.163-0.041} \times 20 \times 5 \div 5 \div (6.941 \times 0.2)$$

$$= 24.799\ U$$

3. **Serum/plasma**

a. Definition: Reacts at 37ºC for 5 minutes, 1μmol/L GSH concentration decreasing (already deduct effect of nonenzymatic reaction) in reaction system per 0.1mL blood serum is considered as 1 enzyme activity unit (U).

b. Formula:

$$\text{Blood serum (or plasma) GSH-PX activity} = \frac{OD_{Nonenzyme} - OD_{Enzyme}}{OD_{Standard} - OD_{Blank}} \times \text{Standard solution concentration } (20\mu mol/L) \times \text{Dilution times } (6^{*}) \times \text{Sample dilution times before assay}$$

Note: * According to operation table of enzymatic reaction, 0.4mL reaction solution is mixed with 2mL Reagent 2, this fact equals to 6 times dilution, so multiply with 6.

c. **Example**: blood plasma optimal sample concentration and optimal sample volume probing:

(1) Sample source：Take whole blood from normal rat eye sockets, get blood plasma anticoagulated by heparin.

(2) Sample pretreatment:

Dilute blood plasma with physiological saline at ratio of 1:1, 1:4, 1:7, 1:14, 1:19 separately in order to make a series of blood plasma of different concentrations. Take 0.1mL a series of blood plasma of different concentrations to assay according to blood plasma (serum) assay operation table.

(3) Result:

OD_{Blank}		0.041	
$OD_{Standard}$		0.163	
Sample concentration	OD_{Enzyme}	$OD_{Nonenzyme}$	Inhibition percentage
1：1	0.117	0.519	77.46%
1：4	**0.283**	**0.520**	**45.58%**
1：7	0.365	0.518	29.53%
1：9	0.397	0.518	23.36%
1：14	0.421	0.516	18.41%
1：19	0.442	0.517	14.51%

(4) Conclusion:

According to data above, optimal sample concentration of 1:4 can make inhibition percentage (inhibition percentage=(($OD_{Nonenzyme}$-OD_{Enzyme})/$OD_{Nonenzyme}$) ×100%) in the range of 45%～55%. As result, it is better to take 0.1mL 1:4 diluted normal rat blood plasma for GSH-PX formal assay.

Take 0.1mL 1:4 diluted rat blood serum to assay. In results, $OD_{Nonenzyme}$ is 0.475, OD_{Enzyme} is 0.293, $OD_{Standard}$ is 0.165, OD_{Blank} is 0.041. Calculate as follows:

$$\text{Rat blood serum GSH-PX activity} = \frac{0.475-0.293}{0.165-0.041} \times 20 \times 6 \times 5 = 880.65\ \text{U}$$

[IMPORTANT NOTE]

1. Hemolysate enzyme activity keeps stable at room temperature for 1 hour. So it is suggested to measure no longer than 1hour after sample dilution.
2. Blood samples must be fresh. Heparin anticoagulated blood can be stored at 4～8ºC in fridge less than 48 hours.
3. Please make sure that erythrocytes are completely haemolyzed before measure blood GSH-PX activity (completely haemolyzed blood appears **transparent** towards light, **if it is not transparent enough, then you can freeze-thaw it once.** But erythrocytes of some rats and swines can not be placed below 0ºC or they will be hard to haemolyze. For example, erythrocytes of diabetic rats and some normal rats are very hard to haemolyze after freezing. It is better to take 1-2 samples to do pretesting before formal experiment.
4. Reagent 1 container should be washed completely, its working solution should be used soon after preparation.
5. 1mmol/L GSH, 100μmol/L GSH, 20μmol/L GSH standard solutions should be used soon after preparation.
6. **Reaction time recording must be accurate** (for example, react in 37ºC water bath for 5 minutes).
7. Taking and measuring of supernatant should be down in same day.
8. There are various methods to measure protein content in tissue, you can follow Experimental Methodology or buy our protein assay kits.

[APPENDIX]

GSH standard curve preparation

1. **Operation table:**

Take 2mL 1mmol/L GSH standard solution, add 18mL GSH standard solvent to adjust volume to 20mL,100μmol/L GSH standard working solution is prepared.

Tube No.	1	2	3	4	5	6
100μmol/L GSH standard（mL）	0	0.4	0.8	1.2	1.6	2
Reagent 2 working solution (mL)	2	1.6	1.2	0.8	0.4	0
Reagent 3 working solution (mL)	2	2	2	2	2	2
Reagent 4 working solution (mL)	0.5	0.5	0.5	0.5	0.5	0.5
Reagent 5 working solution (mL)	0.1	0.1	0.1	0.1	0.1	0.1
Mix sufficiently, place at room temperature for 15 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 412nm (adjust zero by double distilled water)						

2. **Result:**

Tube No.	1	2	3	4	5	6
Corresponding GSH standard concentration(μmol/L)	0	20	40	60	80	100
Referenced OD	0.043	0.165	0.285	0.406	0.525	0.648
Absolute OD	0	0.122	0.242	0.363	0.482	0.605

3. **Draw graph**:

Use corresponding GSH standard concentration as x axis, use absolute OD values as y axis, draw standard curve: