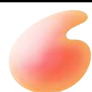
Code : ACB-EC1-E105-EX

Human collagen type1 ELISA

Ver.03

For Research Use Only



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Introduction

Type 1 collagen is the most abundant protein in the connective tissues, especially in tendon, skin and bone. This kit is designed to quantify collagen in various sources such as cell media, ECM (Extra Cellular Matrix) of culture cell and tissue because the kit detects atelo-collagen which is prepared by pepsin digestion.

1. Features

- Short assay time (2 hours 15 minutes).
- Collagen pre-coated microtiter-plate.
- Simultaneous assay of many samples (Assay maximum is 40 samples per 1 kit in duplicate).
- No need of special machines and equipments because of non-isotope assay.
- Partitional use because of split type (8 wells/strip).

2. Principle (Competitive EIA)

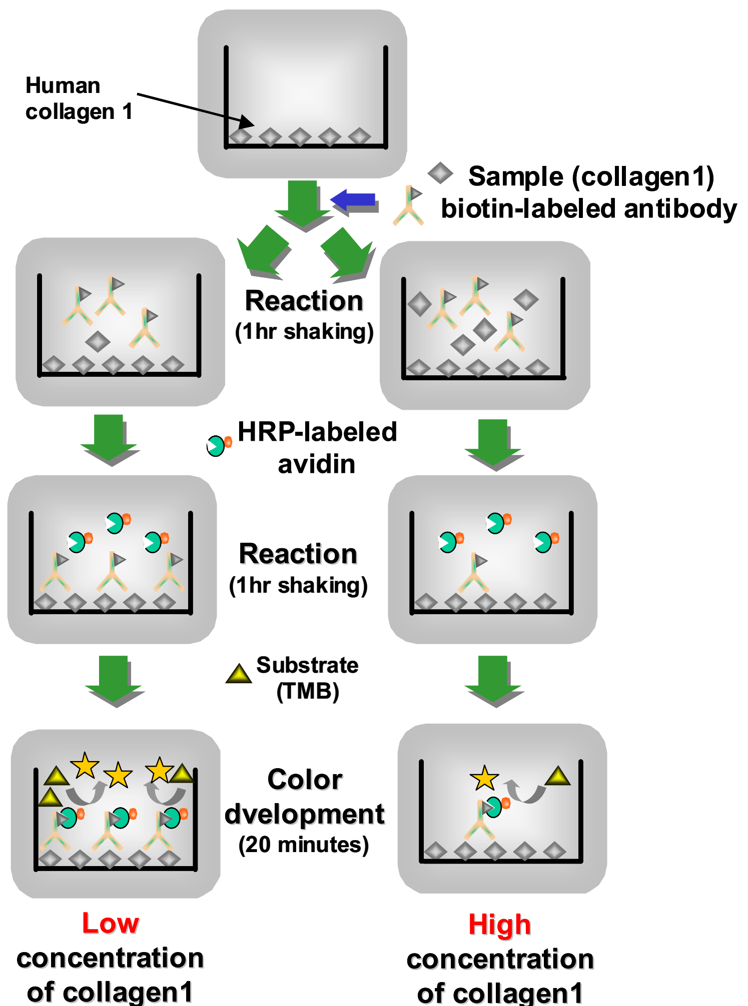
This assay is a competitive EIA in which polyclonal antibody to human type 1 atelo-collagen is used.

Mixture of sample contained atelo-collagen and biotinylated anti-atelo-collagen antibody is added to a well of microtiter-plate on which purified atelo-collagen is immobilized.

After washing, add peroxidase labeled avidin. The avidin reacts with the biotinylated antibody on the microtiter-plate.

After washing, add TMB (3, 3’, 5, 5’-tetramethylbenzidine) which is a substrate of peroxidase. After reaction, measure the absorbance of the color density of the reaction mixture at 450 nm.

In proportion to increase the collagen concentration of samples, the color density decreases since the amount of biotinyleted antibody and peroxidase labeled avidin decreases.



3. Contents

No.	Components	Volume
①	**Collagen coated microtiter plate** Pull out the strips (8 wells/strip) you wish to run from the bag. Return the rests and store in the bag after sealed tightly at 2~8℃. Use within 30 days after unsealed.	96 wells, 12 strips
②	**Collagen standard** Lyophilized. Reconstitute the lyophilized powder with 0.5mL of diluent A（standard/sample diluent）. The concentration of the standard after reconstitution is printed on the label of reagent bottle. Prior to assay, prepare a standard solution set (see “preparation of a standard solution set” (p.5)).	0.5mL
③	**Biotinylated anti-collagen antibody concentrate** Ready for use.	0.8mL
④	**Avidin-HRP conjugate concentrate** Prior to use, dilute 100 times with diluentＢ（see “preparation of avidin-HRP conjugate solution” (p.6)).	0.07mL
⑤	**Diluent A** Use for dilution of the collagen standards and samples.	10mL
⑥	**Diluent B** Use for dilution of avidin-HRP conjugate concentrate.	10mL
⑦	**Wash buffer concentrate （10X concentrated)** Dilute 10 times with distilled water or deionized water (see preparation of wash buffer (p.6)).	50mL
⑧	**TMB substrate** 3, 3’, 5, 5’-tetramethylbenzidine. Ready for use.	7mL
⑨	**Stop solution** １N sulfuric acid. Ready for use.	7mL
⑩	**Plate seal** Cut in an appropriate size for use.	2 sheets

