Total Collagen/Hydroxyproline Assay Kit

Item No. 702440

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
400553	Hydroxyproline Oxidation Buffer	1 vial/14 ml	4°C
400554	Hydroxyproline Assay Reagent 1	1 vial/1.5 ml	-20°C
400555	Hydroxyproline Assay Reagent 2	1 vial/14 ml	-20°C
400556	Hydroxyproline Standard	1 vial/100 µl	-20°C
400557	Collagen Standard	1 vial/300 µl	4°C
700020	Half-Volume 96-Well Clear Plate	1 plate	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Total Collagen/Hydroxyproline Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 560 nm
2. A dry heat plate, capable of heating reaction tubes to 120 and 65°C
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of pure water; glass-distilled or pure water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. 0.2 ml PCR tubes with caps or 0.5 ml microcentrifuge tubes; strips and plates are recommended for higher throughput
6. Materials and reagents needed for hydrolysis; 10N NaOH and 10N HCl (see page 13)

INTRODUCTION

Background

Collagens are a major component of non-specialized and specialized connective tissues, including tendon, organic matter in bone, and corneal tissues, and have unique but interrelated roles that are dependent on their tissue location.[1-3] The collagen family is composed of 29 glycoproteins that are encoded by 40 genes and characterized by three main features: the amino acid repeating sequence $[Gly\text{-}X\text{-}Y]_n$, with occupation of the X and Y positions by proline and its hydroxylated form, hydroxyproline, respectively, and a unique quaternary structure formed by three left-handed polyproline α-chains of identical length. Collagens are grouped into four subfamilies: fibril-forming collagens, basement membrane collagens, short-chain collagens, and collagens with multiple interruptions of their $[Gly\text{-}X\text{-}Y]_n$ motifs.[3] Hydroxyproline is a primary amino acid in collagen and is generated from post-translational hydroxylation of proline in newly synthesized collagen, primarily by collagen prolyl 4-hydroxylase.[2] Mutations in the genes encoding various collagens are associated with a variety of connective tissue disorders, including Ehlers-Danlos syndrome, osteogenesis imperfecta, osteoarthrosis, and Knobloch syndrome, as well as Ullrich congenital muscular dystrophy and autosomal recessive myosclerosis.[4,5]

About This Assay

Cayman's Total Collagen/Hydroxyproline Assay Kit provides colorimetric-based methods for measuring collagen/hydroxyproline in tissue, serum, and urine. Prior to beginning the assay, collagen is hydrolyzed to individual amino acids. Hydroxyproline, found almost exclusively in collagen, makes up 11.3 to 15% of collagen 1 and collagen 3, respectively, by mass of the amino acid composition.[6] In this assay, hydroxyproline is oxidized by Hydroxyproline Reagent 1 to a pyrrole intermediate, which reacts with Hydroxyproline Reagent 2 to create a chromophore with peak absorbance at 560 nm. Both hydroxyproline and collagen standards are included. It is only necessary to use the standard that best fits the needs of your assay.

In studies where hydroxyproline measurement is relevant (*i.e.*, collagen metabolism or biosynthesis) the hydroxyproline standards may be used. The hydroxyproline standards may also be used to calculate collagen levels in appropriate samples by assuming hydroxyproline makes up 11-15% of the amino acid content of collagen by mass. For a direct measurement of collagen, the collagen standard may be used. The collagen standard is hydrolyzed along with the samples to give hydroxyproline.

The range of this assay is 0.02-5 µg for collagen and 0.006-3 µg for hydroxyproline with lower limits of quantification (LLOQ) of 0.5 and 0.047 µg for collagen and hydroxyproline, respectively.

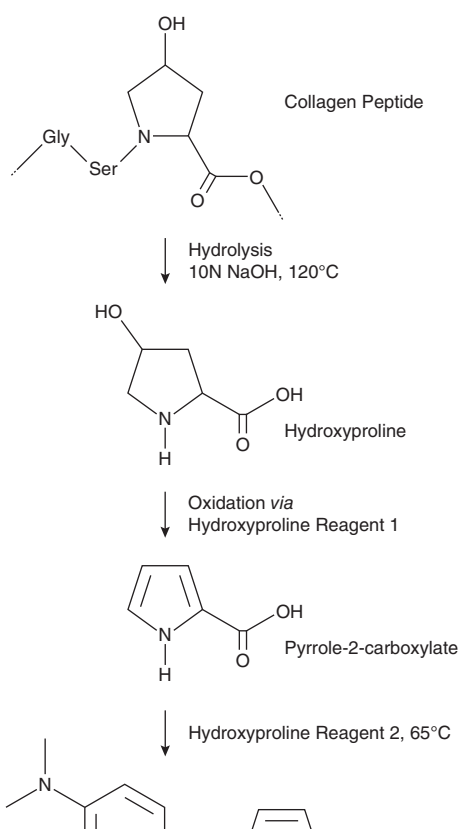


Figure 1. Reaction scheme

PRE-ASSAY PREPARATION

Reagent Preparation

All reagents must be thawed at room temperature prior to the assay.

