# Collagen IV H

An immunoassay for the quantitative determination of human type IV collagen

Dilute Standards and Samples

Add Standards and Samples to Plate

Add Anti-Collagen IV Antibody

Incubate Overnight

Wash Plate

Add Conjugate

Incubate for 60 Minutes

Wash Plate

Develop for 5-10 Minutes

Add Color Stopper

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**Collagen IV H:** A quantitative assay for the determination of human type IV collagen in biological samples origin.

**Technical Background:** Collagen IV is a structural component of basement membranes and other extracellular matrices, and is elaborated in cell culture by various cell types. Collagen IV H is designed to measure human collagen IV in tissue culture samples or biological specimens.

This is a competitive indirect ELISA. To complete the assay, samples containing collagen IV and rabbit anti-collagen IV are added to wells that are pre-coated with (human) collagen IV. The antibody binds either to the collagen IV in the soluble phase or to that of the solid phase, hence the notion of competition. A wash step eliminates reactants in the soluble phase, and an anti-rabbit IgG HRP antibody conjugate is subsequently added. This conjugate binds to the rabbit antibody that is bound to the solid phase. A wash step removes unreacted conjugate and a chromogenic substrate for HRP (horseradish peroxidase) is then added. After a short incubation period, the color development is stopped with acid, and absorbances in the wells are determined at 450 nm using a microtiter plate reader. Because the assay is a competitive one, color intensity is inversely proportional to the logarithm of collagen IV concentration of the sample (in the soluble phase).

The sensitivity and precision of the assay are dependent upon the duration of the primary incubation period. Using an incubation of at least an hour will lead to a usable assay that will measure down to approximately 0.156 ug collagen IV/mL. However, increasing the incubation of specimen and rabbit anti-collagen IV Ab to 24 hours will improve the slope of the dose response, and will increase sensitivity down to 0.078 ug/mL or better.

**Specimen Collection and Storage:** Samples should be collected and frozen/stored at ≤ -50°C (without preservative) until they are analyzed. Assay results are not affected by common extraction reagents or protease inhibitors. They should come to room temperature immediately before analysis. A room temperature waterbath is especially useful for this step. Samples should be vortexed thoroughly after thawing. They should be allowed to settle for an hour, and aliquots for analysis should be drawn from the top of the fluid column to preclude introduction of particulates into the assay plate

**Kit Contents:** Your Collagen IV H kit should contain the following items:

a. 1 Collagen IV H Test Plate
b. 2 NHEBSA (Diluent): 12 mL
c. 1 Human Collagen IV Standard: 0.5 mL
d. 1 Rabbit Anti- Collagen IV Antibody: 6 mL
e. 1 Goat Anti-rabbit IgG HRP Conjugate: 12 mL
f. 1 Color Developer: 12 mL
g. 1 Color Stopper: 12 mL
h. Instruction manual

Human Collagen IV Standard, NHEBSA, Rabbit anti-Collagen IV Antibody and Anti-rabbit IgG HRP Conjugate preparations contain 0.05% Proclin 300 (active components isothiazolones) as preservative. Color Stopper contains dilute (2.0 N) sulfuric acid

All kit reagents are supplied in ready to use liquid form. The collagen IV H plates are precoated and ready to use. A wash buffer must be supplied by the user. The kit is subjected to QC analysis using Exocell EIA Wash Buffer with composition: 0.15 M NaCl, 0.01 M triethanolamine (pH 6.8), 0.05% Tween 20 and 0.05 % Proclin 300 (preservative may be omitted if the buffer is freshly prepared). This buffer is recommended.

The wash buffer must ***not*** contain azide.

Adjustable pipettors are required that are capable of delivering volumes over the range of 10-1000 ul. Multi-channel pipettors capable of delivering 50 and 100 uL are recommended. Finally, a microplate reader equipped to determine absorbance at 450 nm is required.

Dilutions for the assay are prepared in tubes, and 1.5 mL microfuge tubes are suitable for this purpose.

**Assay Procedure:** Allow reagents and samples to come to room temperature before running the assay.

**Standard Dilutions:** This procedure is written to allow the construction of the standard curve run in duplicate wells.

1. Prepare 12 microfuge tubes with 200 uL of NHEBSA per tube.
2. Label the tubes C, 1-11.
3. Transfer 200 uL of Human Collagen IV Standard to tube 1.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 200 uL of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube 11.
8. Tubes 1-11 now contain two-fold, serially diluted human collagen IV covering the concentration range 10-0.009 ug/mL.

**Preparation of Sample Dilutions:** Accurate determination of human collagen IV concentration depends upon proper sample dilution. That said; often tissue culture samples or urine specimens may be analyzed “undilute.”

**Addition of Controls, Standard Collagen IV Dilutions and Samples to the plate:**

This is a suggested experimental design.

