Version 25-04/25

User's Manual

Hydroxyproline Assay Kit

REF **DEIA-XY2132**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

Collagen is the major structural protein of the extracellular matrix in many tissues. Hydroxyproline, a major component of collagen, comprises around 13.5% of its amino acid composition. Due to its highly restricted distribution in collagen, the hydroxyproline content accurately reflects the amount of collagen in the sample. Therefore, quantitating hydroxyproline has been utilized for evaluating tissue fibrosis or collagen deposition (1, 2, 3). However, classic hydroxyproline assays are not useful since it requires cumbersome procedures and special tools. This hydroxyproline assay kit employs an improved assay system that can be operated with ease and precision using 96-well plates.

This kit works for quantitation of total collagen of any type and species in tissue specimens and tissue homogenates.

Reagents And Materials Provided

1. Hydroxyproline Standard: 1 vial, 4 mg/ml x 0.5 ml
2. 10X Chloramine T Concentrate: 1 vial, 1 ml
3. 2X DMAB (dimethylaminobenzaldehyde) Concentrate: 1 vial, 5 ml
4. Solution A- Chloramine T Dilution Buffer: 1 bottle, 10 ml
5. Solution B - DMAB Dilution Buffer: 1 vial, 5 ml
6. ELISA Plate: 1 each, 8-well strips x 12

Materials Required But Not Supplied

Concentrated HCl (10N)

A glass screw-thread vial (1-2 ml) with a teflon cap

Storage

-20°C

Specimen Collection And Preparation

1. Weigh 10 mg of a tissue sample in a glass screw-thread vial.
2. Add 100 µL of distilled water.
3. Mash the tissue sample with a small spatula.

Note: 100 µL of a sample homogenate can be used. Skip step 1-3, then add 100 µL of the sample into a vial.

4. Add 100 µL of concentrated HCl (10N), and tightly screw on the teflon cap.
5. Incubate at 120°C for 24 hours. Mix the sample periodically during incubation.

Note: A heat block or dry bath can be used for the incubation.

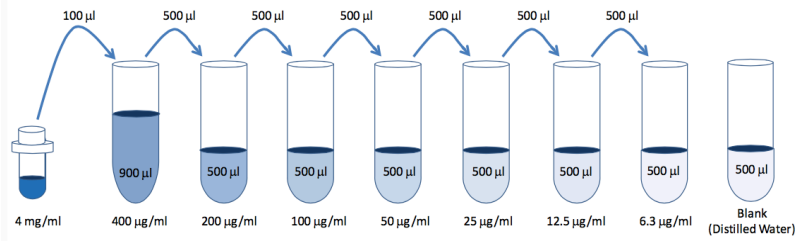
6. Cool down. Do not open the cap before cooling down.
7. If hydrolyzed black residue is still present in the sample, transfer to a microcentrifuge tube and spin at 10,000 rpm for 3 minutes.

Note: Black residue is occasionally produced from tissue samples in the hydrolysis process. However, the sample hydrolyzation should be completed at the end of the incubation.

8. Use supanatant for the assay.

Assay Procedure

1. Prepare Standard Dilutions: Take 100 µL of Hydroxyproline (HP) Standard and add to 900 µL of distilled water to make 400 µg/ ml of the diluted HP standard; then serially dilute it with distilled water. For example, mix 500 µL of the standard (400 µg/ml) with an equal volume of distilled water to make a 200 µg/ml solution, and then repeat it five more times to make 100, 50, 25, 12.5, and 6.3 µg/ ml standards.



2. Prepare Sample Dilutions: The hydrolyzed samples can be used undiluted. If necessary, the samples can be diluted with 5N HCl. If your sample has color (is not clear), Sample Blank wells should be prepared due to the higher background color. See steps 4 and 5 for this process.
3. Prepare Chloramine T solution: Mix 10 µL of 10X Chloramine T solution and 90 µL of Solution A for each well. For example, 10 samples, 7 point standard, one blank (all in duplicate) will require 3.6 ml of the 1X Chloramine T solution. Mix 360 µL of 10X Chloramine T solution with 3.24 ml of Solution A .

Note: Prepare the 1X solution just before use. Do not store and reuse the mixed solution for the next assay.

4. Add Standards and Samples: Choose 4-1 or 4-2 depending on your samples.

4- 1. Colorless samples

Use the plate layout as shown in Figure 1. Add 10 µL of standards, distilled water (blank, B) into the purple wells, and samples into the orange wells in duplicate. For example, add 10 µL of sample 1 into S1 wells, then add 10 µL of sample 2 into S2 wells. Proceed to Step 5-1.

4- 2. Colored samples

Use the plate layout as shown in Figure 2. Add 10 µL of standards, distilled water (blank, B) into the purple wells, and samples into the orange and green wells in duplicate. For example, add 10 µL of sample 1 into S1 and SB1 wells, then add 10 µL of sample 2 into S2 and SB2 wells. Proceed to Step 5-2.

