Chondrex, Inc.

Hydroxyproline Assay Kit

Catalog # 6017

For Research Use Only - Not Human or Therapeutic Use

INTRODUCTION

Collagen is the major structural protein of the extracellular matrix in many tissues. Hydroxyproline, a major component of collagen, makes up about 13.5% of its amino acid composition. Due to its highly restricted distribution in collagen, the hydroxyproline content accurately reflects the amount of collagen. Therefore, quantitating hydroxyproline has been utilized for evaluating tissue fibrosis or collagen deposition (1 - 3). However, conventional hydroxyproline assays are not useful because they require cumbersome procedures and special tools. Chondrex, Inc. offers a hydroxyproline assay kit (catalog # 6017) which employs an improved assay system that can be operated with ease and precision using 96-well plates.

This kit can quantify the total collagen content in any tissue specimen or tissue homogenate, regardless of collagen type or species. Chondrex, Inc. also provides three additional collagen detection kits for different purposes. Firstly, the Sirius Red Total Collagen Detection Kit (catalog # 9062) can be used to quantify solubilized collagen in samples. Secondly, the collagen detection ELISA kits specific to type I or II collagen of various species (catalog # 6012-6016, 6019, 6018, and 6021) are used for distinguishing collagen types and species in samples. Lastly, the Sirius Red/Fast Green Collagen Staining Kit (catalog # 9046) is a semi-quantitative assay to determine the total collagen content in tissue sections.

KIT COMPONENTS

Item	Quantity	Amount	Storage
Hydroxyproline Standard (60171)	1 vial	4 mg/ml x 0.5 ml	-20°C
Solution A- Chloramine T Dilution Buffer (60172)	1 bottle	10 ml	-20°C
Solution B - DMAB Dilution Buffer (60173)	1 vial	5 ml	-20°C
10X Chloramine T Concentrate (60174)	1 vial	1 ml	-20°C
2X DMAB (dimethylaminobenzaldehyde) Concentrate (60175)	1 vial	5 ml	-20°C
ELISA Plate	1 each	96-well (8-well strips x 12)	-20°C

Not included: concentrated HCl (10N) and glass screw-thread vials (1-2 ml) with teflon caps (Example: National Scientific B7999-1)

Standard Assay Layouts of 96-Well Plates

Figure 1 - Colorless (clear) samples

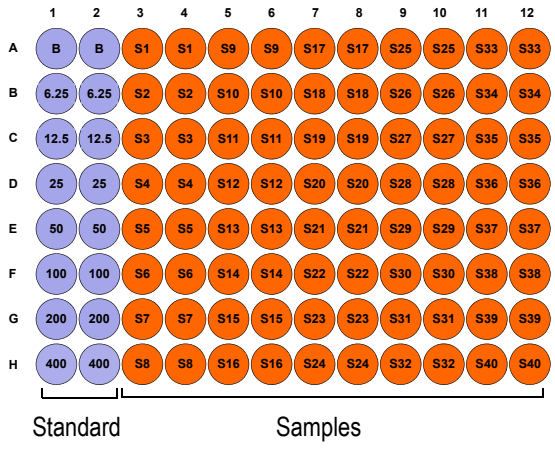
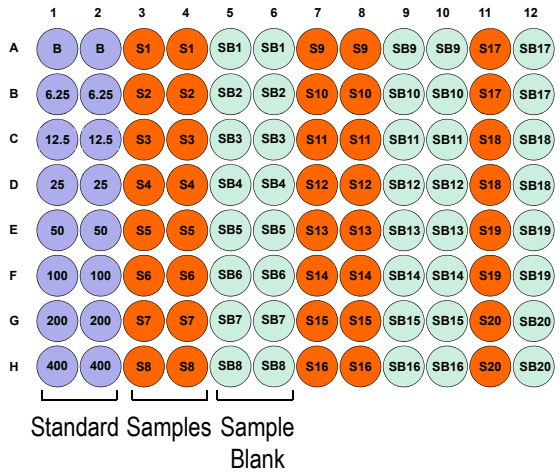


Figure 2 - Colored samples



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NOTES BEFORE USING ASSAY

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 are precipitates in the bottles/vials, it is necessary to warm up the bottles/vials in warm water until the precipitates dissolve completely.

SAMPLE HYDROLYSIS

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.

