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Hydroxy Radical Assay kit

(Spectrophotometric method)

Serial No.: BC000   Pack: 50T/48S

1. Assay principle:

Fenton reaction is the most common hydroxy radical producing chemical reaction, H_2O_2 amount appears direct proportion with OH· amount produced by Fenton reaction. It receives electron acceptor and reacts with Griess reagent to produce red compound, its absorbance appears direct proportion with OH· amount.

This kit can be used for laboratory research only.

2. Reagent composition & preparation:

Reagent 1: 3% H_2O_2 standard stock solution 0.5ml×1 vial, can be stored at 4℃ for 3 months.

0.03% standard working solution: Dilute 3% H_2O_2 standard stock solution with double distilled water at ratio of 1:99, this working solution should be used soon after preparation.

Reagent 2: Substrate stock solution 1ml×1 vial, can be stored at 4℃ for 3 months.

Substrate working solution preparation:

(1) If you use hydroxy radical inhibition sample (ODSample<ODContrast), then dilute substrate stock solution with double distilled water at ratio of 1:99, this working solution should be used soon after preparation.

(2) If you use hydroxy radical producing sample (ODSample>ODContrast), then dilute substrate stock solution with double distilled water at ratio of 1:299, this working solution should be used soon after preparation.

Reagent 3: Solution A stock solution 2ml×1 vial, can be stored at 4℃ for 3 months. When use, dilute stock solution with double distilled water at ratio of 1:9 to make Solution A working solution.

Solution B 7ml×2 vials, can be stored at 4℃ for 3 months.

Reagent 3 working solution preparation: Mix Solution A working solution with Solution B at ratio of 1:1, consider reagent volume according to you require, residuary solution can be stored at 4℃.

Reagent 4: Solution 10ml×1 bottle, can be stored at 4℃ for 3 months. When use, add double distilled water until volume reaches to 100ml, can be stored at 4℃ for 3 months. If crystals seed out, then place container in 37℃ water bath until dissolving completely before dilution.

Reagent 5: Solution 30ml×1 bottle, can be stored at 4℃ away from light for 3 months.

Reagent 6: Solution 30ml×1 bottle, can be stored at 4℃ away from light for 3 months.

Reagent 7: Analytical pure glacial acetic acid, provided by yourself.

Chromogenic agent preparation: Mix Reagent 4, Reagent 5, Reagent 6 & glacial acetic acid at ratio of 8:3:3:2, consider reagent volume according to you require, this chromogenic agent should be used soon after preparation.

Note: Hydroxy radical inhibiting materials: blood serum (or plasma), various tissue homogenates, health care products, etc;

Hydroxy radical producing materials: Neutrocytes, some drugs, some plants, etc.

3. Operation procedure:

Prewarm all prepared working solutions in 37℃ water bath for 3 minutes, then do all steps in 37℃ water bath.

	Blank tube	Standard tube	Contrast tube	Sample tube
Double distilled water (ml)	0.4	0.2	0.2	
0.03% H2O2 standard working solution (ml)		0.2		
Substrate working solution (ml)			0.2	0.2
Sample* (ml)				0.2
Reagent 3 working solution (ml)	0.4	0.4	0.4	0.4
Mix sufficiently, react at 37℃ for 1 minute accurately (count time by seconds-counter), at 1 minute after adding Reagent 3, add chromogenic agent immediately to terminate reaction. You should operate on 1 test tube once.				
Chromogenic agent (ml)	2	2	2	2

Mix sufficiently, place at room temperature for 20 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 550nm (adjust zero by double distilled water).

*** Referenced sample volume: Blood serum (or plasma) sample should be diluted with physiological saline 20 times, then take 0.2ml to assay; if you have precise micropipet, then you can take 0.010ml blood serum (or plasma) directly, add 0.190ml physiological saline. 0.5% tissue homogenate sample should be taken 0.2ml to assay. Extensional sample volume should be**

determinated by you probing (see in Appendix).

4. Calculation:

(1) Blood serum (or plasma) hydroxy radical inhibition capacity assay:

① **Definition:** 1ml blood serum (or plasma) reacts at 37℃ for 1 minute, 1mmol/L H2O2 concentration decreasing in reaction system is considered as 1 hydroxy radical inhibition capacity unit (U).