5. Add 1X Chloramine T Solution:

5- 1. Colorless samples

Add 100 µL of the 1X Chloramine T solution into all wells. Incubate at room temperature for 20 minutes.

5- 2. Colored samples

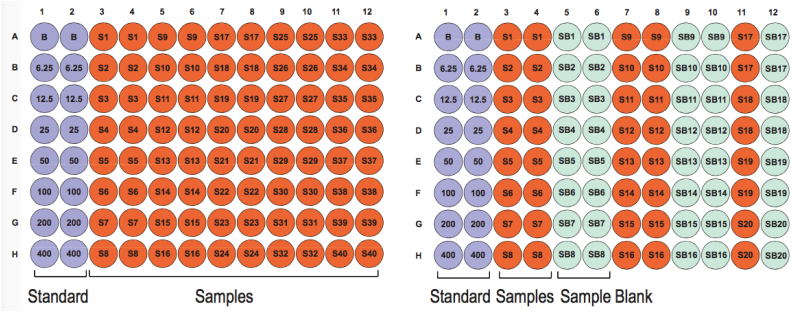
Add 100 µL of the 1X Chloramine T solution into the purple and orange wells, and add 100 µL of Solution A into the green wells. Incubate at room temperature for 20 minutes.

6. Prepare DMAB solution: Mix 50 µL of 2X DMAB solution and 50 µL of Solution B for each well. For example, 10 samples, 7 point standard, one blank (all in duplicate) will require 3.6 ml of the 1X DMAB soluion. Mix 1.8 ml of 2X DMAB solution with 1.8 ml of Solution B.

Note: Prepare the 1X solution just before use. Do not store and reuse the mixed solution for the next assay.

7. Add 1X DMAB solution: Add 100 µL of 1X DMAB solution into all wells and incubate at 60°C for 30 minutes. Note: Mix the plate well by tapping or use a plate shaker.
8. Read Plate: Read the OD values at 530-560 nm. If the OD values of samples are greater than the OD values of the highest standard, re-assay the samples at a higher dilution.

Standard Assay Layouts of 96-Well Plates



Calculation

1. Average the duplicate OD values for the standards, blanks (B), and test samples.
2. Subtract the blank (B) values from the averaged OD values of the standards and test samples.
3. Plot the OD values of the standards on the y-axis and the Hydroxyproline standard concentrations (µg/ml) on the x-axis. Using a log/ log plot will linearize the data. Figure 3 shows a representative experiment where the standard range is from 6.25 to 400 µg/ml.
4. The concentration of Hydroxyproline (µg/ml) in the hydrolyzed samples can be calculated using regression analysis.
5. Choose one of the following equations depending on the type of sample:

5. 1. Solid Samples: Hydroxyproline level in a tissue sample (µg/mg) is determined by the following equation:

Hydroxyproline (µg/ml) x (Distilled Water Volume ml + HCl Volume ml) / Sample Weight (mg) = Hydroxyproline level (µg/mg) in the sample

5. 2. Solution: Hydroxyproline level in a solution sample (mg/ml) is determined by the following equation: Hydroxyproline (µg/ml) x (Sample Volume ml + HCl Volume ml) / Sample Volume (ml) = Hydroxyproline level (µg/mg) in the sample

6. Hydroxyproline level can be converted into collagen level by the following equation (4):

$$\text{Hydroxyproline level (μg/mg)} \times \frac{100}{13.5} = \text{Collagen level (μg/mg)}$$

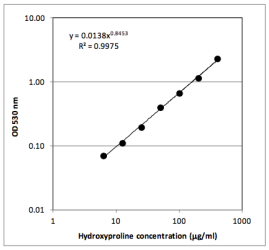
Note: Hydroxyproline accounts for 13.5% of the collagen amino acid composition.

Table 1 - Reproducibility of data assayed by hydroxyproline assay kit

Test	Mouse Kidney	Hydroxyproline 200 μg/ml	Hydroxyproline 12.5 μg/ml
Inter-Assay CV (%)	5.1	6.1	7.6
Intra-Assay CV (%)	5.1	6.3	3.4
Spiking Test*	91 -95 %	-	-

*Standard was mixed with known amounts of mouse kidney samples or hydroxyproline solution.

Figure 3 - A typical standard curve for hydroxyproline assay



Precautions

1. It is recommended that the standards and samples be run in duplicate.
2. Partially used reagents may be kept at -20°C.
3. If there is precipitates in the bottles/vials, it is necessary to warm up the bottles/vials in warm water until the precipitates are dissolved completely.

References

1. Blumenkrantz N, Asboe-Hansen G. A quick and specific assay for hydroxyproline. Anal Biochem. Sep;55(1):288-91 (1973).
2. G. Kesava Reddy, Chukuka S. Enwemeka. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem. Jun;29(3):225-9 (1996).
3. CJ Rogers, JR Kimmel, ME Hutchin. A hydroxyproline method of analysis for a modified gelatin in plasma and urine. J Biol Chem. Feb;206(2):553-9 (1954).
4. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem. May;184(1):299-306 (1950).