QuickZyme Hydroxyproline Assay

QuickZyme Biosciences

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES

Introduction

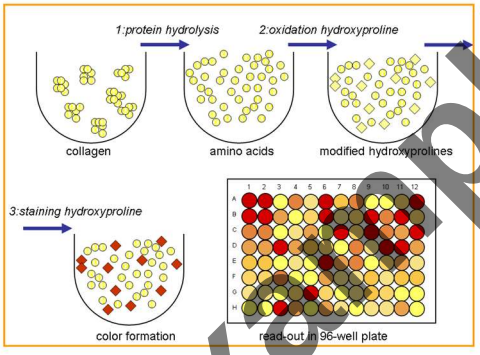
Dysregulation in collagen metabolism may result in pathologies such as fibrosis (too much collagen), or osteoarthritis (too little collagen). Therefore measurement of collagen production is important in many disease related studies.

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 it leads to increased 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.

The QuickZyme Hydroxyproline assay is a modification of the method described by Prockop and Udenfriend (Anal. Biochem.,1960, 1: 228-239). The assay measures the total amount of hydroxyproline present in the sample. If this hydroxyproline is obtained upon hydrolysis of collagen, it 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 assay is developed to measure hydroxyproline in acid tissue and/or protein hydrolysates in such a way that it doesn't need the drying step following acid hydrolysis of collagen for which often special equipment is needed.

Assay principle



What's in the box?

- 2x adhesive plate seals
- Assay buffer
- 2x Detection reagent A
- Detection reagent B
- Hydroxyproline standard (3 mM) in sterile water
- 96-well assay plate
- Assay protocol booklet

Other materials required

The following materials and equipment are required but not supplied:

- 4M HCl for sample and standard dilution
- MilliQ or comparable high quality water
- Single and/or multichannel pipettes
- Eppendorf centrifuge

- Incubator for heating at 60ºC
- Microplate reader capable of measuring at a wavelength between 540 and 580 nm, 570 nm preferred.
- Microplate shaker
- PP-, PE- or glass tubes (no polystyrene)
- Eppendorf tubes

Storage conditions

Unopened kit:
Store at room temperature in the dark. Do not use kit components past kit expiration date.

Opened kit / reconstituted reagents:
The opened standard and assay buffer should be stored light protected at 4ºC. The other opened reagents should be stored light protected at RT and are stable for at least 1 month. The reconstituted detection reagent (A+B) should be used on the day of reconstitution

Precaution

The kit contains n-propanol, perchloric acid, and DMSO. See for relevant MSDS: www.quickzyme.com/products/hydroxyproline-assay.
Wear eye, hand, face, and clothing protection during hydrolysis of the samples and when using the kit. Perform assay in fume hood.

Critical parameters

- This type of assays, like many biochemical 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. Often at least 5-fold dilution is needed (at a 100 mg tissue/ml 6M HCl hydrolysate). Suggested dilutions for several mouse tissues are given in an application note (see product web page). If for your particular sample type the required dilution to avoid matrix effects leads to very low A570 values we recommend using the QuickZyme Sensitive Tissue Hydroxyproline kit.
- The samples used in the assay should be present in a solution containing 4-6M HCl (acid hydrolysis) or 2-4M NaOH (alkaline hydrolysis).
- The incubation time for color development at 60ºC during the last step of the assay is 1 hr. 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.
- When assay buffer is added to 35 µl of the hydrolysate, a cloudy appearance can develop, that will disappear within a minute and does not influence the assay.
- At low temperature the assay buffer may contain some crystals. These can be easily dissolved by warming.
- Just below room temperature Reagent A may become a gel or solid, heating at 37ºC and vortexing will solve this.

Buffer / reagent preparation

Assay buffer is ready for use.
For preparation of the detection reagent mix 2 volumes of detection reagent A with 3 volumes detection reagent B.
Detection reagent B and 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.

The A+B solution should be made in PP, PE or glass tubes. Polystyrene or PET are not recommended. The 96-well plate provided in the kit is resistant to the dilute A+B solution present in the assay.
Detection reagent B and the 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.

Sample preparation

The QuickZyme Hydroxyproline assay is developed to measure hydroxyproline in acid or alkaline hydrolysates, e.g. from cell extracts, tissue homogenates, wet or dry tissue samples. These samples should have been hydrolyzed in 6M HCl (final concentration for acid hydrolysis) or 2-4M NaOH (final concentration for alkaline hydrolysis) according to established procedures. A protocol for acid hydrolysis can be found in the manual of our QuickZyme Total collagen kit (see product web page). After hydrolysis the tubes are cooled down to room temperature. Tubes are centrifuged for 10 min at 13,000 x g in an Eppendorf centrifuge. In acid hydrolysates 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.

