ELK Biotechnology

Hydroxyproline Assay Kit

(Cat/No.:BC126 Size:50T/48S)

1. Measurement principle

The oxidation product of hydroxyproline under the action of an oxidizing agent reacts with dimethylaminobenzaldehyde to produce a purple-red color, and its content can be estimated based on the intensity of the color.

2. Composition and preparation: (Shelf life of the kit is 6 months)

Reagent 1: 1 bottle of powder, 1 bottle of 10mL solution A, and 1 bottle of 20mL solution B. Before use, dissolve the powder in 10mL of solution A until completely dissolved (look from the bottle opening inwards; it should be completely dissolved). Then add 20mL of solution B and mix thoroughly. Store the prepared reagent at 4℃～8℃. [Note]: The powder must be completely dissolved after adding 10mL of solution A before adding solution B.

Reagent 2: 30mL liquid 1 bottle, store at 4℃ protected from light.

Reagent 3: 1 bottle of powder, 1 bottle of 30mL solvent. Before use, add one bottle of powder to 30mL of solvent and dissolve thoroughly. After preparation, store at 4℃ away from light. It is effective for 1 month.

Reagent 4: Hydroxyproline standard 5mg x 3 vials, store at 4℃.

Preparation of 100μg/mL standard stock solution: Before testing, dissolve one standard sample in double-distilled water and bring the volume to 50mL. It is valid for 2 weeks when stored at 4℃.

Preparation of 5μg/mL standard application solution: Take 1mL of 100μg/mL standard stock solution and add double-distilled water to make up to 20mL. Prepare fresh before use.

Sample pretreatment reagents (store at 4℃): 60mL alkaline hydrolysis solution × 1 bottle; 5mL pH indicator × 1 bottle; 60mL pH adjustment solution A × 1 bottle; 30mL pH adjustment solution B × 1 bottle; Activated carbon × 1 bag.

3. Required instruments and reagents

Spectrophotometer and cuvettes (or microplate reader (550±10nm) and 96-well plate), boiling water bath, 60℃ water bath, centrifuge, vortex mixer, distilled water, balance.

4. Determination of hydroxyproline

Sample pretreatment:

1) Sample hydrolysis:

① Serum (plasma): Take 0.5 mL of serum (plasma) and accurately add 1 mL of alkaline hydrolysis solution, mix well. Place in a test tube, cap, and hydrolyze at 95°C or in a boiling water bath for 20 minutes.

② Urine (culture medium): Take 1.0 mL of urine (culture medium) and accurately add 1 mL of alkaline hydrolysis solution, mix well. Place in a test tube, cap, and hydrolyze at 95°C or in a boiling water bath for 20 minutes.

③ Tissue: Accurately weigh 30-100 mg of wet tissue (after cutting it into small pieces) and place it in a test tube. Accurately add 1 mL of alkaline hydrolysis solution and mix well. After capping, hydrolyze at 95°C or in a boiling water bath for 20 minutes (mix once every 10 minutes of hydrolysis to ensure more complete hydrolysis).

2) Adjust the pH value to around 6.0 to 6.8.

After cooling each test tube with running water, add 10 µL of indicator to each tube and

① shake well.

② Add 1.0 mL of pH-adjusting solution A to each tube accurately and mix well (the solution should be red at this point); (Note: If the solution does not turn red after adding pH-adjusting solution A, continue adding a small amount of solution A until the solution turns red).

③ Using a 200µL pipette, carefully add pH-adjusting solution B dropwise to each tube, mixing well after each drop*, until the indicator in the liquid turns yellow-green** (i.e., the red color disappears). At this point, the pH value is approximately 6.0–6.8 (approximately 100µL–500µL of pH-adjusting solution B has been added).

* When adding pH-adjusting solution B, mix well after each drop. To prevent spillage, if you do not have a glass test tube with a cap and ground glass joint, you can use a regular glass test tube instead. Each time you mix, you can cover the mouth of the test tube with plastic film or refrigerator plastic wrap and vortex thoroughly to mix.

** If your sample is a cell culture medium, because it contains phenol red, the mixed indicator in the liquid will be orange-yellow-red at a pH of around 6.0 to 6.8, rather than yellow-green.

④ Then add double-distilled water to bring the volume to 10 mL (this volume can be adjusted as needed; for example, if the hydroxyproline content is low, it can be brought to 5 mL or other volumes, and the variables should be substituted into the calculation), and mix well.

⑤ Take 2-4 mL of the hydrolysate after adjusting the volume, add an appropriate amount of activated carbon (about 20-30 mg, until the supernatant is clear and colorless after centrifugation), mix well, centrifuge at 3500 rpm for 10 minutes, and carefully take 1 mL of the supernatant for testing.

Note 1: Reference sampling amounts for alkaline hydrolysis: 0.03-0.05 g wet weight of skin tissue, 0.08-0.1 g wet weight of cartilage, 0.08-0.1 g wet weight of liver tissue, 0.5 mL of cell culture medium, and 0.5 mL of serum (plasma).

Note 2: The addition of hydrolysate and pH-adjusting solution A must be accurate, otherwise it will be difficult to adjust the pH value. If the solution does not turn red after adding pH-adjusting solution A (it is still the original yellow), you can add a little more pH-adjusting solution A until the solution turns red (but remember the volume of the extra solution A added).

Note 3: The sample must be weighed very accurately to avoid affecting the test results. First, cut the tissue into pieces with a wet weight of about 10mg on the plastic wrap, then weigh the tissue along with the plastic wrap. After weighing, put the sample into a test tube, weigh the plastic wrap again, and calculate the net weight of the tissue.

