COSMO BIO CO., LTD.

Collagen Quantitation Kit

Cat. No. CSR-COL-001E

Edition Date : 2015 / 04 / 02

【I-1.】Background

Collagen is the one of the main components of extracellular matrix, and accounts for about 30% of the whole human protein. Recent studies, the production of collagen are reduced in aged skin, the degradation and the accumulation of collagen found in a particular disease, such as has been shown. Therefore, it has become important in accurately quantifying technology health maintenance and disease diagnosis collagen.

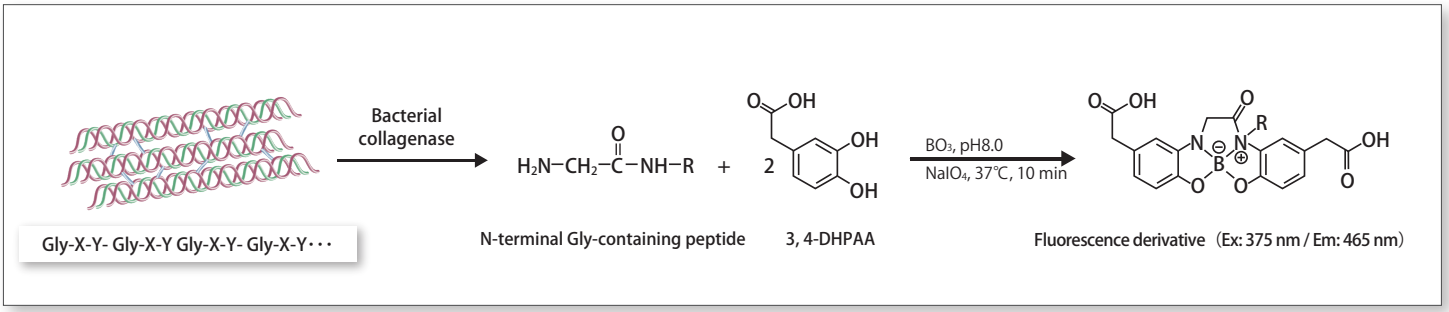


Figure 1　Principle of collagen determination

Collagen contains approximately 30 % Glycine (Gly) of whole amino acid residues in each molecule, and constitutes a repeated amino acid sequence, -Gly-X-Y- in which Pro or Hyp frequently appears at the X or Y position. The proposed assay method takes an advantage of this unique sequence of collagen as well as the remarkable specificity of bacterial collagenase that cleaves all collagen types at the position of N-terminal side of the Gly residue. Therefore, abundant N-terminal Gly-containing peptides such as Gly-Pro-Hyp, Gly-Pro-Ala, Gly-Pro-Pro, and Gly-Pro-Z (Z is other amino acid or oligopeptide) are produced from one molecule of each collagen, and greatly amplified fluorescence signals from these products are obtained by the reaction with 3,4-Dihydroxyphenylacetic acid (3,4-DHPAA).

This kit is a highly specific and sensitive method for the assay of whole collagen in biological samples using a fluorogenic reagent, 3,4-Dihydroxyphenylacetic acid (3,4-DHPAA). The 3, 4-DHPAA reagent can selectively detect N-terminal Gly-containing peptides.

【I-2.】Features

This kit can quickly measure the collagen in the food and cosmetics. Compared to the existing hydroxyproline method, there is no need of hydrochloric acid hydrolysis, it can be determined in a safe and simple procedure with a small amount of sample. This kit is suitable for quality control and product development during the manufacturing process.

- Simple procedure: takes only 2 - 3 hours
- Fast and convenient
- The kit can measure 96 tests

For research use only, Not for diagnostic use.
Please read this manual thoroughly before use.

