ELISA PRODUCT INFORMATION & MANUAL

Human Collagen Type I ELISA Kit (Colorimetric)

NBP2-30102

Sample Insert for reference use only

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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Protocol

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Important Note!
The Protocol listed on the website should NOT be used as a final document.
Please refer to the Protocol that is supplied with the kit and specific lot number.

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content. It is naturally found exclusively in metazoa, including sponges. In muscle tissue it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscles. The gelatin used in food and industry is derived from the partial hydrolysis of collagen. Collagen type I (COL1) is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth.

Intended use:
The Human Collagen Type I ELISA Kit (Colorimetric) is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of COL1 in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

Test Principle:
The microtiter plate provided in this kit has been pre-coated with an antibody specific to COL1. Standards, control or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific to COL1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain COL1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of COL1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Kit Components:
*Microtiter Strips, 1x96wells, Pre-coated, ready to use
*Standard, 2x1vial
Standard Diluent, 1x20ml
*Detection Reagent A (green), 1x120ul
*Detection Reagent B (red), 1x120ul
Assay Diluent A, 1x12ml
Assay Diluent B, 1x12ml
TMB Substrate, 1x9ml
*Positive Control, 0.98ng/ml, 1x1vial (Lyophilized)
Stop Solution, 1x6ml
Wash Buffer, 30X, 1x20ml

Precaution:
The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Storage and Stability:
Store Microtiter strips, Standard, Detection Reagent A and B, and Positive Control at -20°C. After reconstitution of *Positive Control*, store at 4°C and use within 5 days. Store all the other components at 4°C. Unused kit is stable for 6 months after receipt. Once kit components are opened, it is highly recommended to use remaining reagents within 1 month provided this is within the expiration date of the kit. For maximum recovery of product, centrifuge the original vials after thawing and prior to removing the cap.

Materials Required But Not Supplied:
1. Microplate reader with 450 ± 10nm filter
2. Precision single or multi-channel pipettes and disposable tips
3. Eppendorf Tubes for diluting samples
4. Deionized or distilled water
5. Absorbent paper for blotting the microtiter plate
6. Container for Wash Solution

Sample Preparation and Storage:

Serum:
Allow samples to clot for two hours at RT or overnight at 4°C before centrifugation for 20 minutes at approximately 1000xg. Assay freshly prepared serum immediately or store samples in aliquots at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Plasma:
Collect plasma using EDTA or heparin as anticoagulant. Centrifuge samples for 15 minutes at 1000xg at 4°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquots at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note: Serum/plasma samples require about a 40-fold dilution. *See Sample Preparation*.

Tissue Homogenates:
The preparation of tissue homogenates will vary depending upon tissue type.
1. Rinse tissue thoroughly in ice-cold PBS (0.02M pH 7.0-7.2) to remove excess blood and weigh before homogenization.
2. Mince the tissue to small pieces and homogenize in fresh lysis buffer (1ml lysis buffer per 20-50mg tissue sample) using a glass homogenizer on ice (micro tissue grinders may also be used).
Note: Use lysis buffer composition that is appropriate for the sample; choice depends on the sub-cellular localization of the target protein.
3. Subject the resulting suspension to sonication using an ultrasonic cell disrupter until a clarified mixture is obtained.
4. Centrifuge the homogenate for 5 minutes at 10,000 x g. Collect the supernatant and assay immediately or aliquot and store at -20°C or lower.

Cell Lysates:
Cells must be lysed before assaying according to the following directions.
1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in fresh lysate buffer with concentration of 10e7 cells/ml. If necessary, the cells could be subjected to ultrasonication until solution is clarified.
4. Centrifuge at 1500xg for 10 minutes at 4°C to remove cellular debris.
5. Assay immediately or aliquot and store at -20°C or lower.

Cell Culture Supernates and Other Biological Fluids:
Centrifuge samples for 20 minutes at 1000xg. Remove particulates and assay immediately or store samples in aliquot at -20°C or -70°C. Avoid repeated freeze/thaw cycles.