② **Formula:**

$$\text{Hydroxy radical inhibition capacity (U/ml)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Standard} - OD_{Blank}} \times \text{Standard concentration (8.824mmol/L)} \times \frac{1ml}{\text{Sample volume}} \times \text{Sample dilution times before assay}$$

③ **Example:**

Take 0.2ml 20 times diluted (by physiogical saline) blood serum to measure hydroxy radical inhibition capacity, in result, ODContrast is 0.785, ODSample is 0.464, ODStandard is 0.443, ODBlank is 0.003, H2O2 standard concentration is 8.824mmol/L, calculate as follows:

$$\text{Hydroxy radical inhibition capacity (U/ml)} = \frac{0.785 - 0.464}{0.443 - 0.003} \times 8.824 \times \frac{1}{0.2} \times 20 = 643.75U/ml$$

(2) Tissue hydroxy radical inhibition capacity assay:

① **Definition:** 1mg tissue protein reacts at 37℃ for 1 minute, 1mmol/L H2O2 concentration decreasing in reaction system is considered as 1 hydroxy radical inhibition capacity unit (U).

② **Formula:**

$$\text{Hydroxy radical inhibition capacity (U/mgprot)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Standard} - OD_{Blank}} \times \text{Standard concentration (8.824mmol/L)}) \div \left[\text{Sample protein concentration (mgprot/ml)} \times \text{Sample volume (0.2ml)} \right]$$

③ **Example:**

Take 0.1ml 5% mouse liver tissue homogenate, dilute 10 times by physiogical saline to make 0.5% mouse liver tissue homogenate, take 0.2ml to measure hydroxy radical inhibition activity, in results, ODContrast is 0.785, ODSample is 0.347, ODStandard is 0.443, ODBlank is 0.003, H2O2 standard

concentration is 8.824mmol/L, protein concentration in 0.5% mouse liver tissue homogenate is 0.486mg/ml, calculate as follows.

$$\text{Hydroxy radical inhibition capacity (U/mgprot)} = \frac{0.785-0.443}{0.347-0.003} \times 8.824 \div (0.486 \times 0.2) = 90.37\,U/mgprot$$

(3) Hemolysate hydroxy radical inhibition capacity assay:

① **Definition:** 1mg hemoglobin reacts at 37℃ for 1 minute, 1mmol/L H2O2 concentration decreasing in reaction system is considered as 1 hydroxy radical inhibition capacity unit (U).

② **Formula:**

$$\text{Hydroxy radical inhibition capacity (U/mgHb)} = \frac{OD_{Contrast}-OD_{Sample}}{OD_{Standard}-OD_{Blank}} \times \text{Standard concentration (8.824mmol/L)} \times \frac{1ml}{\text{Sample volume}} \times \text{Sample dilution times before assay} \div \text{Hemoglobin content (mgHb/ml)}$$

③ **Examples:**

a. Take 0.2ml anticoagulated RBC, add 0.8ml cold double distilled water, mix sufficiently by vortex for 1 minute to make hemolysate, hemoglobin content is 41.182mgHb/ml. Take 0.01ml hemolysate, add 5.99ml double distilled water, mix sufficiently, take 0.2ml to measure hydroxy radical inhibition capacity, in results, ODContrast is 0.785, ODSample is 0.615, ODStandard is 0.443, ODBlank is 0.003, H2O2 standard concentration is 8.824mmol/L, calculate as follows:

$$\text{Hydroxy radical inhibition capacity (U/mgHb)} = \frac{0.785-0.615}{0.443-0.003} \times 8.824 \times \frac{1}{0.2} \times 600 \div 41.182 = 248.36\,U/mgHb$$

b. Take 0.2ml anticoagulated RBC, add 0.8ml cold double distilled water, mix sufficiently by vortex for 1 minute to make hemolysate, hemoglobin content is 53.684mgHb/ml. Take 0.01ml hemolysate, add 5.99ml double distilled water, mix sufficiently, take 0.2ml to measure hydroxy radical inhibition capacity, in result, ODContrast is 0.785, ODSample is 0.304, ODStandard is 0.443, ODBlank is 0.003, H2O2 standard concentration is 8.824mmol/L, calculate as follows:

$$\text{Hydroxy radical inhibition capacity (U/mgHb)} = \frac{0.785-0.304}{0.443-0.003} \times 8.824 \times \frac{1}{0.2} \times 600 \div 53.684 = 539.06\,U/mgHb$$

(4) Hydroxy radical producing capacity assay:

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① **Definition:** 1ml or 1mg material or (10^6 cells in $1cm^3$) reacts at 37℃ for 1 minute, 1mmol/L H_2O_2 concentration increasing in reaction system is considered as 1 hydroxy radical producing capacity unit (U).