【 I - 3. 】 Kit Components

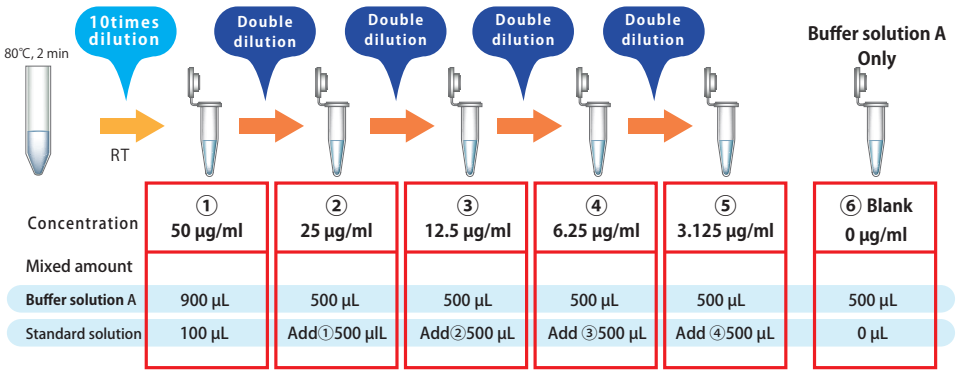
No.	Component	Content	Quantity	Storage
1	Enzyme Reagent (Collagenase)	200 μL	1	4℃ -10℃
2	Standard solution （500 μg/mL Collagen）	300 μL	1	
3	Buffer A	30 mL	1	
4	Fluorescence Reagent (3,4-DHPAA)	500 μL	1	
5	Buffer B	15 mL	1	
6	$NaIO_4$ solution	5 mL	1	

Required but not provided

PBS(-) (without Mg and Ca)
1.5 mL and 500 μL micro-test tube
96 well Black Microplate
96 well Fluorescent Microplate Reader (Ex: 375 nm/Em: 465 nm)
※ Please prepare a microcell cuvette if you are using a fluorescence spectrophotometer.

【 II-1. 】 Standards Preparation

Heat Standard solution (500 μg / mL collagen) for 2 minutes at 80 ℃ and then cool it down to room temperature (as the Calibrator stock). Dilute 100 μL of Calibrator stock with 900 μL of Buffer A, constitute ① highest concentration (50 μg / mL). The toal quantity of Stp 1 will be 1 mL, and it can be prepared 50 μg/mL in a tube x 20. The total quantity of collagen standrd solution is 300 μL, Hence it can be prepared 60 times (20 x 3).
Following, prepare ② 25 μg / mL, ③ 12.5 μg / mL, ④ 6.25 μg / mL, ⑤ 3.12 5 μg / mL, and ⑥ blank (BufferA only).



Concentration	① 50 μg/ml	② 25 μg/ml	③ 12.5 μg/ml	④ 6.25 μg/ml	⑤ 3.125 μg/ml	⑥ Blank 0 μg/ml
Mixed amount						
Buffer solution A	900 μL	500 μL	500 μL	500 μL	500 μL	500 μL
Standard solution	100 μL	Add①500 μL	Add②500 μL	Add ③500 μL	Add ④500 μL	0 μL

【 II-2. 】 Sample Preparation

1) Cell layer
Remove culture medium. Wash out each well with PBS(-) and homogenize the cell layer. (ex: 24 well plate: 500 μL - 1 mL of PBS(-).) Transfer the cell lysate into micro-test tube and heat at 80 ℃ for 2 minutes.

2) Foods/Cosmetics
Sample of solid and jelly-like is weighed and measure it after being dissolved or extracted to collagen by adding a certain amount of Buffer A. As collagen concentration at the time of measurement is within the calibration curve, adjust the sample concentration with Buffer A in advance.

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【 II-3. 】 Reagent Preparation

● **Enzyme Solution Preparation**

Dilute Enzyme Reagent with Buffer A (1:19)

● **Fluorescent Solution Preparation**

Dilute Fluorescent Reagent (3,4-DHPAA) with Buffer B (1:19)

【 III. 】 Measurement Protocol

1. Transfer 25 μL of prepared diluted standard solutions or samples into 500 μL micro-test tube.
2. Add 25 μL of the Enzyme Solution (diluted Enzyme Reagent with Buffer A (1:19) into the micro-test tube.
   In totally, it can be prepared the quantity 4 mL of Enzyme Reagent (200 μl x the dilution range 20 times). It can be tested 160 times (25 μL in a test x 160 tests = 4mL
3. Heat the tubes at 37 ℃ for 1 hour.
4. Add 100 μL of the Fluorescent Solution (Diluted Fluorescent Reagent (3,4-DHPAA) with Buffer B (1:19) into each tube. In totally, it can be prepared the quantity 10 mL of Enzyme Reagent (500 μL x the dilution range 20 yimes). It can be tested 100 times (100 μL in a test x 100 tests = 10 mL)
5. Add 50 μL of $NaIO_4$ solution into each tube. (It contains 5 mL in a kit, hence it can be used for 100 tests.)
6. Heat the tubes at 37 ℃ for 10 minutes.
7. Transfer the 100 μL of the content of each tube into the wells of 96 well black microplate and then measure the fluorescence using fluorescence plate reader set at a wavelength (Ex:375 nm /Em:465 nm)
8. Create a standard curve by serial dilution of standard solutions as indicated in the below. Draw a smooth curve through these points to construct the calibration curve. Read the concentration in the samples from the calibration curve.

