Inhibition and produce superoxide anion assay kit

(CAT/NO.:BC101 Size:50T/48S)

1. Assay principle (Colorimetric Method)

This kit imitates xanthine-xanthine oxidase reaction system to produce superoxide anion radical (O2), then electron transfer matter & Gress's chromogenic reagent is added in order to make reaction system presents prunosus color. It is able to calculate inhibition & production of superoxide anion radical by measuring OD values. If sample contains O2· inhibitor, then ODSample tube is lower than ODContrast; if sample contains O2·producer, the ODSample tube is higher than ODContrast. It is able to calculate sample's effect to O2· by using vitamin C as standard.

2. Composition and preparation (The kit is valid for 6 months)

(1) Reagent 1: Solution 5ml×1 bottle (Crystals may seed out in cold days, so please dissolve them by hot water bath before use). When use, add distilled water until 50ml, can be stored at room temperature for 1 year (take care of mould).

(2) Reagent 2: Solution 5ml×1 bottle,store at 2℃~8

(3) Reagent 3: Solution 5ml×1 bottle, store at 2℃~8℃

(4) Reagent 4: Solution 350μl×1 vial, can be stored at 4℃ (No freezing!) Diluent 5ml×1 bottle, can be stored at 2℃~8℃ for 6 months. When use, dilute solution with diluent at ratio of 1:14, consider volume according to requirement. Prepared Reagent 4 can be stored at 2℃~8℃ for 1 month. (No freezing!)

Note: All tips used in operation must be used specially and autoclaved.

(5) Reagent 5: Powder ×1 vial, add 37.5ml 70℃~80℃ hot distilled water to dissolve before use. Prepared reagent can be stored at 2℃~8℃ away from light.

(6) Reagent 6: Powder ×1 vial, add 37.5ml distilled water to dissolve before use, prepared reagent can be stored at 2℃~8℃ away from light.

Chromogenic reagent preparation: Mix Reagent 5, Reagent 6 and acetic acid at ratio of 3 : 3 :2, prepared chromogenic reagent can be stored at 2℃~8℃ for 3 months .

(7) Reagent 7: Vc standard 4 vials.

Vc standard stock solution preparation: Dissolve 1 vial of Vc standard in distilled water, adjust volume to 5ml (prepared Vc standard should be used at same day).

0.15mg/ml Vc standard working solution preparation: Mix 1ml stock solution and 4ml distilled water, 5 times diluted standard should be used soon after preparation.

Note: Prepared Vc standard decomposes quickly under light, so prepared 0.15mg/ml Vc standard working solution should be used for assay in 30 minutes.

3. Required instruments and reagents

Visible spectrophotometer and 1cm path length cuvette (or ELISA reader (550nm) and 96-well plate), 37℃ water bath (or incubator), vortex mixer, electric furnace (for heating reagents), distilled water, glacial acetic acid (analytical grade, acetic acid concentration ≥99.5%), physiological saline, chloroform (analytical grade, for high-fat samples), protein assay reagent (for tissue samples, available from our company).

4. Sample pretreatment

① Serum (plasma) samples: (Hemolysis must be avoided during blood collection) Serum (plasma) should be used directly. Samples should ideally be stored at 4℃ and tested on the same day. If this is not possible, they can be frozen below 0℃; the lower the temperature, the longer the storage time.

② Tissue samples: Accurately weigh the tissue to be tested. Add 9 times the volume of physiological saline at a weight (g):volume (mL) ratio of 1:9. Homogenize mechanically under ice-water bath conditions to prepare a 10% tissue homogenate. Centrifuge at 2500 rpm for 10 minutes, and use the supernatant for analysis. (Take a portion of the supernatant to determine protein concentration. Protein quantification kits are available in our facility; we recommend using our Coomassie Brilliant Blue method protein quantification kit.)

③ Cell Samples: Collect cells (diet with trypsin or scrape off cells, transfer to centrifuge tubes, centrifuge at 1000-2000 rpm for 5 minutes, discard the

supernatant and keep the precipitate; for suspended cells, transfer directly and centrifuge to collect the precipitate). Wash 1-2 times with 0.5-1 mL of PBS, centrifuge at 1000-2000 rpm to collect the precipitated cells, then add 0.3-0.5 mL of 0.1M pH 7.4 isotonic PBS buffer to suspend the cells. Sonicate or manually grind the cells to break them up. Take the cell suspension (if the suspension contains obvious particles or flocculent matter, centrifuge at 2000 rpm for 10 minutes and use the supernatant) for testing.

