

Quantikine™ ELISA

Human Pro-Collagen I α 1/COLIA1

Catalog Number DPCA00

For the quantitative determination of human Pro-Collagen I alpha 1 (Pro-Collagen I α 1) concentrations in cell culture supernates, cell lysates, tissue extracts, serum, plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Type I collagen is the most abundant structural protein of connective tissues such as skin, bone and tendon. It is synthesized as a pro-collagen molecule which is characterized by a 300 nm triple helical domain flanked by globular N- and C-terminal propeptides (1). The triple helical domain contains Gly-Xaa-Yaa triplets where Xaa and Yaa are frequently proline and hydroxyproline, respectively. The non-helical propeptides are removed by pro-collagen N- and C-proteinase activities so that the mature triple helices can self-assemble into collagen fibrils that provide tensile strength to tissues (1). Type I collagen is a heterotrimer that consists of two alpha 1(I) chains and one alpha 2(I) chain, although homotrimers consisting of three identical alpha 1(I) chains have also been described (2).

The Quantikine™ Human Pro-Collagen I alpha 1/COLIA1 (Pro-Collagen I α 1) Immunoassay is a 3.5 hour solid-phase ELISA designed to measure human Pro-Collagen I α 1 in cell culture supernates, cell lysates, tissue extracts, serum, plasma, saliva, and urine. It contains recombinant human Pro-Collagen I α 1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Pro-Collagen I α 1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Pro-Collagen I α 1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Pro-Collagen I α 1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Pro-Collagen I α 1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Pro-Collagen I α 1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Pro-Collagen I α 1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

Pro-Collagen I α 1 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Pro-Collagen I α1 Microplate	899350	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Pro-Collagen I α1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Pro-Collagen I α1 Standard	899352	2 vials of recombinant human Pro-Collagen I α1 in a buffered protein base with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	Use a new standard for each assay. Discard after use.
Human Pro-Collagen I α1 Conjugate	899351	21 mL of a monoclonal antibody specific for human Pro-Collagen I α1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26	895525	21 mL of a concentrated buffered protein base with preservatives. <i>Use diluted 1:4 in this assay.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples
- Human Pro-Collagen I α1 Controls (optional; R&D Systems®, Catalog # QC299)

If using cell lysate samples, the following is also recommended:

- Lysis Buffer 17 (R&D Systems, Catalog # 895943)

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

Tissue Extracts - Preparation of tissue extracts will vary depending on the tissue type.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 50-fold dilution, due to high endogenous levels. A suggested 50-fold dilution is 10 μ L of sample + 490 μ L of Calibrator Diluent RD5-26 (diluted 1:4)*.

Saliva samples require a 5-fold dilution, due to high endogenous levels. A suggested 5-fold dilution is 40 μ L of sample + 160 μ L of Calibrator Diluent RD5-26 (diluted 1:4)*.

Urine, cell culture supernate, cell lysate, and tissue extract samples may require dilution due to high endogenous levels.

Multiple dilutions are recommended for unknown samples.

For cell lysate samples, quantitation of protein sample concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 0.1-0.4 μ g/well.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: High concentrations of Pro-Collagen I α 1 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

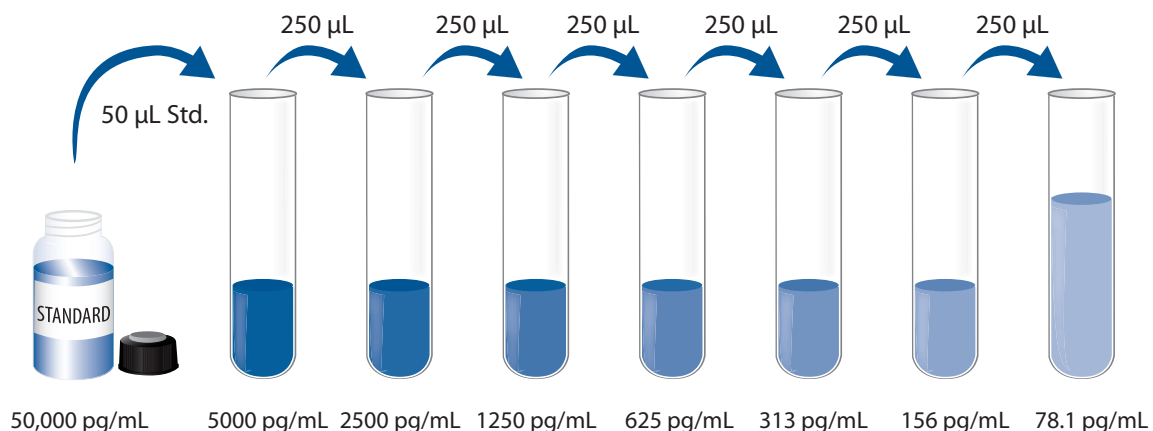
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 10 mL of Calibrator Diluent RD5-26 to 30 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Human Pro-Collagen I α 1 Standard - Refer to the vial label for reconstitution volume.