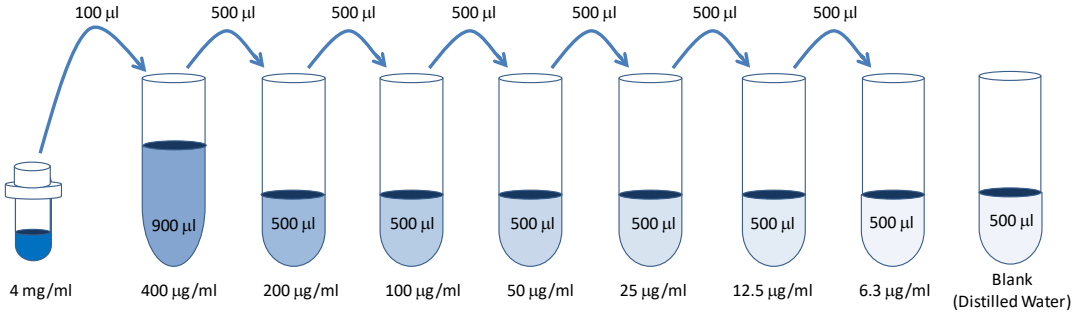
   Note: 100 µl of a sample homogenate can be used. Skip steps 1-3, then add 100 µl of the sample homogenate to a vial.
4. Add 100 µl of concentrated HCl (10N), and tightly screw on the teflon cap.
5. Incubate at 120°C for 24 hours. Mix the sample periodically during incubation.

   Note: A heat block or dry bath can be used for 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, sample hydrolyzation should be complete by the end of the incubation period.
8. Use the supernatant for the assay.

ASSAY PROCEDURE

1. **Prepare Standard Dilutions**: Take 100 µl of the 4 mg/ml Hydroxyproline (HP) Standard and add to 900 µl of distilled water to make a 400 µg/ml HP standard solution. 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 standard solutions.



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 potential for high background color. See steps 4 and 5 for this process.

3. **Prepare Chloramine T solution**: Mix 10 µl of the 10X Chloramine T solution with 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 the 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.

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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 the S1 wells, then add 10 µl of sample 2 into the 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 the S1 and SB1 wells, then add 10 µl of sample 2 into the 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 with 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 the 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 by tapping gently or using 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 hydrolyzed samples at a higher dilution using 5N HCl.

CALCULATING HYDROXYPROLINE LEVELS

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 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. Multiply the results by the dilution factors if the hydrolyzed samples were diluted.

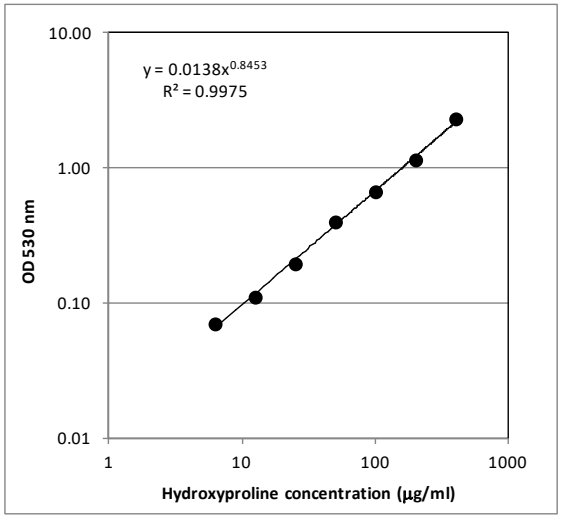

Figure 3 - A typical standard curve for hydroxyproline assay

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5. Choose one of the following equations depending on the sample type:

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

$$\frac{\text{Hydroxyproline (μg/ml) x (Distilled Water Volume (ml)+ HCl Volume (ml))}}{\text{Sample Weight (mg)}} = \text{Hydroxyproline level (μg/mg) in the sample}$$

   5.2. **Solution or Homogenate:** Hydroxyproline level in a solution sample (mg/ml) is determined by the following equation:

$$\frac{\text{Hydroxyproline (μg/ml) x (Sample Volume (ml) + HCl Volume (ml))}}{\text{Sample Volume (ml)}} = \text{Hydroxyproline level (μg/ml) in the sample}$$

6. Hydroxyproline levels can be converted into collagen levels with the following equation (4):

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

   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.

REFERENCES

1. Blumenkrantz N, Asboe-Hansen G. A quick and specific assay for hydroxyproline. *Anal Biochem.* **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.* **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.* **206(2)**, 553-9 (1954).
4. Neuman RE, Logan MA. The determination of hydroxyproline. *J Biol Chem.* **184(1)**, 299-306 (1950).

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