4. Preparation of equipments and reagents

- Micropipettes and tips (10～100 μL、100～500 μL)
- 1.5ｍL tubes
- Measuring cylinder (500 mL)
- Distilled or deionized water
- Microtiter-plate shaker
- Microtiter-plate reader capable of reading absorbance at 450 nm
- Pepsin (powder)
- 150mM Acetic acid solution (for preparation of assay sample of culture media)
- 50mM Acetic acid solution (for preparation of assay sample of extra-cellular matrix)
- Neutralizing solution (200 mM Tris, 150 mM NaCl)

5. Preparation of assay samples

(1) Cell culture : Quantification of collagen in culture media

When collagen production cell (fibroblast) is cultured, both pro-collagen and collagen (mature type) are secreted into culture media. Collagen in the culture media can be quantified by pepsin digestion.

① Prepare pepsin solution (Dissolve pepsin powder into 150 mM acetic acid solution to final concentration 0.6 mg/mL).

② Add 0.1 mL of pepsin solution (see above) to 0.2 mL of culture media and mix well.

③ Incubate for 2 hours at room temperature (or for overnight at 4 ℃).

④ Add 0.1 mL of neutralization solution (200 mM Tris, 150 mM NaCl) and mix well. This mixture can be used as assay sample.

(2) Cell culture : Quantification of collagen in extra-cellular matrix (ECM)

When collagen production cell (fibroblast) is cultured in petri-dish, collagen in culture media deposit as ECM. The deposited collagen can be quantified as follows.

① Prepare pepsin solution (Dissolve pepsin powder into 50 mM acetic acid solution to final concentration 0.1 mg/mL).

② Remove the culture media from the petri-dish and detach cells from the bottom of the petri-dish using a cell-scraper.

③ Add equal volume of the pepsin solution to the petri-dish (i.e. : When cells proliferate to 100% confluent, 0.5 mL for a 24 wells-plate, 5 mL for a 6 cm petri-dish).

④ Shake for overnight at 4˚C.

⑤ Centrifuge for 10 miniutes at 10,000 g.

⑥ Recover the supernatant.

⑦ Add 1/3 volume of neutralization solution (200 mM Tris, 150 mM NaCl) to pepsin solution and mix well .

⑧ The mixture can be used as assay sample.

6. Assay procedure

Read followings carefully before starting the assay.

(1) Preparation of standard solution set

Follow carefully the assay procedure to have good results.

① Dissolve the collagen standard (lyophilized) completely with 0.5 mL of Diluent A (diluent for standards or samples) .The rest of the standard solution should keep at -80℃ in frozen condition and can be used only once again.
Caution : The collagen concentration of each standard is printed on the reagent bottle.

Note: Be sure to use 0.5 mL of Diluent A for dissolving the collagen standard. Do not change the dilution volume or use different diluent to avoid incomplete dissolution. In case of foaming, keep still for few minutes to remove the foams.

② Dilute the standard solution (above) with the Diluent A and prepare standard dilution sets (See below). Each diluted sample should be mixed well by tapping the tube.

Note: Prepare a standard set for each assay.

Preparation of standard set

Standard sets	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7
Collagen standard solution (Dissolve completely with 500ul of Diluent A)	20 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Diluent A volume	380 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Final volume	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	400 µL

(2) Preparation of assay mixture（Mixture of standard solution / assay sample and biotinylated-anti collagen antibody solution）

Standard solution (prepared by following the method "6.Preparation of assay standard (1)") / assay sample (prepared by following the method "5. Preparation of assay samples (1) / (2)") and biotinylated anti-collagen antibody solution are mixed well with the ratio 9 : 1.

(In case of duplicate assay, 126 µL of assay sample/standard solution and 14µL of biotinylated collagen antibody solution are mixed).

Note: Add the mixture above to the well as soon as possible after preparation (Recommend within 1 hour).

(3) Preparation of Avidin – HRP conjugate solution

Note : Do not mix the solution vigorously to avoid foaming in the mixture.

Add 60μL of Avidin-HRP conjugate concentrate to 6mL of Diluent B and mix well.

Note: When using partitional strips of plate, calculate the appropriate volume of the reagents (i.e. Add 20μL of Avidin-HRP conjugate concentrate to 2mL of Diluent B for 4 strips).

(4) Preparation of wash buffer

① Add the whole volume of the wash buffer concentrate (10X concentrated) to the 500mL of the measuring cylinder and adjust the final volume to 500mL with de-ionized water.

② Cover the measuring cylinder with parafilm and mix thoroughly.

③ Store at 2 –8°C for up to 30 days.

(5) Assay procedure

All reagents must equilibrate to room temperature prior to use.

① Prepare standard solution sets (See "(1) Preparation of standard solution set").

② Prepare assay mixture (See "(2) Preparation of assay mixture").

