QuickZyme

Sensitive Tissue Collagen Assay

for small tissue samples

QuickZyme Biosciences

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NOT FOR USE IN DIAGNOSTIC PROCEDURES

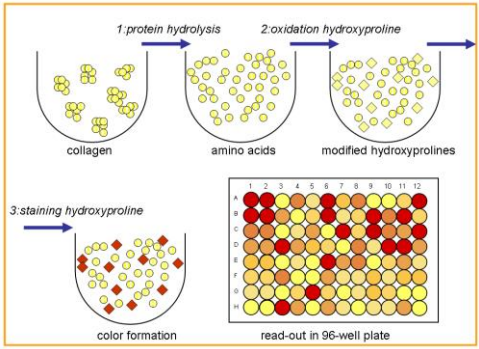
Introduction

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 Sensitive Tissue Collagen assay is based on the detection of hydroxyproline. Hydroxyproline is a non-proteinogenic amino acid, which in mammals occurs mainly in collagen. Hydroxyproline in tissue hydrolysates can be used as a direct measure of the amount of collagen present in the tissue.

The measurement of collagen is started by complete hydrolysis of tissue samples in 6M HCl at 95°C. 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.
In contrast to other hydroxyproline-based assays, the QuickZyme Sensitive Tissue Collagen assay has no matrix effect and no need for dilution of the hydrolysate enabling measuring in small samples with low collagen concentrations. The assay is simple and 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
- 2x Enhancer solution
- 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 95^{o}C (not higher!)
- Incubator for heating at 60^{o}C
- 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 (RT) in the dark. Do not use kit components past kit expiration date.

Opened kit / reconstituted reagents:
The opened collagen standard, assay buffer and enhancer solution should be stored light protected at 4^{o}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 our product web page.
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

A common complication in biochemical assays, including hydroxyproline based colorimetric assays applied to tissue samples, is the occurrence of so called matrix effects, caused by non-identified components in the sample. The matrix effect can result in erroneous high or low values. Matrix effects can often be prevented by dilution of the sample. This dilution, however, results in lower OD's, which is particularly problematic for samples with low collagen content. In contrast to other hydroxyproline-based collagen assays, the QuickZyme Sensitive Tissue Collagen assay shows no matrix effect and no need for further dilution of the hydrolysate enabling measuring in small samples with low collagen concentrations. The assay is simple and it doesn't need the drying step following acid hydrolysis for which often special equipment is needed.

- 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 35 µl of the (diluted) hydrolyzed sample is added to the assay buffer, a cloudy appearance can develop, that will disappear within a minute and does not influence the assay.
- Also upon addition of the enhancer solution a cloudy appearance develops. This does not affect the assay, and will disappear in later steps of the assay.
- At low temperature the assay buffer may contain some crystals. These can easily 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. Reagent A+B solution should not be made polystyrene or PET tubes.
  The 96-well plate provided in the kit is resistant to the dilute A+B solution.
  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)

- Tissues

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. See also critical parameters above.

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 ~100 mg tissue/ml. A minimum volume of 100 µl is advised. Close tubes tightly (see Critical parameters and Sample preparation (2)) before placing in oven.

Sample preparation (2)

Tubes must be closed very tightly (rubber ring should become well visible from above) and incubated for 20 hrs at 95°C in a calibrated oven or thermoblock (do not incubate at higher temperature). After incubation the tubes are cooled down 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 type. 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 (if needed) 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 95^{o}C (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. Remainder of hydrolyzed standard can be used for future assays.

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 and dilutions as described in 'sample preparation'.
2. Prepare the collagen standard as described in 'standard preparation'.
3. Pipette 35µl standard into appropriate wells of the assay microplate.
4. Pipette 35 µl of the diluted hydrolyzed unknown samples into the appropriate wells.
5. Add 75 µl assay buffer to each well and mix.
6. Add 25 µl Enhancer solution to each well and mix. A cloudy appearance will develop which will disappear in later steps.
7. Cover the plate with an enclosed adhesive plate seal and incubate 20 minutes at room temperature, while shaking the plate.
8. 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).
9. Carefully remove the plate seal

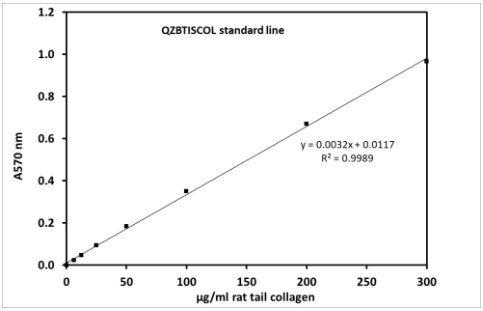
10. Add 75 µl detection reagent to each well.
11. Cover the plate with an enclosed adhesive plate seal.
12. Mix well by shaking the plate. Incubate 60 minutes at 60°C in an oven or incubator (do not use higher or lower temperature).
13. Cool the plate on ice for max. 5 minutes to room temperature.
14. Mix the plate and carefully remove the plate seal.
15. Clean the bottom of the plate and read the plate at 570 nm (540-580 nm acceptable but with slightly lower response) and perform data analysis.

Data analysis

Several options are available for the data analysis. 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- 3.13 – 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, this concentration should be multiplied with the dilution factor to give the collagen concentration in the hydrolyzed sample.

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

- Total Collagen assay
- Hydroxyproline assay
- Sensitive Tissue Hydroxyproline assay
- Soluble Collagen assay
- Total Protein assay
- Granzyme B activity Reagent Set
- 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

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