COSMO BIO CO., LTD.
Inspiration for Life Science

ACEL ECM Reagent Series

Human Collagen Type1 ELISA

(without pepsin)

Cat: ACE-EC1-E105-EX

Ver.03

For Research Use Only

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Introduction

Type 1 collagen is the most abundant protein in connective tissues, especially in tendon, skin and bone.

This kit is designed to quantify collagen in samples such as cell media, ECM (Extra Cellular Matrix) of culture cell and tissues.

1. Features

- Short assay duration (2 hours 15 minutes).
- Collagen pre-coated microtiter-plate.
- Simultaneous assay of many samples is feasible (Maximum number of samples per assay is 40 samples per 1 kit, carried out in duplicate).
- No special machines and equipments are necessary as assay is non-isotopic.
- Partial use is possible because of split type (8 wells / strip).

2. Principle（Competitive EIA）

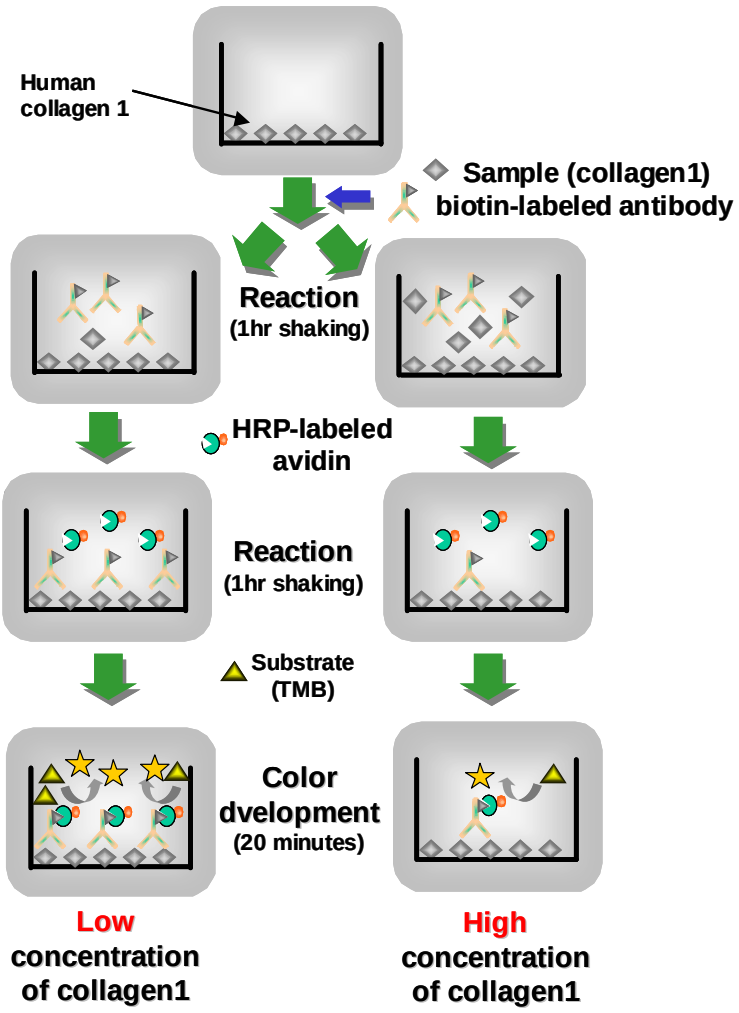
This assay is a competitive EIA where the polyclonal antibody to human type 1 atelo-collagen is used.

Mixture of sample containing atelo-collagen and biotinylated anti-atelo-collagen antibody is added to each well of the microtiter-plate for which purified atelo-collagen is immobilized.

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

After washing, TMB (3, 3', 5, 5'-tetramethylbenzidine), a substrate of peroxidase is added. After the reaction, the absorbance of the color density of the reaction mixture is measured at 450 nm.

The higher the collagen concentration in the samples, the lower the color density due to less amount of biotinyleted antibody and peroxidase labeled avidin.