③ Prepare the number of strips (8wells/strip) you wish to run. Each sample should be assayed in duplicate.

④ Add 200μL of the wash buffer (See "(4) Preparation of wash buffer") to each well and wash all wells 3 times.

⑤ Add 50μL of the assay mixture (See "(2) Preparation of assay mixture") to each well.

⑥ Cover the plate with a seal and incubate at room temperature (20-28°C) for 1 hour with shaking moderately using a microtiter-plate shaker.

⑦ Add 200μL of the wash buffer (See "(4) Preparation of wash buffer") to each well and wash all wells 3 times.

⑧ Add 50μL of avidin-HRP conjugate solution (See "(3) Preparation of avidin-HRP conjugate solution") to each well.

⑨ Cover the plate with a seal and incubate at room temperature (20-28°C) for 1 hour with shaking moderately using a plate shaker.

⑩ Add 200μL of the wash buffer (See " (4) Preparation of wash buffer") to each well and wash all wells 3 times.

⑪ Add 50μL of TMB substrate to each well.

⑫ Incubate for 15 minutes at room temperature (20-28°C).Do not shake the plate.

⑬ Add 50μL of stop solution to each well.

⑭ Cover the plate with a seal and shake for 1 minute moderately at room-temperature.

⑮ Determine the optical density at 450nm within 10 minutes.

7. Data processing

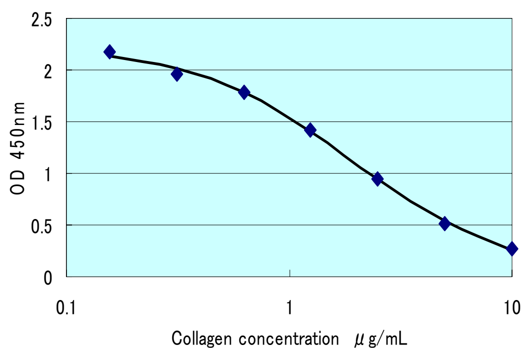
Calculate the collagen concentration in the samples using standard curve.

Note 1 : A standard curve should be generated by each assay.

Note 2 : In case of high concentration of samples beyond its assay range, assay again after dilution of the samples with Diluent A.

(1) Calculation model

① Show the model of standard curve fitted by 4 parameter logistic model.



$Y=((A-D)/(1+(X/C)^{B})+D$

X: Collagen concentration in samples

Y: Optical density of samples(OD_{450})

A: The maximum OD at lowest value of X axis

B: The curve slope at the inflection point

C: The X analyte concentration giving 50% inhibition

D: The minimum OD at highest value of X axis

＊ Analyze by SOFTmax (Molecular Devices Corp.).

② Calculate collagen concentration by interpolating sample OD value to the standard curve.

＜Example＞

- Change the formula of the regression curve as follows.

  $X=C\times((A-Y)/(Y-D))^{\wedge}(1/B)$

- Calculate collagen concentration in assay mixture by interpolating the sample OD value to the formula above.

  Example : OD value of a sample＝1.0
  In case of A=2.244, B=1.190, C=1.938, D=0.019,
  $X=1.938\times(2.244-1.0)/(1.0-0.019)^{\wedge}1/1.190 = 2.0$ μg/mL

- Multiple the calculated values by dilution rate.

  Example : In case the sample is diluted to half by pepsin treatment and neutralization.
  $X=2.0\times2=4.0$ μg/mL

8. Assay performance

(1) Cross reactivity

Table 1 Cross reactivity

Sample	Cross reactivity (%)
Bovine type1	0.0
Human type2	5.6
Human type3	10.0

(2) Assay limit

Minimum : 0.02 μg/mL

Maximum : 40 μg/mL

9. General notes

- This assay kit is for research use only. Do not use for human and animal diagnostic use.
- Wear suitable protective clothing and avoid contact skin and eyes for handling the reagents. In case of accident, wash immediately with sufficient water and follow medical advice.
- Avoid foaming of reagent by vigorous stirring.
- Avoid touching the bottom and tops of the plate through whole assay. Stain of bottom of plate or contamination may cause false results.
- Assay in duplicate is recommended for standards and samples.
- Run a separate standard curve for each assay.
- Wash step is especially important. Wash wells thoroughly and uniformly.
- Use same reagent lot for each assay. Avoid mixing reagents from different lots.
- Avoid using expired reagents.

10. Prior to inquiry

(1) Low optical density

① Check reader wavelength at 450nm.
② Check incubation time and temperature.
③ Check reagents have been equilibrated to room temperature.
④ Check reagents have been correctly prepared.
⑤ Check reagents have been correctly stored.
⑥ Ensure reagents have not been expired.

(2) High optical density

① Check wash step have been correctly performed.
② Check incubation time and temperature.
③ Check reagents have been correctly prepared.
④ Check reagents have been correctly stored.
⑤ Ensure reagents have not been expired.

(3) Poor precision or replication

① Check pipette calibration.
② Check standard solution have been correctly prepared.
③ Ensure that tips have been changed for each reagent. Avoid mixing reagents from different kit lots.
④ Check wash step have been correctly performed.
⑤ Check shaker have been correctly worked.

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