1. **Hydroxyproline Oxidation Buffer (1X) - (Item No. 400553)**

   This vial contains 14 ml of Hydroxyproline Oxidation Buffer (1X), pH 6.5. It is ready to be used as supplied. It will be stable for at least one year when stored at 4°C.

2. **Hydroxyproline Standard - (Item No. 400556)**

   This vial contains 100 µl of 3 mg/ml hydroxyproline in water, which is ready to use as supplied. It will be stable for at least one year when stored at -20°C.

3. **Collagen Standard - (Item No. 400557)**

   This vial contains 300 µl of 3 mg/ml collagen in acetic acid. It is ready to be used as supplied. It will be stable for at least one year when stored at 4°C.

4. **Hydroxyproline Assay Reagent 1 (10X) - (Item No. 400554)**

   This vial contains 1.5 ml of Hydroxyproline Assay Reagent 1 (10X). If any crystals are present, vortex until dissolved. Immediately prior to use, add 1.2 ml of Hydroxyproline Assay Reagent 1 (10X) to 10.8 ml of Hydroxyproline Oxidation Buffer (1X). Vortex to mix. This volume of the Hydroxyproline Assay Reagent 1 (1X) is sufficient for 100 reactions. Adjust accordingly if a smaller number of reactions is planned. Hydroxyproline Assay Reagent (1X) will be stable for one hour at room temperature.

5. **Hydroxyproline Assay Reagent 2 (1X) - (Item No. 400555)**

   This vial contains 14 ml of Hydroxyproline Assay Reagent 2 (1X). It is ready to use as supplied. If any crystals are present, vortex to dissolve prior to the assay. Avoid prolonged exposure to light.

Sample Preparation

Tissue Homogenate

Tissue should be minced into small pieces. Prepare a fine homogenate of 50 mg of minced tissue in 500 µl of pure water. Depending on the nature of the tissue, mechanical homogenization may be necessary. Hydrolyze all tissue samples according to the hydrolysis protocol on page 13.

Serum and Urine

Serum and urine can be tested immediately after collection or stored at -20°C or -80°C. Hydrolyze all serum and urine samples according to the hydrolysis protocol on page 13 using 100 µl of each sample.

ASSAY PROTOCOL

Plate Set Up

The oxidation reaction is performed in 0.5 ml microcentrifuge tubes or 0.2 ml PCR tubes. Samples and standards are transferred to a plate for measuring absorbance at the conclusion of the assay. There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 2, below. It is suggested that the contents of each tube and the corresponding well are recorded on the template sheet provided (see page 28).

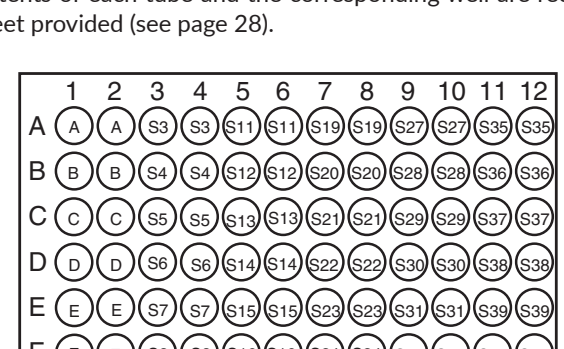


Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume in the assay is 200 µl in all of the reaction tubes. 100 µl will be transferred to a 96-well plate for measurement.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- Hydrolysis is performed at 120°C. The oxidation reaction is performed at room temperature, and development is performed at 65°C.
- Monitor the absorbance at 560 nm.

Hydrolysis

Hydrolysis is necessary for all tissue, urine, and serum samples and the collagen standard. It is not necessary for the hydroxyproline standards. Use the same heat block for all the samples and standards to avoid variabilities in measurements.