Hydrolyzed samples will need to be diluted. Samples hydrolyzed in 6M HCl have to be diluted with water: 1 volume sample + 0.5 volume water (e.g. 200 µl hydrolysate + 100 µl water). The sample is now in 4M HCl. Often further dilution with 4M HCl is needed to prevent matrix effect (see Critical parameters in this manual). Samples from alkaline hydrolysis have to be diluted at least six-fold with 4M HCl.
35 µl of the (diluted) hydrolysate is used for analysis in the assay.

Standard preparation

The hydroxyproline standard is provided as
a stock solution of 3 mM in sterile water.
For a standard line 8 Eppendorf tubes are labeled as S1-S8. S1 to S7 are dilutions of the stock solution and S8 is a blank. The standard dilutions are made with 4M HCl according to the scheme below.
This results in a standard line as follows:
300 µM (S1); 200 µM (S2); 150 µM (S3); 100 µM (S4); 50 µM (S5); 25 µM (S6); 12.5 µM (S7); 0 µM (S8). Mix all the standards well upon dilution.
35 µl of each standard is used for analysis in the assay.

Standard label	*Sample from*	*4M HCl*	*Conc (µM)*
S1	30 µl stock	270 µl	300
S2	120 µl S1	60 µl	200
S3	45 µl S1	45 µl	150
S4	90 µl S2	90 µl	100
S5	90 µl S4	90 µl	50
S6	90 µl S5	90 µl	25
S7	90 µl S6	90 µl	12.5
S8	0 µl	90 µl	0

Pipetting scheme for the preparation of the samples for the hydroxyproline standard line

Assay procedure

It is recommended that all samples and standards are assayed in duplicate

1. Prepare the samples as described in 'sample preparation'
2. Prepare the hydroxyproline standard as described in 'standard preparation'
3. Pipet 35 µl standard into appropriate wells of the assay microplate
4. Pipet 35 µl of each (diluted) sample (in 4M HCl) into the appropriate wells.
5. Add 75 µl assay buffer to each well and mix well
6. Cover the plate with an enclosed adhesive plate seal and incubate 20 minutes at room temperature, while shaking the plate
7. Prepare a volume of detection reagent sufficient for the number of wells to be tested (75 µl/well) by mixing detection reagents A and B 2:3 (resp 30 µl + 45 µl/well)
8. Carefully remove the plate seal
9. Add 75 µl detection reagent to each well
10. Cover the plate with an enclosed adhesive plate seal
11. Mix well by shaking the plate. Incubate 60 minutes at 60°C in an oven or incubator (do not use higher or lower temperature).
12. Cool the plate on ice for max. 5 minutes to room temperature
13. Mix the plate and carefully remove the plate seal
14. Clean the bottom of the plate and read the plate at 570 nm (540-580 nm acceptable although with slightly lower A values) and perform data analysis.

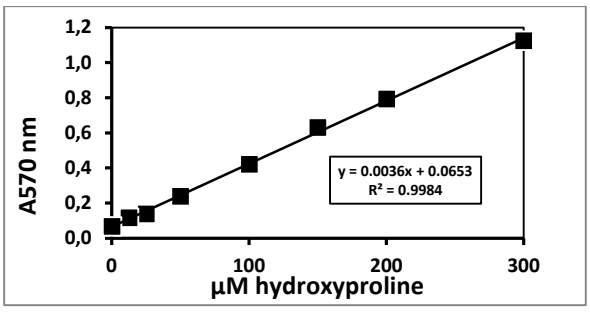
Data analysis

Several options are available for the calculation of the hydroxyproline concentration in the assay samples. It is recommended that the data are handled by a software package utilizing a regression curve fitting program. If not available, the hydroxyproline concentration can be calculated manually as follows.

- Average the duplicate readings for each standard or sample and subtract the average blank from all readings.
- Create a standard curve by plotting the mean A_{570} (minus blank) of each standard on the y-axis against the hydroxyproline content on the x-axis (0- 12.5 – 25 - 50 – 100 – 150 - 200 - 300 µM hydroxyproline). Draw a best-fit linearized curve through the points on the graph. Use this standard curve to convert the A_{570} values of the test samples to µM hydroxyproline. This gives the hydroxyproline concentration in the hydrolysate. If after hydrolysis a dilution step is included, the concentration should be multiplied with the dilution factor to give the hydroxyproline concentration in the hydrolysate. Depending on the sample preparation the amount of hydroxyproline in the original samples can be calculated.

Typical data

The shown data curve (see below) is provided for demonstration only. The exact A_{570} values can vary slightly per experiment



A typical hydroxyproline standard curve (the background is not subtracted).

Related products
- Total Collagen assay
- Sensitive Tissue Collagen assay
- Sensitive Tissue Hydroxyproline assay
- Soluble Collagen assay
- Total Protein assay
- Human MMP-2 activity assay
- Human MMP-7 activity assay
- Human MMP-8 activity assay
- Human MMP-9 activity assay
- Mouse MMP-9 activity assay
- Human MMP-14 activity assay
- Granzyme B activity Reagent Set
- Human TIMP-1 ELISA
- Human Urokinase (uPA) ELISA

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