5. Operation procedure

Reagent	Control tube	Standard tube	Sample tube
Reagent 1（ml）	1.0	1.0	1.0
Distilled water（ml）	0.05		
0. 15mg/ml Vc standard（ml）		0.05	
Sample（ml）			0.05
Reagent 2（ml）	0. 1	0. 1	0. 1
Reagent 3（ml）	0. 1	0. 1	0. 1
Reagent 4（ml）	0. 1	0. 1	0. 1

Mix sufficiently by vortex, place in 37℃thermostatic waterbath for 40 minutes.

Chromogenic reagent（ml）	2.0	2.0	2.0

Mix sufficiently, place for 10 minuts, transfer in cuvette of 1cm light path, adjust zero by distilled water, measure OD values at 550nm.。

Note: Optimal sample volume: Different matters have different O2 · in hibition capacities under

formulary substrate concentration in reaction system, thus sample concentration should not be too high or too lower or it will disturb result. Please do pretest before formal experment in order to get optimal sample concentration.(according to Appendix II)

5. Calcultion

(Different matters have different definitions and different formulas as follows):

(1-1) Anti-superoxide anion radical activity definition in blood serum (or plasma)

In 37℃ reaction system, superoxide anion radical inhibition per liter blood serum (or plasma) in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 anti-superoxide anion radical activity unit.

(1-2) Formula:

$$\text{Blood serum (or plasma) anti-superoxide anion radical activity (U/L)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Contrast} - OD_{Standard}} \times \text{Standard concentration (0.15mg/ml)} \times 1000\text{ml} \times \frac{\text{Sample dilution times before assay}}{}$$

(1-3) Example:

Take 50μl blood serum to do anti-superoxide anion radical assay , in result, ODContrast is 0.570, ODSample is 0.230, ODStandard is 0.256, standard tube's concentration is 0. 15mg/ ml Vc standard. Calculate as follows:

$$\text{Blood serum (or plasma) anti-superoxide anion radical activity (U/L)} = \frac{0.570 - 0.230}{0.570 - 0.256} \times 0.15 \times 1000 \times 1 = 162.42(U/L)$$

(2-1) Anti-superoxide anion radical activity definition in tissue sample:

In 37 ℃ reaction system, superoxide anion radical inhibition per mg tissue protein in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 anti-superoxide anion radical activity unit.

(2-2) Formula:

$$\text{Tissue anti-superoxide anion activity unit (U/gprot)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Contrast} - OD_{Standard}} \times \text{Standard concentration (0.15mg/ml)} \times 1000\text{ml} \div \frac{\text{Protein concentration}}{\text{(gprot/L)}}$$

(2-3) Example:

Take 50μl 1% rat gastric mucosa homogenate to do anti-superoxide anion radical assay , in result, ODContrast is 0.506, ODSample is 0.267, ODStandard is 0.265, protein concentration is 0.657mgprot/ml, standard tube's concentration is 0. 15mg/ ml Vc standard. Calculate as follows:

$$\text{Tissue anti-superoxide anion activity unit (U/gprot)} = \frac{0.506-0.267}{0.506-0.265}\times 0.15\times 1000 \div 0.657 = 226.42(U/gprot)$$

5. This kit also applies to measure superoxide anion radical production such as leukocytes and some medicines, calculate as follows:

(1) Definition: In 37℃ reaction system, superoxide anion radical production per 1L(or 1g) matter in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 superoxide anion radical production activity unit.

(2) Formula: ①

$$\text{Superoxide anion production activity unit (U/L)} = \frac{OD_{Contrast}-OD_{Sample}}{OD_{Contrast}-OD_{Standard}} \times \text{Standard concentration} \times 1000ml \times \text{Sample dilution times before assay}$$

(0.15mg/ml)

②

$$\text{Superoxide anion production activity unit (U/g)} = \frac{OD_{Contrast}-OD_{Sample}}{OD_{Contrast}-OD_{Standard}} \times \text{Standard concentration} \times 1000ml \div \text{Sample concentration (g/L)}$$

(0.15mg/ml)

(1-3) Example:

Take 50μl blood serum to do anti-superoxide anion radical assay , in result, ODContrast is 0.570, ODSample is 0.230, ODStandard is 0.256, standard tube's concentration is 0. 15mg/ ml Vc standard. Calculate as follows:

$$\text{Blood serum (or plasma) anti-superoxide anion radical activity (U/L)} = \frac{0.570-0.230}{0.570-0.256}\times 0.15\times 1000\times 1 = 162.42(U/L)$$

(2-1) Anti-superoxide anion radical activity definition in tissue sample:

In 37 ℃ reaction system, superoxide anion radical inhibition per mg tissue protein in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 anti-superoxide anion radical activity unit.