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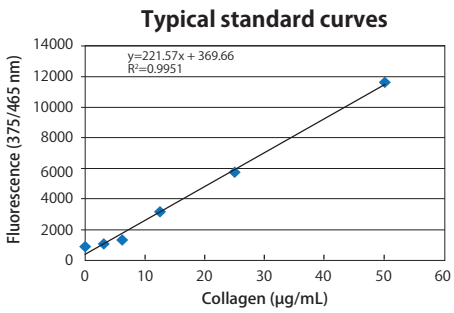
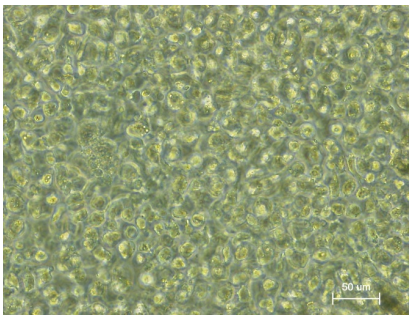
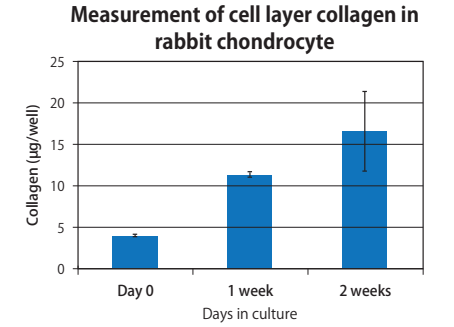
【IV.】Assay examples

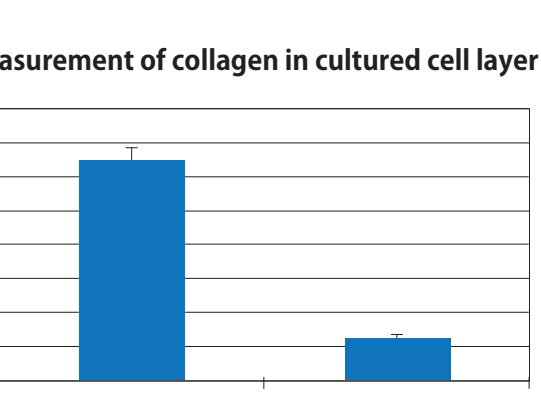
Measurement of cell layer collagen in a rabbit chondrocyte

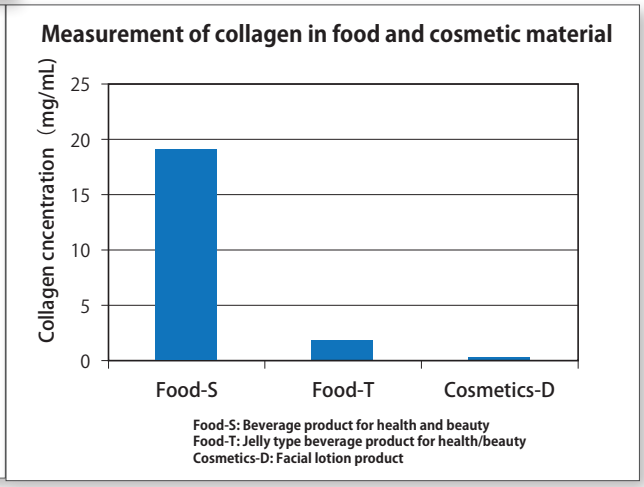




Rabbit chondrocyte (phase contrast microscope)







【V.】References

[1] Selective and sensitive determination of peptides using 3, 4-dihydroxyphenylacetic acid as a fluorogenic reagent. Hasina Yasmin, Takayuki Shibata, Mohammed Shafikur Rahman, Tsutomu Kabashima, Masaaki Kai, *Analytica Chimica Acta*, **721** (2012) 162-166

[2] Amplified and selective assay of collagens by enzymatic and fluorescent reactions. Hasina Yasmin, Tsutomu Kabashima, Mohammed Shafikur Rahman, Takayuki Shibata, Masaaki Kai, *Scientific Reports* (May 2014) | 4 : 4950 | DOI: 10.1038/srep04950

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