Notes:
1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the results so hemolyzed specimens should not be used.
3. When performing the assay, bring samples to RT.

Reagent Preparation:
1. Bring all kit components and samples to RT before use.

2. Standard
a) Reconstitute Standard with 500ul of Standard Diluent and let stand for 10 minutes at RT; shake gently, taking care not to foam. The concentration of the standard in the stock solution is 10ng/ml.
b) Prepare 7 tubes containing 250ul Standard Diluent and produce a double dilution series. Set up 7 points of standards such as 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, 0.156ng/ml (see table below). Mix each tube thoroughly before the next transfer. The last tube (#7) is the blank tube and will contain Standard Diluent only.

Tube #	Vol. Standard	Vol. Std. Diluent	Final Concentration
Stock	10ng (lyo)	500ul	10ng/ml
1	250ul from Stock	250ul	5ng/ml
2	250ul from Tube 1	250ul	2.5ng/ml
3	250ul from Tube 2	250ul	1.25ng/ml
4	250ul from Tube 3	250ul	0.625ng/ml
5	250ul from Tube 4	250ul	0.312ng/ml
6	250ul from Tube 5	250ul	0.156ng/ml
7	0ul	250ul	0ng/ml

3. Detection Reagent A (green) and Detection Reagent B (red)

Briefly spin or centrifuge the stock Detection Reagent and Detection Reagent B before use. Dilute to the working concentration with Assay Diluent A or Assay Diluent B respectively (1:100).

4. Wash Buffer, 30X
Dilute 20ml of Wash Buffer, 30X with 580ml of ddH2O to prepare 600ml of 1X Wash Solution.

5. TMB Substrate
Aspirate the needed dosage of TMB Substrate with sterilized tips. Do not put any residual solution back into the vial.

6. Positive Control, 0.98ng/ml (Lyophilized):
Reconstitute with 150ul of Standard Diluent and let stand for 10 minutes. Mix gently before use. Use 100ul in appropriate well of plate and test concentration along with standards and samples. Store at 4°C for up to 5 days.

Notes:
1. Do NOT perform the serial dilution directly in the wells.
2. Prepare standard within 15 minutes before assay. Do not dissolve the reagents at 37°C directly.
3. Detection Reagents A and B are sticky solutions. Pipet slowly to reduce volume errors.
4. Carefully reconstitute Standards, Positive Control and dilute working Detection Reagents A and B according to the instructions. Avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipeting, use small volumes and ensure that pipetors are calibrated. It is recommended to aspirate more than 10ul for one pipeting.
5. The reconstituted Standards, Positive Control, and working Detection Reagents A and B can be used only once.
6. If crystals have formed in the 30X Wash Buffer, warm to RT and mix gently until the crystals are completely dissolved.
7. Contaminated water or containers for reagent preparation will influence the detection results.

Sample Preparation:
1. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. Serum/plasma samples require about a 40-fold dilution. A suggested 40-fold dilution is 10ul sample + 390ul PBS. Samples can be diluted by 0.01M PBS (pH 7.0-7.2).
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
6. Cell culture supernatant samples may not be detected by the kit because of factors including cell viability, cell number or sampling time.
7. Fresh samples that have not been stored for a long time are recommended for the test; otherwise, protein degradation and denaturation may occur and lead to incorrect results.

Assay Procedure:
1. Determine the number of wells required for standards, blank, control and samples. Add 100ul each of standards (see Reagent Preparation), blank, control and samples into the appropriate wells. Cover with the plate sealer. Incubate for 1 hour at 37°C.
2. Remove the liquid of each well; do not wash.
3. Add 100ul of Detection Reagent A working solution to each well. Incubate for 1 hour at 37°C after covering it with the plate sealer.
4. Aspirate the solution and wash each well for 1-2 minutes with 350ul of 1x Wash Solution using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Wash for a total of 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100ul of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the plate sealer.
6. Repeat the aspiration/wash process 5 times as conducted in step 4.
7. Add 90ul of TMB Substrate to each well. Cover with a new plate sealer. Incubate for 10-20 minutes at 37°C (Do not exceed 30 minutes). Protect from light. Addition of Substrate Solution will turn the liquid blue.
8. Add 50ul of Stop Solution to each well. The liquid will turn yellow on addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Read the absorbance at 450nm immediately.