Reconstitute the Human Pro-Collagen I α 1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 450 μ L of Calibrator Diluent RD5-26 (diluted 1:4) into the 5000 pg/mL tube. Pipette 250 μ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μL of Assay Diluent RD1W to each well.
4. Add 50 μL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μL of Human Pro-Collagen I $\alpha 1$ Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

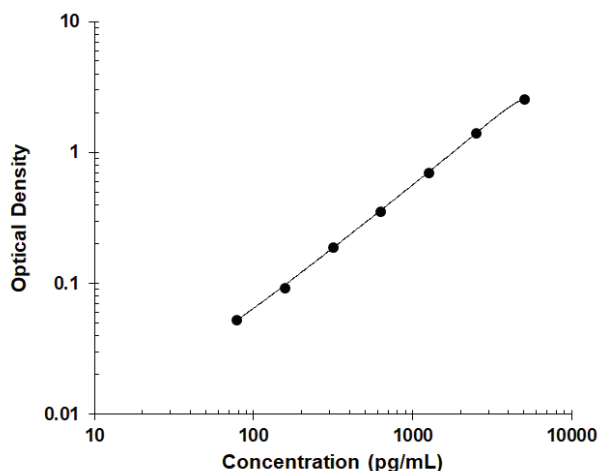
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Pro-Collagen I α 1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.011 0.011	0.011	—
78.1	0.049 0.057	0.053	0.042
156	0.084 0.102	0.093	0.082
313	0.180 0.199	0.190	0.179
625	0.337 0.376	0.357	0.346
1250	0.689 0.721	0.705	0.694
2500	1.401 1.411	1.406	1.395
5000	2.503 2.611	2.557	2.546

PRECISION

Intra-Assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	442	1410	2669	448	1378	2677
Standard deviation	17.8	32.4	50.1	28.9	49.8	109
CV (%)	4.0	2.3	1.9	6.4	3.6	4.1

RECOVERY

The recovery of human Pro-Collagen I $\alpha 1$ spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Media (n=4)	107	97-118%
Lysis buffer (n=1)	97	94-101%
Extraction buffer (n=1)	105	101-110%
Saliva (n=4)	97	87-114%
Urine (n=4)	90	79-111%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Pro-Collagen I $\alpha 1$ were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

Samples		Cell culture supernates (n=8)	Cell lysates (n=3)	Tissue extracts (n=2)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	94	95	101	103	104	104	98	100
	Range (%)	86-101	93-96	101-101	98-105	102-105	102-107	96-100	98-104
1:4	Average % of Expected	97	99	104	105	107	108	103	105
	Range (%)	88-105	92-104	100-109	102-106	106-110	104-113	101-106	96-112
1:8	Average % of Expected	104	103	111	107	105	107	107	106
	Range (%)	91-116	91-114	98-124	105-109	102-109	104-111	99-113	89-121
1:16	Average % of Expected	102	105	110	99	99	101	104	113
	Range (%)	92-110	91-121	96-124	86-109	92-106	90-112	98-115	112-113

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human Pro-Collagen I α 1 ranged from 1.74-8.75 pg/mL. The mean MDD was 3.51 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified recombinant human Pro-Collagen I α 1 manufactured at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Pro-Collagen I α 1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=30)	95,355	53,905-179,132	32,684
EDTA plasma (n=30)	91,272	50,544-143,534	28,421
Heparin plasma (n=30)	95,599	54,640-176,613	32,389
Saliva (n=10)	4031	877-8443	3010
Urine (n=10)	945	97-3982	1429

Cell Culture Supernates:

Cardiomyocytes were generated from pluripotent stem cells using the StemXVivo™ Cardiomyocyte Differentiation Kit (R&D Systems®, Catalog # SC032B). After 12 days of differentiation, cells were maintained in Cardiomyocyte Differentiation Base Media II. Cells were left unstimulated or stimulated with 1 μ g/mL LPS for 24 hours. An aliquot of the cell culture supernate was removed, assayed for human Pro-Collagen I α 1, and measured.

Condition	(pg/mL)
Unstimulated	13,092
Stimulated	16,196

Cell Lysates - IMR-90 human fetal lung fibroblast cells were grown in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate until they reached confluency. Cells were dislodged and then washed with PBS before solubilizing the cells in Lysis Buffer 17 (R&D Systems®, Catalog # 895943) using 3-5X the cell pellet volume. An aliquot of the cell lysates was removed, assayed for human Pro-Collagen I α 1, and measured at 4923 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Pro-Collagen I α 1.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Pro-Collagen I α 1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

BMP-1
COL-13A1
COL-15A1
COL-18A1
COL-23A1
COL-25A1
COL2A1
COL-3A1
COL-4A1
MFAP-4
MRC-2

REFERENCES

1. Canty, E.G. *et al.* (2005) *J. Cell Sci.* **118**:1341.
2. Han, S. *et al.* (2008) *J. Mol. Biol.* **383**:122.

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