1. Using a polypropylene screw top vial, add 100 µl of tissue homogenate/ serum/urine/collagen standard to 100 µl of 10N NaOH. Vortex thoroughly.
2. Incubate the tightly sealed vial at 120°C for 1 hour.
3. Allow the sample to cool briefly on ice.
4. Neutralize the sample with 100 µl of 10N HCl. Vortex thoroughly.
5. If any particulate remains in the tissue sample, centrifuge and transfer the supernatant into a clean tube for assaying. The hydrolysate may be stored at -20°C for at least one month.

Standard Preparation

Hydroxyproline Standards

Label six clean glass or polystyrene test tubes A-F. Pipette 180 µl of pure water to tube A and 100 µl of pure water to tubes B-F. Transfer 20 µl of the Hydroxyproline Standard (3 mg/ml) to tube A. Mix gently. Serially dilute the standard by removing 100 µl from tube A and placing it into tube B. Mix gently. Repeat the process for tubes C-E. Do not add any standard to tube F. This tube is the zero point, which is the lowest point of the standard curve

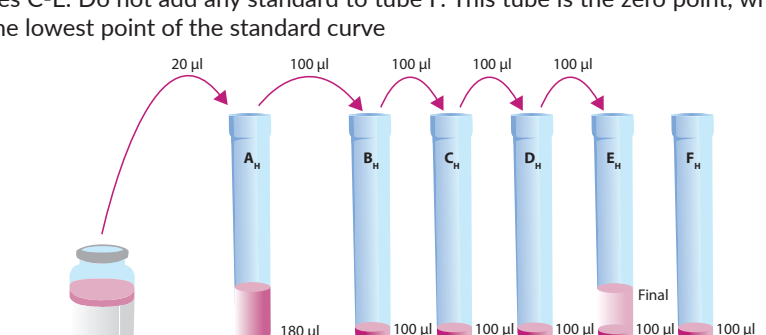


Figure 3. Preparation of the hydroxyproline standards

Collagen Standards

The Collagen Standard must be hydrolyzed prior to the beginning of the assay (see page 13). Use the collagen hydrolysate (1 mg/ml) to prepare the standards.

Label six tubes A-F. Pipette 100 µl of pure water to tube A and 100 µl to tubes B-F. Transfer 100 µl of the collagen hydrolysate (1 mg/ml) to tube A. Mix gently. Serially dilute the standard by removing 100 µl from tube A and placing it into tube B. Mix gently. Repeat the process for tubes C-E. Do not add any standard to tube F. This tube is the zero point, which is the lowest point of the standard curve.

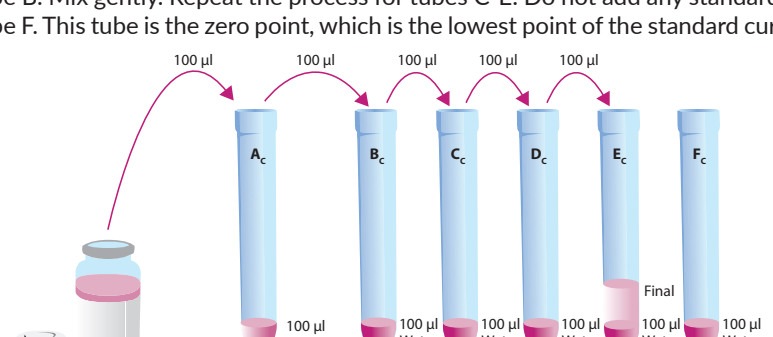


Figure 4. Preparation of the collagen standards

Hydroxyproline Standards		
Concentration (µg/ml)	Volume Added to Tubes (µl)	Final Amount in Assay (µg)
300	10	3
150	10	1.5
75	10	0.75
37.5	10	0.38
18.75	10	0.19
0	10	0
Collagen Standards		
Concentration (µg/ml)	Volume Added to Tubes (µl)	Final Amount in Assay (µg)
500	10	5
250	10	2.5
125	10	1.25
62.5	10	0.63
31.25	10	0.31
0	10	0

Table 1. Standards summary

Performing the Assay

1. **Standard and Sample Incubation**

   NOTE: It is not necessary to use both hydroxyproline and collagen standards in the same experiment. Use the standard that best fits your needs.

   Standards: Add 10 µl of each prepared standard to designated 0.5 ml microcentrifuge tubes or 0.2 ml PCR tubes in duplicate.