Notes:
1. *Assay preparation:* Keep appropriate numbers of wells for 1 experiment and remove the extra wells from the microtiter plate. These extra wells should be resealed and stored at -20°C.
2. *Samples or reagents addition:* Use freshly prepared Standard. Carefully add samples to wells and mix gently to avoid

foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separate reservoirs for each reagent.
3. *Incubation:* To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. *Washing:* The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. *Controlling of reaction time:* Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), if the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. Protect it from light.
7. An environment humidity less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used under this condition.

Calculations:
Average the duplicate readings for each standard and samples and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with COL1 concentration on the Y-axis and absorbance on the X-axis. Use of plotting software is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical Data:
In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. The O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects); plotting log of the data to establish standard curve for each test is recommended. A typical standard curve is provided below and is shown for reference only (user must generate their own standard curve with every assay).

Detection Range:
0.156-10ng/ml. The standard curve concentrations used for ELISA were 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, 0.156ng/ml

Sensitivity:
The minimum detectable dose of human COL1 is typically less than 0.055ng/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD), is defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Specificity:
This assay has high sensitivity and excellent specificity for detection of COL1. No significant cross-reactivity or interference between human COL1 and analogues was observed.
Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between human COL1 and all the analogues; therefore, cross-reaction may still exist.

Recovery:
Matrices listed below were spiked with certain level of recombinant COL1 and the recovery rates were calculated by comparing the measured value to the expected amount of COL1 in samples.

Matrix	Recovery Range (%)	Average (%)
Serum (n=5)	87-96	91
EDTA plasma (n=5)	84-104	96
Heparin plasma (n=5)	82-101	93

Linearity:
The linearity of the kit was assayed by testing samples spiked with appropriate concentration of COL1 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16

Serum (n=5)	82-103%	98-109%	79-90%	87-97%
EDTA plasma (n=5)	79-91%	90-99%	86-96%	78-101%
Heparin plasma (n=5)	80-99%	81-104%	90-103%	83-94%

Precision:
Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level COL1 were tested 20 times on one plate, respectively.

Intra-Assay: CV<10%

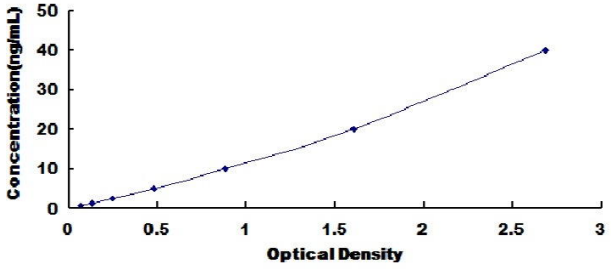
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level COL1 were tested on 3 different plates, 8 replicates in each plate.

Inter-Assay: CV<12%

CV(%) = (SD/mean) X 100

Assay Procedure Summary:
1. Prepare all reagents, samples and standards.
2. Add 100ul standard or sample to each well. Incubate 1 hour at 37°C.
3. Aspirate and add 100ul prepared Detection Reagent A. Incubate 1 hour at 37°C.
4. Aspirate and wash 3 times.
5. Add 100ul prepared Detection Reagent B. Incubate 30 minutes at 37°C.
6. Aspirate and wash 5 times.
7. Add 90ul TMB Substrate. Incubate 10-20 minutes at 37°C.
8. Add 50ul Stop Solution. Read at 450nm immediately.

Images



Typical standard curve for Human Collagen Type I ELISA Kit (Colorimetric) (shown for reference only; user must generate their own standard curve with each assay).

References