Hydroxyproline (Total Collagen) Assay Kit

Hydroxyproline (Total Collagen) Assay Kit is a detection kit for the quantification of Hydroxyproline in tissue and cell extracts which can be used as a direct measure of the amount of collagen present.

Catalog number: ARG82320

Package: 96 wells

For research use only. Not for use in diagnostic procedures.

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: 9F.-7, No. 12, Taiyuan 2nd St., Zhubei City,

Hsinchu County 302082, Taiwan

Phone: +886 (3) 622 1320

Fax: +886 (3) 553 0266

Email: info@arigobio.com

INTRODUCTION

Collagen is the main structural protein in the extracellular matrix found in the body's various connective tissues. As the main component of connective tissue, it is the most abundant protein in mammals, making up from 25% to 35% of the whole-body protein content. Collagen consists of amino acids bound together to form a triple helix of elongated fibril known as a collagen helix. It is mostly found in connective tissue such as cartilage, bones, tendons, ligaments, and skin. The collagen protein is composed of a triple helix, which generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2). The amino acid composition of collagen is atypical for proteins, particularly with respect to its high hydroxyproline content. The most common motifs in the amino acid sequence of collagen are glycine-proline-X and glycine-X-hydroxyproline, where X is any amino acid other than glycine, proline or hydroxyproline. [provide by Wikipedia: Collagen] Dysregulation in collagen production results in pathologies such as fibrosis (too much collagen), or osteoarthritis (too little collagen). Therefore measurement of collagen production is important in many disease related studies.

This Hydroxyproline (Total Collagen) Assay Kit is based on the detection of hydroxyproline. Hydroxyproline is a non-proteinogenic amino acid, which in mammals occurs in elastin and collagen. Its presence is mainly limited to the triple helix of collagen, where its presence increasesthe triple helix stability. Hydroxyproline is formed post-translationally from specific proline residues by action of the enzyme prolylhydroxylase. Hydroxyproline in tissue hydrolysates can be used as a direct measure of the amount of collagen present.

PRINCIPLE OF THE ASSAY

This Hydroxyproline (Total Collagen) Assay Kit is based on the detection of hydroxyproline. Samples are completely hydrolysis in 6M HCl at 95°C. The hydroxyproline residues in the hydrolysate are quantified using a modified method based on the protocol described by Prockop and Udenfriend (Anal. Biochem.,1960, 1: 228-239). The assay measures the total amount of hydroxyproline present in the sample, which represents all the types of collagen present in the sample without discriminating between the types of collagen and between procollagen, mature collagen and collagen degradation products. The assay is simple and results in a chromogen with an absorbance maximum at 570 nm. The hydroxyproline in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

The kit is shipped with blue ice. Unopened kit can be stored at room temperature (RT) in the dark. Do not use kit components past kit expiration date.

Component	Quantity	Storage information
Microplate	96 wells plate	RT
Standard (collagen) (1200 µg/ml)	1 vial	RT/4°C
Assay buffer	1 vial (Ready-to-Use)	RT/4°C
Detection reagent A	2 vial	RT/4°C
Detection reagent B	1 vial	RT/4°C
Screw-capped tubes	100 ea.	RT
Adhesive plate seals	2 pieces	RT

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at between 540 and 580 nm, 570 nm is preferred
- PP-, PE-or glass tubes(no polystyrene)
- Pipettes and pipette tips
- Deionized water or distilled water or demineralized water
- Horizontal orbital microplate shaker
- 12M and 6M HCl for sample hydrolysis
- 4M HCl for sample and standard dilution
- Incubator (or thermoblock or calibrated oven) for heating at 95°C
- Incubator/ oven at 60°C
- Eppendorf centrifuge

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- The kit is shipped with blue ice. Unopened kit can be stored at room temperature (RT) in the dark. Do not use kit components past kit expiration date.
- After opening, the opened collagen standard and assay buffer should be stored light protected at 4°C. The other opened reagents should be stored light protected at RT and are stable for at least 1 month. The mixed detection reagent (A and B) solution should be used on the day of preparation.
- If crystals are observed in the assay buffer, warm to RT or until the crystals are completely dissolved.
- Detection reagent A may become a gel or solid at RT or below RT, heating at 37°C and vortexing will solve this.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended that the standards and samples be assayed in duplicates. A separate standard curve must be run on each plate.
- Change pipette tips between the addition of different reagent or samples.
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Sample collection:

Cell extracts – Wash the cells once with PBS and then collect the cell into a centrifuge tube. Discard the supernatant after centrifugation. Add lysis buffer with 1% Triton X-100 and 0.5% SDS and protease inhibitor. Sonicate the samples with power 20%, sonication 3s, intervention 10s, repeat 30 times or homogenize by a Dounce homogenizer passing for 10-20 times on ice. Keep the samples on ice while sonication or homogenization. Centrifuge samples at 10,000 x g for 20 minutes at 4°C. Collect the supernatant for assay. 50 –250 µl of the cellular extracts supernatant are transferred to the screw-capped tubes and are diluted 1:1 (v/v) with 12M HCl (final concentration 6M HCl). A minimum of 50 µl sample and 50 µl 12M HCl is advised. Close tubes tightly before placing in oven.