   Samples: Following hydrolysis, add hydrolyzed samples in duplicate to the corresponding tubes. It is recommended to assay each sample at more than one volume in the range of 2-20 µl to ensure that the values will fall within the range of the assay. *e.g.*, Add 2.5, 5, 10, and 20 µl of hydrolyzed samples to the designated tubes.

   Allow samples to completely evaporate on dry heat block at 65°C. This generally takes 45-90 minutes.

2. **Oxidation**

   Remove samples from heat and allow to cool to room temperature.

   Add 100 µl of Hydroxyproline Assay Reagent 1 (1X) to all tubes. Cap the tubes and mix thoroughly.

   Incubate tubes for 20 minutes at room temperature.

3. **Development**

   Add 100 µl of Hydroxyproline Assay Reagent 2 (1X) to all tubes. Cap the tubes and mix thoroughly.

   Incubate the tubes on the dry heat block at 65°C for 20 minutes. Do not exceed 20 minutes for maximum absorbance.

4. **Reading the Plate**

   Quench the reaction by placing tubes very briefly on ice, or in room temperature water.

   Transfer 100 µl of each sample to the 96-well plate.

   Read absorbance at 560 nm.

	Standard Wells (µl)	Sample Wells (µl)
Hydroxyproline Standards	10	--
Collagen Standards	10	--
Sample hydrolysate	--	2-20 µl to fall within the range of the assay
Evaporate at 65°C for 45-90 minutes		
Hydroxyproline Assay Reagent 1 (1X)	100	
Incubate at room temperature for 20 minutes		
Hydroxyproline Assay Reagent 2 (1X)	100	
Incubate at 65°C for 20 minutes		
Quench reaction on ice; transfer 100 µl to 96-well plate		
Read plate at 560 nm		

Table 2. Pipetting summary

ANALYSIS

Calculations

1. Determine the average absorbance of each standard and sample well.
2. Subtract the absorbance value of the zero-point standard from itself and all other standards and samples. This is the corrected signal (CS).
3. Plot the CS values (from step 2 above) of each standard as a function of the final amount of collagen or hydroxyproline from Table 1, page 16. See Figures 5 and 6, on pages 21 and 22, for typical standard curves.
4. Calculate the hydroxyproline or collagen amount in the wells using the equation obtained from the linear regression of the corresponding standard curve substituting the CS for each sample.

$$\text{Hydroxyproline/Collagen (µg)} = \left[\frac{\text{CS - (y-intercept)}}{\text{Slope}}\right]$$

To calculate collagen (µg) from hydroxyproline:

$$\text{Collagen (µg)} = \frac{\text{Hydroxyproline (µg)}}{0.11^*}$$

To calculate hydroxyproline (µg) from collagen:

$$\text{Hydroxyproline (µg)} = \text{Collagen (µg)} \times 0.11^*$$

**Assuming hydroxyproline comprises 11% of total collagen by weight. This proportion may range between 11–15% depending on the collagen source.*

Performance Characteristics

The assay range describes the lowest and the highest concentrations in which collagen and hydroxyproline can be reliably detected. The assay range is 0.02-5 µg for collagen and 0.006-3 µg for hydroxyproline.

The lower limit of quantification (LLOQ) for the collagen assay and hydroxyproline assay are 0.5 and 0.047 µg, respectively.

Precision:

When a series of 24 mouse liver homogenate measurements were performed on the same day, the intra-assay coefficient of variation was 15%. When a series of five mouse liver homogenate measurements were performed on different days under the same experimental conditions, the inter-assay coefficient of variation was 5%.