② **Formula:**

$$\text{Hydroxy radical producing capacity (U/ml)} = \frac{OD_{Sample} - OD_{Contrast}}{OD_{Standard} - OD_{Blank}} \times \text{Standard concentration (8.824mmol/L)} \times \frac{1ml}{\text{Sample volume}} \times \text{Sample dilution times before assay}$$

③ **Example:**

Take 0.2ml 10 times diluted traditional Chinese drug material to measure hydroxy radical producing capacity, in result, ODContrast is 0.217, ODSample is 0.621, ODStandard is 0.443, ODBlank is 0.003, H_2O_2 standard concentration is 8.824mmol/L, calculate as follows:

$$\text{Hydroxy radical producing capacity (U/ml)} = \frac{0.621 - 0.217}{0.443 - 0.003} \times 8.824 \times \frac{1}{0.2} \times 10 = 405.10U/ml$$

5. Announcements:

（1）Operate on 1 test tube once separately, and reaction time must be 1 minute accurately.

（2）Add reagents according to operation table strictly, DO NOT make mixed reagents.

（3）You can use physiological saline, distilled water, acetic acid or dehydrated alcohol as sample solvent/medium, but never use phosphate buffer solution (PBS).

（4）This method is relatively sensitive, if you assay sample except blood serum (or plasma) & tissue, then it is suggested to take undiluted sample and diluted samples of different ratios (such as 5 times, 10 times) to do probing. If sample tube's color is too light, then dilute it with solvent until color becomes deep enough. Our Institute has measured hydroxy radicals in pollen extract, 150 times diluted sample appears good chromogenic behavior.

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APPENDIX: Hydroxy radical optimal sample concentration/volume probing

1. Sample pretreatment:

(1) Blood serum (or plasma): Dilute blood serum (or plasma) with physiological saline at ratio of 1:1, 1:4, 1:9, 1:19 to prepare a series of blood serum (or plasma) of different concentrations, take 0.1ml blood serum (or plasma) of different concentrations separately to do assay according to blood serum assay operation table.

(2) Tissue homogenates, cells, mitochondria and cell membrane, etc: Dilute tissue homogenate with physiological saline to a series of tissue homogenates such as 10%、5%、2%、1%、0.5%、0.1%, etc, take 0.1ml blood serum (or plasma) of different concentrations separately to do assay according to tissue assay operation table.

2. Operation procedure (use blood serum sample)

(1) Sample source: Normal rat whole blood (from eye sockets), use heparin for anticoagulation, do hydroxy radical assay.

(2） Sample dilution: Dilute blood serum (or plasma) with physiological saline at ratio of 1:1, 1:4, 1:9, 1:19, 1:49, 1:99 to make a series of blood serum (or plasma) of different concentrations, take 0.2ml blood serum (or plasma) of different concentrations separately to do assay according to blood serum assay operation table

3. Operation table:

	Blank tube	Standard tube	Contrast tube	Sample tube
Double distilled water (ml)	0.4	0.2	0.2	
0.03% H2O2 standard working solution (ml)		0.2		
Substrate working solution (ml)			0.2	0.2
Sample* (ml)				0.2
Reagent 3 working solution (ml)	0.4	0.4	0.4	0.4
Mix sufficiently, react at 37℃ for 1 minute accurately (count time by seconds-counter), at 1 minute after adding Reagent 3, add chromogenic agent immediately to terminate reaction. You should operate on 1 test tube once.				

Chromogenic agent (ml)	2	2	2	2

Mix sufficiently, place at room temperature for 20 minutes, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 550nm (adjust zero by double distilled water).

4. Result:

Contrast	0.785		Blank	0.003	Standard	0.443
Dilution times	1:1	1:4	1:9	1:19	1:49	1:99
OD value	0.105	0.132	0.188	0.408	0.677	0.741
Inhibition ratio	86.59%	83.16%	76.01%	47.99%	13.71%	5.66%

(5) Conclusion:

According to the data above, inhibition ratio (((ODContrast-ODSample)/ODContrast)×100%) of 45%～55%'s corresponding optimal sample concentration is 1:19. As result, take 0.2ml 1:19 diluted normal rat blood plasma to do formal hydroxy radical assay.

(6) Discussion:

Before you do formal assay, please take 2~3 samples from each batch to do probing (pretest) in order to determine optimal sample concentration/volume. Please make sure inhibition ratio is between 20% and 50%, there should also be differences between batches. If you have doubt, then do probing again.