Tissue lysate-

A. Tissue homogenates

Tissue homogenates (50 –250 µl) are transferred to the screw-capped tubes and are diluted 1:1 (v/v) with 12M HCl (final concentration 6M HCl). A minimum of 50 µl sample and 50 µl 12M HCl is advised. Close tubes tightly before placing in oven.

B. Tissue samples

Tissue samples (either wet or dried) are weighed and transferred to the screw-capped tubes. The amount of tissue needed is highly dependent on the collagen level in the tissue. As an indication, dependent on the

type and amount of tissue, add 6M HCl to obtain 50-300 mg tissue/ml. A minimum volume of 100 µl is advised.

Hydrolysis

1. Tubes must be closed very tightly (rubber ring should become well visible from above)
2. Incubated the tubes for 20 hrs at 95°C in a calibrated oven or thermoblock (do not incubate samples at higher temperature).
3. After incubation the tubes are cooled to room temperature. (Do not open tubes until they have reached room temperature.)
4. Tubes are centrifuged for 10 min at 13,000 x g in an Eppendorf centrifuge. Collect the supernatant is used for further analysis.
   Note: Brown or black particles resulting from degradation of fat and carbohydrate may be present that are difficult to remove completely by centrifugation. The amount of particles depends on the sample. Try to avoid pipetting the particles upon transferring the supernatant. Apart from blocking the light path, the particles do not interfere with the assay.
5. First dilute the hydrolyzed sample with demineralized water: 1 volume of sample + 0.5 volume of water (e.g. 200 µl hydrolysate + 100 µl water). The sample is now in 4M HCl.
6. All further dilutions that might be required (see note in "Sample collection" at below) should be performed using 4M HCl.
7. 35 µl of the diluted hydrolyzed sample is used for analysis in the assay.

Note:

1. Hydrolysis takes place at 95°C (higher temperature is not recommended!) for 20 hrs. The screw-capped tubes should be tightly closed by hand. If

tubes are not tightly closed, hydrolysis solution will evaporate.

2. This type of assays can show a matrix effect: disturbing factors in the sample which affect the signal. This effect can be avoided by dilution of the hydrolysate. If a sample type is used for the first time, various dilutions should be tested until linearity of A570 with dilution is obtained.
3. To avoid matrix effect, add a fixed amount of standard or hydroxyproline in the unknown dilution ration samples. Serial dilute the hydrolysates samples in 4 M HCl are recommended to be made from 2 to 512 fold dilution at starting assay. 35 μl samples of the several hydrolysate dilutions were used in the total collagen assay according to the manual.
4. For example: If the recovery of hydroxyproline was very poor at low dilutions (2 – 8 fold dilution) but from dilutions of 16-fold or more a linear relationship with dilution was found. This is in excellent agreement with the results from the hydrolysates. The suitable dilution of this sample would be at least 16X or more dilution.
5. The amounts of collagen in the various tissues are vary per organ, for example in the mouse tissues, tendon (~300 μg/mg) is having the highest collagen content followed by skin (~150 μg/mg), lung (~10 μg/mg), spleen (~5 μg/mg), kidney (~5 μg/mg), heart (~4 μg/mg) and liver (~1 μg/mg).
6. If the tissue was hydrolysed at 100 mg/ml in 6 M HCl (minimum volume of HCl 200 μl), the hydrolysate have to be diluted with 4M HCl before assay. And the started dilution ratio for mouse samples are as below:

   Tendon, Skin: 100-1000

   Lung, Kidney, Spleen: 10-50

   Heart: 5-25

Liver: 5-10

7. In liver tissue hydrolysate the dilution factor is 5-10, due to matrix effect occurring in less diluted samples. This results in low OD values (around or below lowest concentration in standard line). Therefore this type of tissue may be not suitable for this assay kit.

REAGENT PREPARATION

- **Mixed detection reagent:** It is recommended to prepare this reagent immediately prior to addition to the wells. Add **2 volumes** of **detection reagent A** with **3 volumes detection reagent B** to generate a mixed detection reagent working solution. (For one well, add 30 µl of detection reagent A + 45 µl of detection reagent B, mix well.) **75 µl** of **Mixed detection reagent** is needed for one well.

  Note:

  1. The detection reagent B and the mixed detection reagent A+B in concentrated form may attack certain types of plastics. For pipetting these solutions use PP or PE pipet tips, or glass pipets is recommended.
  2. The detection reagent A+B mixed solution should be made in PP, PE or glass tubes. Polystyrene or PET are not recommended.
  3. The 96-well plate provided in the kit is resistant to the dilute mixed detection reagent A+B solution present in the assay.
  4. Detection reagent B and the detection reagent A+B mixture are corrosive and should be handled with care. Work in a fume hood, use proper eye and face protection and wear gloves.