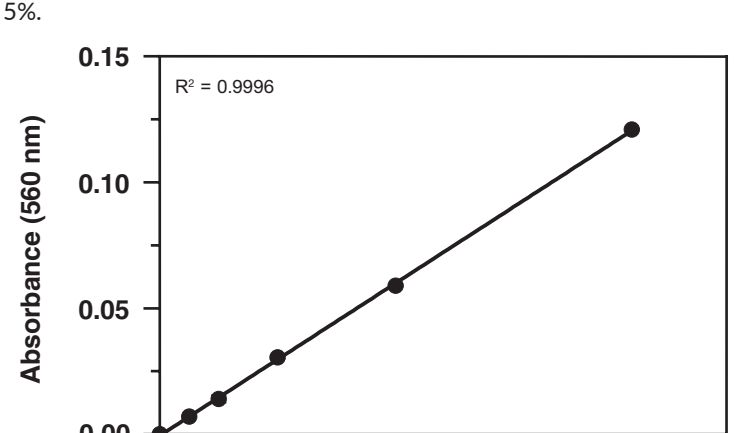


Figure 5. Collagen standard curve

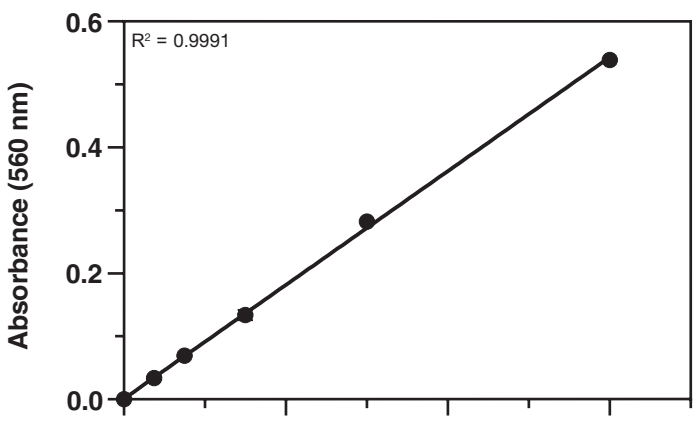


Figure 6. Hydroxyproline standard curve

Sample Matrix Properties

Spike and Recovery

Urine was spiked with different amounts of hydroxyproline, processed as described in the **Sample Preparation** section and evaluated using the Collagen/ Hydroxyproline Assay Kit. The results are shown below. The error bars represent standard deviations obtained from different amounts of hydrolysate added to the reaction.

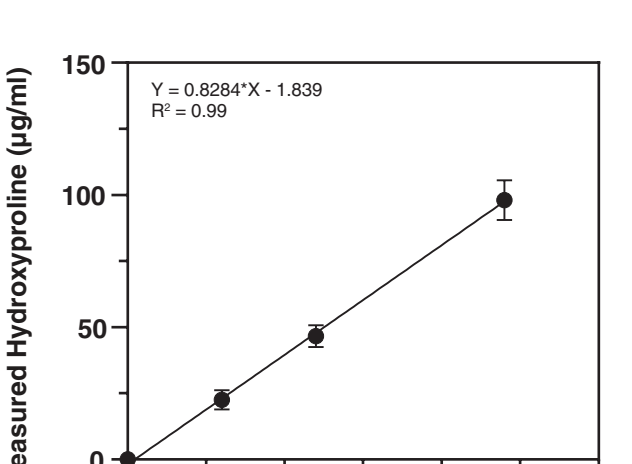


Figure 7. Spike and recovery of hydroxyproline in urine

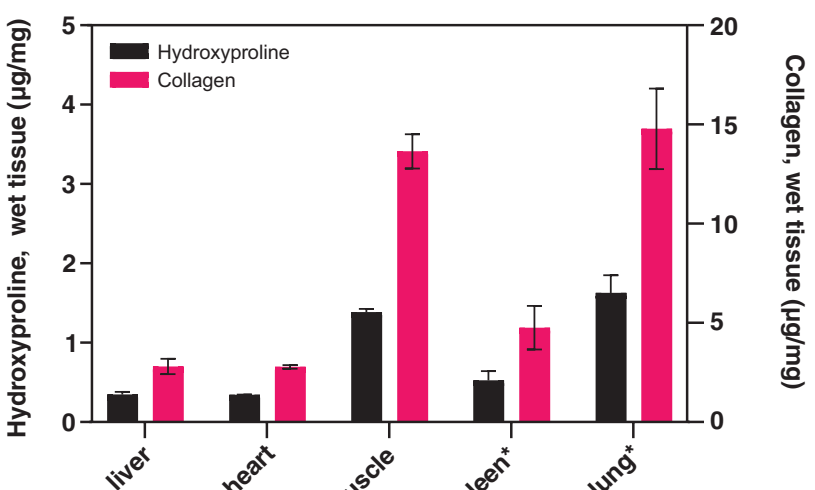


Figure 8. Measurement of total collagen and hydroproline in various tissues
Error bars represent the standard deviations between different amounts of the tissue sample hydrolysate added to the reaction. In samples where both collagen and hydroxyproline were measured using each corresponding standard, correlation between the measured levels of collagen and the levels calculated based on measured hydroxyproline concentration was 0.987.
*Collagen calculated from hydroxyproline standard curve assuming 11% by weight

Interferences

The following reagents were tested for interference in the assay.

