QuickZyme

Total Collagen

Assay

QuickZyme Biosciences

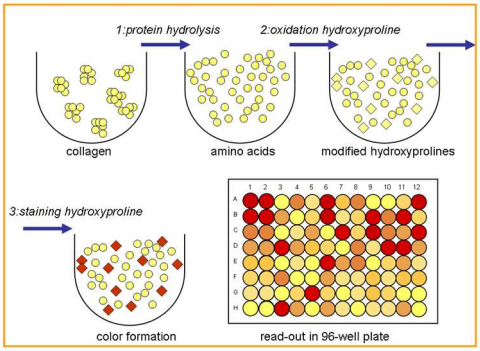
Introduction

Collagen is one of the main components of extracellular matrix. 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.

The QuickZyme Total Collagen assay 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 increases the 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 measurement of collagen is started by complete hydrolysis of tissue samples in 6M HCl at 95ºC. In the hydrolysate hydroxyproline residues are quantified using 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, 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 assay is developed in such a way that it doesn't need the drying step following acid hydrolysis for which often special equipment is needed.

Assay principle



What's in the box?

- 100 special screw-capped tubes needed for sample hydrolysis
- 2 Adhesive plate seals
- Assay buffer
- 2x Detection reagent A
- Detection reagent B
- Collagen standard (1200 µg/ml) in 0.02M acetic acid
- 96-well assay plate
- Assay protocol booklet

Other materials required

The following materials and equipment are required but not supplied:

- 12M and 6M HCl for sample hydrolysis
- 4M HCl for sample and standard dilution
- Single and/or multichannel pipettes
- Eppendorf centrifuge
- Incubator (or thermoblock or calibrated oven) for heating at 95ºC (not higher!)
- 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)

Storage conditions

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

Opened kit / reconstituted reagents:
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 reconstituted detection reagent (A+B) should be used on the day of reconstitution.

Precaution

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

Critical parameters

- 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. Suggested dilutions for several mouse tissues are given in the application note (see product page on our web site). 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 Collagen kit.
- Hydrolysis takes place at 95°C (not higher!) for 20 hrs. *The screw-capped tubes should be tightly closed by hand. If tubes are not tightly closed, hydrolysis solution will evaporate.*
- The incubation time for color development at 60°C 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 (diluted) 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 dissolved by warming.
- Just below room temperature Reagent A may become a gel or solid, heating at 37°C 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 (1)

- Cell extracts

Cellular extracts (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. See also critical parameters above.

- Tissues

A. Tissue homogenates

Tissue homogenates are treated similarly as described above for conditioned culture medium and cell extracts.

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.

Sample preparation (2)

Tubes must be closed very tightly (rubber ring should become well visible from above) and incubated for 20 hrs at 95ºC in a calibrated oven or thermoblock (do not incubate at higher temperature). After incubation the tubes are cooled to room temperature. Do not open tubes until they have reached room temperature. Tubes are centrifuged for 10 min at 13,000 x g in an Eppendorf centrifuge. The supernatant is used for further analysis. 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.

First dilute the hydrolyzed sample with demi water: 1 volume sample + 0.5 volume water (e.g. 200 µl hydrolysate + 100 µl water). The sample is now in 4M HCl. All further dilutions that might be required (see remarks in “Critical parameters”) should be performed using **4M** HCl. 35 µl of the diluted hydrolyzed sample is used for analysis in the assay.

Standard preparation

The collagen standard is provided as a stock of 1200 µg/ml in 0.02M acetic acid.

For a standard line 125 µl of this standard is transferred to a screw-capped tube and mixed with an equal volume (125 µl) of **12M** HCl (final concentration 600 µg/ml in **6M** HCl). The tube is closed very tightly (rubber ring should become well visible) and incubated for 20 hrs at 95ºC (not higher!) in a calibrated oven or thermoblock. After incubation the tube is cooled to room temperature and centrifuged for 10 min in an Eppendorf centrifuge at 13,000 x g. The supernatant is used for further analysis.

Label 8 Eppendorf tubes as S1-S8. S1 to S7 are dilutions of the hydrolyzed stock and S8 is a blank. The first dilution is made with water and 4M HCl to adjust to a 4M HCl concentration. Further dilutions are made in 4M HCl, according to the scheme below.

This results in a standard line as follows:

300 µg/ml (S1); 200 µg/ml (S2); 100 µg/ml (S3); 50 µg/ml (S4); 25 µg/ml (S5); 12.5 µg/ml (S6); 6.25 µg/ml (S7); 0 µg/ml (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*	*H_2O*	*Conc (µg/ml)*
S1	*125 µl hydr. stock*	*62.5µl*	*62.5µl*	*300*
S2	*120 µl S1*	*60 µl*	*-*	*200*
S3	*90 µl S2*	*90 µl*	*-*	*100*
S4	*90 µl S3*	*90 µl*	*-*	*50*
S5	*90 µl S4*	*90 µl*	*-*	*25*
S6	*90 µl S5*	*90 µl*	*-*	*12.5*
S7	*90 µl S6*	*90 µl*	*-*	*6.25*
S8	*0 µl*	*90 µl*	*-*	*0*

Pipetting scheme for the preparation of the samples for the collagen 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 collagen standard as described in 'standard preparation'.
3. Pipet 35 µl standard into appropriate wells of the assay microplate.
4. Pipet 35 µl of the diluted hydrolyzed unknown samples (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 (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) and perform data analysis.

Data analysis

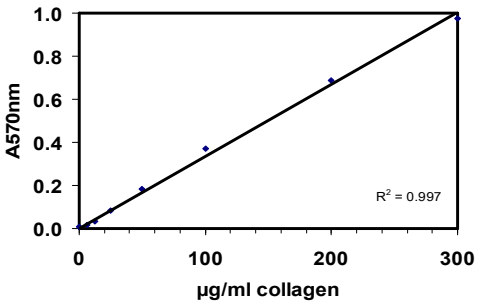
Several options are available for the calculation of the collagen concentration in the assay samples. It is recommended that the data are handled by a software package utilizing a regression curve fitting program. If not available, the collagen concentration can be calculated manually as follows.

- 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 collagen content on the x-axis (0 - 6.25 – 12.5 – 25 - 50 – 100 – 200 - 300 µg collagen/ml hydrolysate). 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 µg/ml collagen. This gives the collagen concentration in the hydrolyzed sample. If after hydrolysis a dilution step

is included, the concentration should be multiplied with the dilution factor to give the collagen concentration in the hydrolyzed sample. Depending on the sample preparation the amount of collagen in the original samples can be calculated.

Typical data

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



A typical collagen standard curve

Related products

- Sensitive Tissue Collagen assay
- Hydroxyproline 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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NOT FOR USE IN DIAGNOSTIC PROCEDURES

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

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