- **Standards**: The standards have to through hydrolysis process before serial dilution and it is the same as sample preparation as below:
  1. Add **125 µl** of **standard** concentrate (1200µg/ml) and an **equal volume (125µl)** of **12M HCl** into a screw-capped tube, mix well, to yield a standard stock concentration of 600 µg/ml in 6M HCl.
  2. The screw-capped tube must be closed very tightly (rubber ring should become well visible)
  3. Incubated the tubes for 20 hrs at 95°C in a calibrated oven or thermoblock (do not incubate samples at higher temperature).
  4. After incubation the tubes are cooled to room temperature. (Do not open tubes until they have reached room temperature.)
  5. Tubes are centrifuged for 10 min at 13,000 x g in an Eppendorf centrifuge. Collect the supernatant is used for further dilution.
  6. Diluted 125 µl of standard stock from step 5 with 62.5 µl of 4M HCl and 62.5 µl of distilled water to yield a S1 standard concentration of **300 µg/ml** in 4M HCl.
  7. 4M HCl serves as zero standard (0 µg/ml), and the rest of the standard serial dilution can be diluted with 4M HCl as according to the suggested concentration below: **300 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml.**

Dilute collagen standard as according to the table below:

Standard#	Standard Conc. (µg/ml)	µl of 4M HCl	µl of water	µl of standard
S1	300	62.5 µl	62.5 µl	125 (600 µg/ml Hydr. Stock)
S2	200	60	-	120 (S1)
S3	100	90	-	90 (S2)
S4	50	90	-	90 (S3)
S5	25	90	-	90 (S4)
S6	12.5	90	-	90 (S5)
S7	6.25	90	-	90 (S6)
S0	0	90	-	0

ASSAY PROCEDURE

Samples and standards should be assayed in duplicates.

1. Add **35 µl** of **Standards** into appropriate wells of the assay microplate.
2. Add **35 µl** of the **diluted hydrolyzed samples** (in 4M HCl) into appropriate wells of the assay microplate.
3. Add **75 µl** of **assay buffer** into each well and mix well.
4. Cover the plate with an enclosed adhesive plate seal and incubate **20 minutes at room temperature** on orbital microplate shaker.

   Note: When assay buffer is added to 35 µl of the (diluted) hydrolysate, a cloudy appearance can develop, that will disappear within a minute and does not influence the assay.
5. Prepare a volume of detection reagent sufficient for the number of wells

to be tested (**75 µl/well**) by mixing detection reagent A and B with 2:3 ratio (Ex: per well: **30 µl** of **detection reagent A** + **45 µl** of **detection reagent B**).

6. Carefully remove the plate seal.
7. Add **75 µl** of **mixed detection reagent** into each well. Cover the plate and mix well by briefly shaking the plate.
8. Incubate the plate for **60 min at 60°C** in an oven (*do not use higher or lower temperature*).

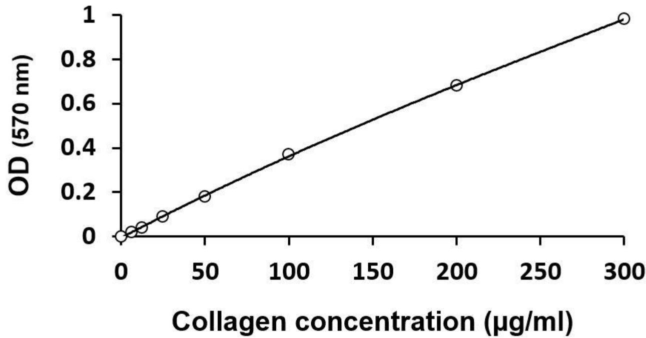
   Note: This incubation time for color development at 60 ℃ is 60 min. This is based on incubation in an oven. When incubation is performed in a plate incubator (with tight contact between incubator and plate) a reduced incubation time (20-30 min) is sufficient.
9. Cool the plate on ice for **max 5 minutes to room temperature**
10. Mix the plate by briefly shaking the plate and carefully remove the plate seal.
11. Clean the bottom of the plate and **read the OD** with a microplate reader at **570 nm** (540-580 nm acceptable) immediately.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, blanks and samples.
2. Subtract all OD values of blank wells
3. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. Use this standard curve to convert the A570 values of the test samples to µg/ml collagen. This gives the collagen concentration in the hydrolyzed sample.
4. Automated method: The results in the IFU have been calculated automatically using a linearized curve fit.
5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
6. If the samples have been diluted after hydrolysis, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.
7. The amounts of collagen in the various tissues are vary per organ, in mouse tissues, tendon (~300 µg/mg) is having the highest collagen content following by skin (~150 µg/mg), lung (~10 µg/mg), spleen (~5 µg/mg), kidney (~5 µg/mg), heart (~4 µg/mg) and liver (~1 µg/mg).

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of Hydroxyproline (Total Collagen) ranged from 6.25-300 µg/ml. The mean MDD was 2.5 µg/ml.