Reagent		Will Interfere
Buffer	RIPA Buffer	No
Protease Inhibitors	Antipain (50 µg/ml)	No
	Bestatin (40 µg/ml)	No
	Chymostatin (60 µg/ml)	No
	E-64 (10 µg/ml)	No
	Leupeptin (0.5 µg/ml)	No
	Pepstatin (0.7 µg/ml)	No
	Phosphoramidon (330 µg/ml)	No
	Pefabloc SC (1 mg/ml)	No
	Aprotinin (2 µg/ml)	No

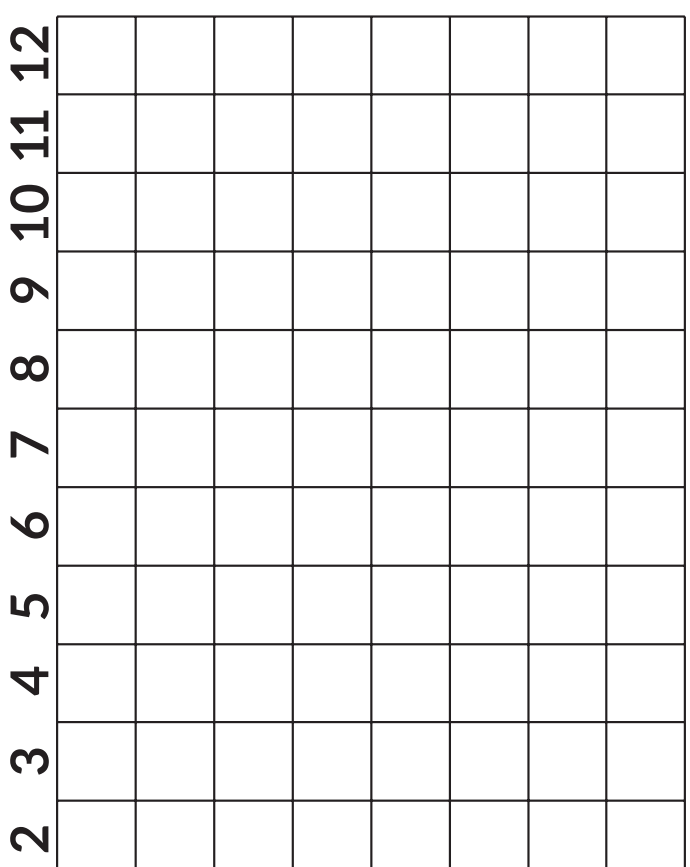
Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Poor pipetting/technique B. Bubble in the well(s) C. Spilling of the sample or reaction mixture when handling D. Improper mixing of reagents E. Uneven heating	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles C. Tightly cap the solutions before mixing and during incubations D. Vortex the components thoroughly after each addition E. Ensure tubes have a snug fit in the heat block and incubation times are exact
No absorbance was detected above the background in the sample wells	The analyte concentration is too low to detect or does not contain target	Reassay the sample by adding a larger volume of hydrolysate
Absorbance of the sample wells is higher than the absorbance of standards	Sample is too concentrated	Re-assay the sample using a reduced volume of hydrolysate or dilute the hydrolysate in water

Assay Summary

Hydrolysis: Incubate 100 µl of prepared sample and Collagen Standard (if using) with 100 µl of 10N NaOH for 1 hour at 120°C. Cool then neutralize with 100 µl of 10N HCl.
Prepare Standards: Follow Figures 3 and 4 on pages 14-15 to prepare either hydroxyproline or collagen standards.
Add Standards to Tubes: Add 10 µl of standards to tubes.
Add Samples to Tubes: Add 2-20 µl of hydrolyzed samples to tubes.
Evaporate standards and samples to dryness on a heat block at 65°C (~45-90 minutes).
Allow standards and samples to **cool** to room temperature.
Prepare Hydroxyproline Assay Reagent (1X): Dilute the 10X reagent 10-fold in Hydroxyproline Oxidation Buffer (1X).
Add 100 µl of **Hydroxyproline Assay Reagent 1 (1X)** to tubes. Mix thoroughly.
Incubate 20 minutes at room temperature.
Add 100 µl of **Hydroxyproline Assay Reagent 2 (1X)** to tubes. Mix thoroughly.
Incubate 20 minutes on a heat block at 65°C.
Quench the reaction briefly on ice.
Transfer 100 µl of each tube to a 96-well plate.
Read the absorbance at 560 nm.

Table 3. Assay summary



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NOTES

Warranty and Limitation of Remedy

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