Hydroxyproline Assay Kit

Catalog # 6017

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INTRODUCTION

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. Chondrex also provides three additional collagen detection kits for different purposes. Firstly, the Sirius Red Total Collagen Detection Kit (catalog # 9062) can be used to quantitate solubilized collagen in samples. Secondly, the collagen detection ELISA kits specific to type I or II collagen of various species (catalog # 6008, 6012-6016, 6018) 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	1 vial	4 mg/ml, 0.5 ml	-20°C
10X Chloramine T Concentrate	1 vial	1 ml	-20°C
2X DMAB (dimethylaminobenzaldehyde) Concentrate	1 vial	5 ml	-20°C
Solution A- Chloramine T Dilution Buffer	1 bottle	10 ml	-20°C
Solution B - DMAB Dilution Buffer	1 vial	5 ml	-20°C
ELISA Plate	1 each	96-well (8-well strips x 12)	-20°C

Not included in the kit:
Concentrated HCl (12N)
A glass screw-thread vial (1-2 ml) with a teflon cap (example: National Scientific B7999-1)

Standard assay layouts of 96-well plates

Figure 1 - Colorless (clear) samples

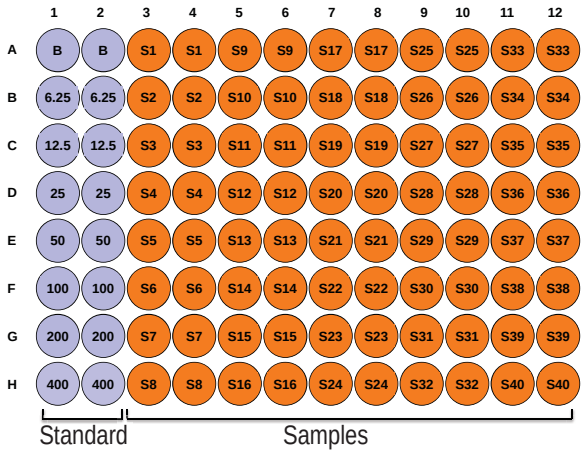
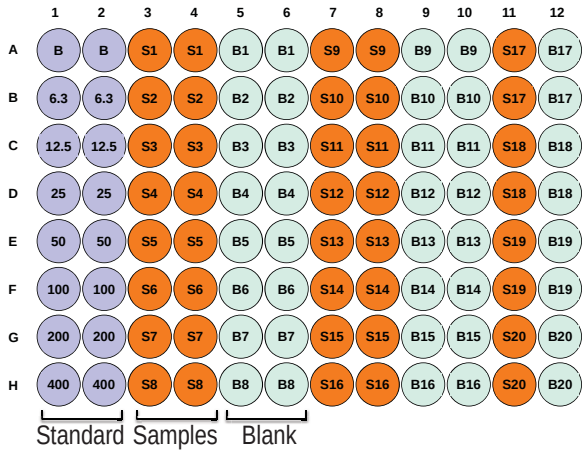


Figure 2 - Colored samples



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NOTES BEFORE USING ASSAY

Note 1: It is recommended that the standards and samples be run in duplicate.

Note 2: Partially used reagents may be kept at –20°C.

Note 3: If there is precipitate in the bottles/vials, it is necessary to warm up the bottles/vials in warm water until the precipitate is dissolved.

SAMPLE HYDROLYSIS

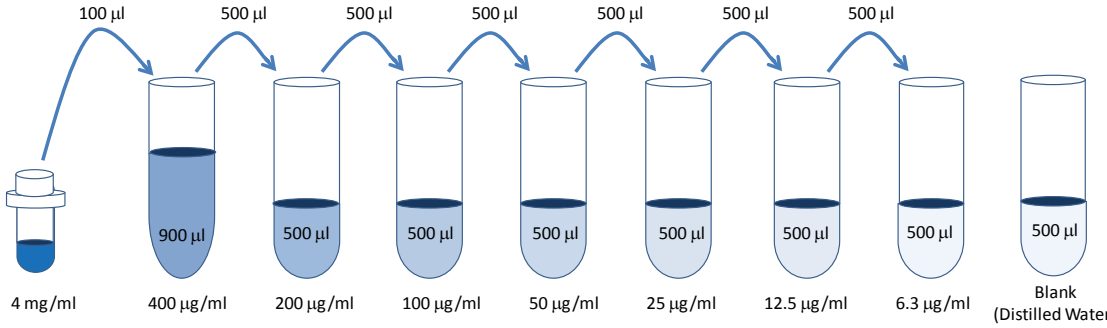
1. Weigh 10 mg of a tissue sample in a glass screw-thread vial.
2. Add 100 µl of distilled water.
3. Mash the tissue sample with a small spatula.

   Note: 100 µl of a sample homogenate can be used. Skip step 1-3, then add 100 µl of the sample into a vial.
4. Add 100 µl of concentrated HCl (12N), and tightly screw on the teflon cap.
5. Incubate at 120°C for 4-24 hours. Mix the sample periodically during incubation.

   A heat block can be used for the incubation.
6. Cool down. *Do not open the cap before cooling down.*
7. If hydrolyzed residue is still present in the sample, transfer to a microcentrifuge tube and spin at 10,000 rpm for 3 minutes.
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 with distilled water. For example, mix 500 µl of the standard (400 µg/m) with an equal volume of distilled water to make 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 6N 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 Chloramin T solution**: Mix 10 µl of 10X Chloramin 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 Chloramin 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**: Add 10 μl of standards, distilled water (blank) and samples to wells in duplicate (Figure 1). For colored samples, use a plate layout in Figure 2. Colored samples will utilize a corresponding Blank well in which only the sample and Solution A are added.

5. **Add 1X Chloramine T solution**: Add 100 μl 1X Chloramine T solution into all wells and incubate at room temperature for 20 minutes. For colored samples, Solution A should be added instead of the 1X Chloramine T solution.

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.

CALCULATION OF HYDROXYPROLINE LEVEL

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 standards against the μg/ml of Hydroxyproline standard. 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. Hydroxyproline (μg/ml) of hydrolyzed samples can be calculated using a regression analysis.

5.1. Hyrdoxyproline level in a tissue sample (μg/mg) is determined by the following equation.

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

5.2. Hyrdoxyproline level in a homogenate sample (mg/ml) is determined by the following equation.

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

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

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

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

Figure 3 - A typical standard curve for hydroxyproline assay

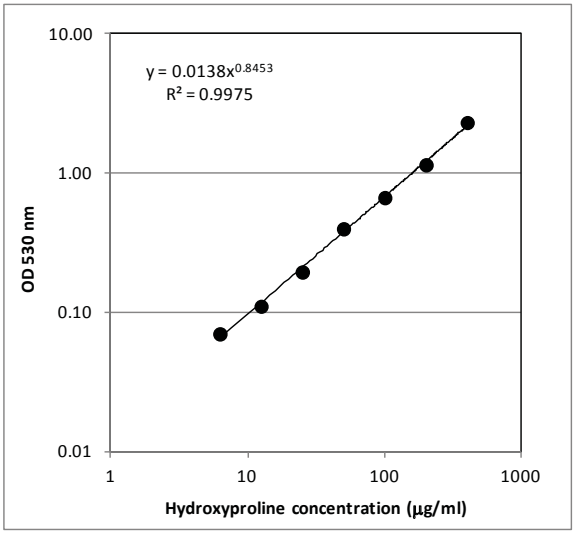


Table 1 - Reproducibility of data assayed by Hydroxyproline assay kit

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

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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).

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4. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem. May;184(1):299-306 (1950).