(2-2) Formula:

$$\text{Tissue anti-superoxide anion activity unit (U/gprot)} = \frac{OD_{Contrast}-OD_{Sample}}{OD_{Contrast}-OD_{Standard}}\times \text{Standard concentration (0.15mg/ml)} \times 1000\text{ml} \div \text{Protein concentration (gprot/L)}$$

(2-3) Example:

Take 50μl 1% rat gastric mucosa homogenate to do anti-superoxide anion radical assay , in result, ODContrast is 0.506, ODSample is 0.267, ODStandard is 0.265, protein concentration is 0.657mgprot/ml,

standard tube's concentration is 0. 15mg/ ml Vc standard. Calculate as follows:

$$\text{Tissue anti-superoxide anion activity unit (U/gprot)} = \frac{0.506-0.267}{0.506-0.265}\times 0.15\times 1000\div 0.657 = 226.42(U/gprot)$$

5. This kit also applies to measure superoxide anion radical production such as

leukocytes and some medicines, calculate as follows:

(1) Definition: In 37℃ reaction system, superoxide anion radical production per 1L(or 1g) matter in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 superoxide anion radical production activity unit.

(2) Formula: ①

$$\text{Superoxide anion production activity unit (U/L)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Contrast} - OD_{Standard}} \times \text{Standard concentration (0.15mg/ml)} \times 1000ml \times \text{Sample dilution times before assay}$$

②

$$\text{Superoxide anion production activity unit (U/g)} = \frac{OD_{Contrast} - OD_{Sample}}{OD_{Contrast} - OD_{Standard}} \times \text{Standard concentration (0.15mg/ml)} \times 1000ml \div \text{Sample concentration (g/L)}$$

6. Conclusion

According to standard curve above, inhibition ratio presents linear relation in the range of 10%~60%. Take the tube which inhibition ratio is between **40%～50%** as **optimal concentration,** If inhibition ratio is higher than 60% (in flat stage of the curve), then please decrease sample concentration before assay again.If inhibition ratio is lower than 10%, then please increase sample concentration before assay again.

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

Appendix II: Lipid-containing sample assay

1. Operation table:

Reagent	Contrast tube	Standard tube	Sample tube	Blank tube
Reagent 1（ml）	1.0	1.0	1.0	1.0
Distilled water（ml）	0.05			
0. 15mg/ml Vc standard（ml）		0.05		
Sample（ml）			0.05	
Reagent 2（ml）	0. 1	0. 1	0. 1	0. 1
Reagent 3（ml）	0. 1	0. 1	0. 1	0. 1
Reagent 4（ml）	0. 1	0. 1	0. 1	0. 1
Mix sufficiently by vortex, place in 37℃thermostatic waterbath for 40 minutes				
Chromogenic blank（ml）	2.0	2.0	2.0	2.0
Sample（ml）				0.05
Trichloromethane（ml）	0.05	0.05	0.05	0.05

Mix sufficiently, centrifugate at 3500rpm for 10 minutes, transfer supernatant in cuvette of 1cm lightpath, adjust zero by distilled water, measure OD values at 550nm.

2. Calculation:

(1). Definition: In 37℃ reaction system, superoxide anion radical inhibition per liter blood serum (or plasma) in 40 minutes which equals to superoxide anion radical inhibition caused by 1mg Vc is considerred as 1 anti-superoxide anion radical activity unit.

(2) Formula:

$$\text{Blood serum (or plasma) anti-superoxide anion radical activity (U/L)} = \frac{OD_{Blank} - OD_{Sample}}{OD_{Contrast} - OD_{Standard}} \times \text{Standard concentration (0.15mg/ml)} \times 1000ml \times \text{Sample dilution times before assay}$$

(3) Example:

Take 0.05ml goat milk enzymolysate to do anti-superoxide anion radical assay, in result, ODSample is 0.346, ODBlank is 0.539, ODContrast is 0.549, ODStandard is 0.293, standard tube's concentration is 0. 15mg/ ml Vc standard. Calculate as follows:

$$\text{Superoxide anion production activity unit (U/L)} = \frac{0.539 - 0.346}{0.549 - 0.293} \times 0.15 \times 1000 \times 1 = 113.0859(U/L)$$

3. Announcements:

(1) Optimal sample volume: Different matters have different O2 · inhibition capacities under formulary substrate concentration in reaction system, according to Lambert-Beer law, sample concentration should not be too high or too lower or it will disturb result. Please do

Pretest before formal experment in order to get optimal sample concentration.

(2)This method applies to majority of lipid-containing samples because it can ignore lipid disturation to assay.

(3) Trichloromethane should be prepared by user, some "hyperlipid" samples may need large amount of trichloromethane (equal to sample volume or even higher), add enough trichloromethane, do

mixing and centrifugation until supernatant becomes limpid.