3. Contents

No.	Components	Volume
①	**Collagen coated microtiter plate** Pull out the strips (8 wells/strip) you wish to run. Store the rest in a tightly sealed bag at 2~8℃ and use within 30 days.	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 diluent B (see “preparation of avidin-HRP conjugate solution” (p.6)).	0.07mL
⑤	**Diluent A** Use to dilute the collagen standards and samples.	10mL
⑥	**Diluent B** Use to dilute 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** 1 N Sulfuric acid. Ready for use.	7mL
⑩	**Plate seal** Cut to appropriate size for use.	1 sheet

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
- 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 culture media can be quantified *directly* (No pepsin digestion necessary). The culture media can be used as an assay sample.

If the concentration of collagen is high, dilute with Diluent A, PBS or TBS.

6. Assay procedure

Please read instructions carefully before starting the assay.

(1) Preparing the standard solution set

① Dissolve the whole bottle of 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℃ and can only be used one more time.

Note :

The collagen concentration of each standard is printed on the reagent bottle.

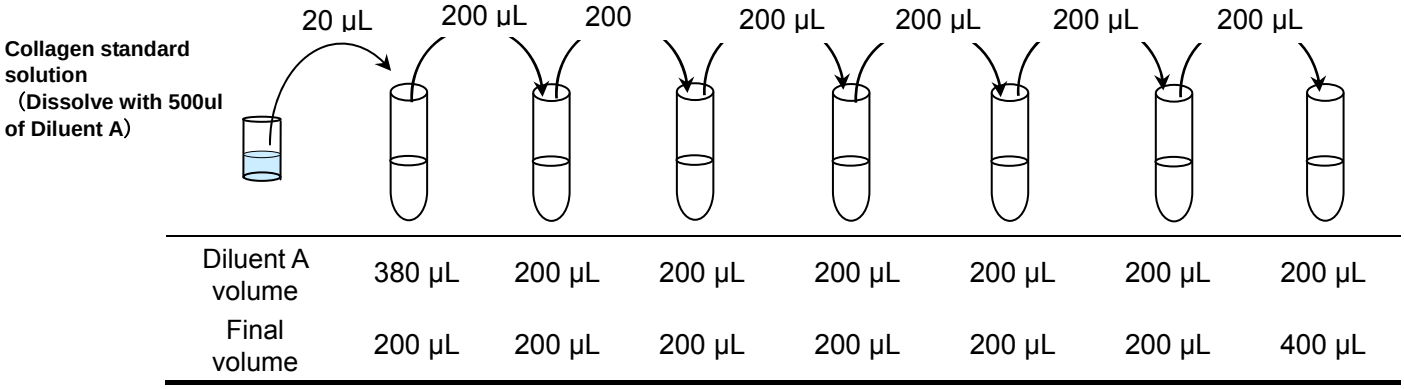
Do not change the amount of Diluent A, or dilution volume as this will result in incomplete dissolution.

In case foams appear, wait for a few minutes until they are gone.

② Dilute the standard solution (above) with the Diluent A and prepare standard dilution sets as shown in figure 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
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）

Mix the Standard Solution and Biotinylated anti-collagen antibody solution in a ratio of 9:1.

Standard solution: prepared by following the method “7.Preparation of assay solution (1)”) / assay sample (prepared by following the method “6. Preparation of assay samples (1) / (2)”)

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

Note: Add the mixture above to wells as soon as possible after preparation (Within 1 hour of preparation).

(3) Preparation of Avidin – HRP conjugate solution

Note：Mix gently. Do not mix the solution vigorously to avoid appearance of foam.

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

Note: When using partial strips of the 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 (8 wells/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, on 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 on 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

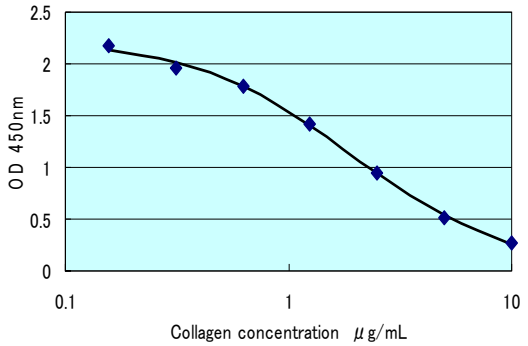
Calculate the collagen concentration in the samples using standard curve.

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×((A-Y)/(Y-D))^(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×(2.244 - 1.0)/(1.0 - 0.019)^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＝2.0×2＝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 a 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． Toubleshooting

(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.

Manufactured by **ACEL, Inc.**