Note 4: If your sample size is small, you can reduce the sample size proportionally in the sampling, hydrolysis, and pH adjustment processes without affecting the results.

Note 5: Each time you mix, you can use plastic film or refrigerator plastic wrap to cover the mouth of the test tube and vortex thoroughly to prevent liquid from splashing out during the mixing process.

Note 6: For 95℃ or boiling water bath, a glass test tube with a cap and ground glass joint can be used, or a regular glass test tube can be used instead. Seal the tube tightly with plastic wrap and prick a small hole with a needle. We recommend using a regular glass test tube as it is safer than a glass test tube with a cap and ground glass joint.

Operation table:

	Blank well	Standard well	Sample well
Double distilled water(mL)	1.0		
5μg/mL standard application solution(mL)		1.0	
test solution(mL)			1.0
Reagent 1(mL)	0.5	0.5	0.5
Mix well and let stand for 10 minutes.			
Reagent 2(mL)	0.5	0.5	0.5
Mix well and let stand for 5 minutes.			
Reagent 3(mL)	0.5	0.5	0.5
Mix well, incubate at 60℃ for 15 minutes, cool, centrifuge at 3500 rpm for 10 minutes, take the supernatant and measure the absorbance value A of each tube at a wavelength of 550 nm (or take 200 μL of reaction solution from each tube, add it to a 96-well plate, and read the value at 550 nm using an ELISA reader).			

5. Calculations and examples

Calculation and examples of serum (plasma) or urine:

1) Calculation formula:

$$\text{Glycogen content } (\mu g/mL) = \frac{A_{Sample} - A_{Blank}}{A_{Standard} - A_{Blank}} \times C_{Standard} \times \frac{V_{Hydrolysate}}{V_{Sample}}$$

C Standard: Standard solution concentration, 5μg/mL;

V Hydrolysate: Total volume of hydrolysate, 10mL;

V Sample: Sample volume, serum (plasma) 0.5mL, urine 1mL.

2) Calculation example:

Example 1: Accurately aspirate 0.5 mL of normal rat serum (if the sample volume is small, the amount can be reduced proportionally during sampling, hydrolysis, and pH adjustment). Perform the test according to the operating procedures. The absorbance of the blank tube is 0.016, the absorbance of the standard tube is 0.587, and the absorbance of the test tube is 0.408. The calculated result is:

$$\text{Glycogen content } (\mu g/mL) = \frac{0.408 - 0.016}{0.587 - 0.016} \times 5 \times \frac{10}{0.5} = 68.531\ \mu g/mL$$

Example 2: Accurately collect 1.0 mL of normal human urine (if the sample volume is small, the amount can be reduced proportionally during sampling, hydrolysis, and pH adjustment). Perform the test according to the operating procedures. The absorbance of the blank tube is measured to be 0.016, the absorbance of the standard tube is 0.587, and the absorbance of the test tube is 0.128. The calculated result is:

$$\text{Glycogen content } (\mu g/mL) = \frac{0.128 - 0.016}{0.587 - 0.016} \times 5 \times \frac{10}{1}$$
$$= 9.807\, \mu g/mL$$

Organizational calculations and examples:

1) Calculation formula:

$$\text{Glycogen content } (\mu g/\text{mg tissue}) = \frac{A_{\text{Assay}} - A_{\text{Blank}}}{A_{\text{Standard}} - A_{\text{Blank}}} \times C_{\text{Standard}} \times \frac{V_{\text{Hydrolysate}}}{W}$$

C standard: standard solution concentration, 5 µ g/mL;

V hydrolysis solution: total volume of hydrolysis solution, 10mL;

W: Organizational quality, mg.

2) Calculation example:

Accurately weigh 61.4 mg of normal rat liver tissue (if the sample size is small, the amount can be reduced proportionally during sampling, hydrolysis, and pH adjustment). Perform the test according to the operating procedures. The absorbance of the blank tube was 0.015, the absorbance of the standard tube was 0.538, and the absorbance of the test tube was 0.161. The calculated results are as follows:

$$\text{Glycogen content } (\mu g/\text{mg tissue}) = \frac{0.161 - 0.015}{0.538 - 0.015} \times 5 \times \frac{10}{61.4}$$
$$= 0.227\, \mu g/\text{mg tissue}$$

6. Significance of the measurement

Hydroxyproline accounts for 13.4% of collagen, a very small amount in elastin, and is absent in other proteins. Since collagen is mostly distributed in the skin, tendons, cartilage, and blood vessels, the amount of hydroxyproline can reflect the collagen metabolism status in connective tissue diseases.

Some people use the measurement of hydroxyproline in the skin as an indicator for screening anti-aging drugs. During the developmental stage, hydroxyproline excretion in urine increases with age, but stabilizes after development ceases. Older adults have lower levels of hydroxyproline than younger adults.

In mice with spontaneous and secondary hypertension, the urinary hydroxyproline content increased.

Surgical trauma can lead to increased urinary hydroxyproline excretion. High-fat, low-protein diets, alcohol poisoning, toxins, malnutrition, or hepatocyte degeneration and necrosis can cause inflammation, increasing fibrosis, dividing liver lobules, and leading to cirrhosis. In liver fibrosis, the main component increasing in the liver is collagen fibers. Hydroxyproline is unique to collagen fibers; measuring the hepatic hydroxyproline content can be converted into hepatic collagen content to reflect the degree of liver fibrosis. Collagen fiber breakdown mainly relies on the action of collagenase and other proteases. Its breakdown product, hydroxyproline, is excreted in the urine. Measuring the urinary hydroxyproline content reflects the extent of collagen degradation. By measuring the hydroxyproline content in tissues and urine, the degree of fibrosis can be determined, and preventative and therapeutic drugs can be screened.