1. Label the strips A-H (or 1-12) with an indelible marker.
2. Use a plate washer or wash plates by hand as follows:
   a. Remove fluids from the well, ie. aspirate off fluids or flip them out into a sink.
   b. Fill wells to over-flowing with the recommended wash buffer.
   c. Remove fluids as before.

d. "b" and "c" constitute a wash cycle.

e. Repeat the process to yield a total of 10 wash cycles.

f. Invert the plate on a paper towel and tap gently to remove adherent fluids.

3. Add a 100 uL aliquot of NHEBSA from tube C to well A1. This is the negative control "C0" and will be used to standardize or "blank" the microplate reader.

4. Add a 50 uL aliquot of NHEBSA from tube C to well B1. This is the positive control “C1,” and serves as a qualitative indicator of assay performance.

5. With a fresh tip, pre-wet and transfer 50 uL aliquots of standard dilution tube 11 to wells A12 and B12.

6. With the same tip, pre-wet and transfer 50 uL aliquots from standard dilution tube 10 to wells A11 and B11.

7. Continue adding aliquots of standard dilutions in this fashion.

8. In a similar manner, add 50 uL aliquots of sample to wells C1 and D1.

9. Using a fresh tip, pre-wet and transfer 50 uL aliquots from the next sample to wells C2 and D2.

10. Continue adding diluted samples to the plate in this fashion.

**Primary Incubation:** Reaction with Rabbit Anti-Collagen IV Antibody:

1. Add 50 uL of rabbit Anti-Collagen IV Antibody to Wells A2-A12, and B1-H12 (i.e. antibody is omitted from well A1, all others get primary antibody)

2. Incubate the plate in a humid atmosphere at room temperature. A food storage container (with cover) with a water moistened paper towel placed in the bottom works well as an incubation chamber.

3. Incubate at least 60 minutes. ***Overnight incubation will improve sensitivity.***

**Secondary Incubation:** Reaction with Anti-rabbit IgG HRP Conjugate:

1. Wash the plate as described.

2. Add 100 uL of goat Anti-rabbit IgG HRP Conjugate to each well on the plate.

3. Incubate for 60 minutes.

**Color Development:**

1. Wash the plates as described.

2. Add 100 uL Color Developer to each well.

3. Develop 5- 10 minutes

4. Add 100 uL Color Stopper to each well.

5. Determine absorbance at 450 using C0, in well A1, as a blank.

**Analysis:**

1. Prepare a spreadsheet using a suitable software package.

   a. Lable the first column “Sample” and enter the name i.e. standard number or sample number.

   b. Label the second column “1/Dilution” and enter reciprocal dilution for samples in the second column; if the samples were analyzed undiluted, enter “1.” If they were diluted 1:2, enter “2,” and so forth.

   c. Label the third column “[ ]” (concentration), and enter the concentrations for the standard dilutions.

   d. Label the fourth column “Log [ ],” and use the log function to calculate the log of the concentrations indicated in the third column.

   e. Label the fourth and fifth columns OD1 and OD2 (OD=Optical density or absorbance) respectively. Enter in the measured absorbances in these columns.

   f. Label the sixth column “Mean OD,” and use the appropriate function to calculate the average OD entered in this column.

   g. Label the seventh column “est [ ],” for estimated concentration.

   h. Label the eight column “undilute [ ],” to account for dilution.

   i. Label the ninth column “Comment.”

2. Use the graphing feature to prepare a graph of the log [ ] vs average OD (absorption). These will be plotted on linear axes, but it is a semi-logarithmic plot.

   a. A line may be drawn through many of the values for the standard curve.

   b. Determine the dilutions which do not fall on this line; these are “OR” (Out-of-Range).

   c. Return to the spreadsheet and enter “OR” in the comments column for OD values that are not on the line.

3. The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.

4. Subject these data to semi-logarithmic analysis to yield a mathematical model, of the form

$$\log_{10}[\text{Human collagen IV}] = m\,A_{450} + b$$

A linear regression model relating log [ ] to absorbance may be used to advantage, but remember you will calculate the log of concentration so must take an anti-log for the final value.

5. Determine the estimated [Human collagen IV] for each experimental sample.

6. Multiply by reciprocal dilution for undiluted concentration.

**Records Keeping:** It is good laboratory practice to record the lot numbers and dates of the kit components and reagents for each assay.

**Sample Handling:** The samples should be secured, processed and stored as discussed above. Experimental samples often contain particulate matter that presents potential sources for error. Centrifugation to clarify samples is recommended.

Dilute Standard and Samples carefully. For the standards, a single tip may be used to prepare the dilution series. For the experimental samples, a fresh tip should be used for each specimen.

**Limitations:** It is the responsibility of the investigator to determine if the presence of experimental compounds or their metabolites in the samples will affect the assay results.

Gross microbiological contamination may affect assay results.

**Trouble Shooting:**

1. No color appears after adding Color Developer: One or more reagents may have been adversly affected by storage above 8°C. One or more reagents may not have been added. Repeat assay. Be sure to store the kit appropriately.

2. Color in wells too light: Longer incubation with Color Developer may be required. If the color is still too light after 20 minutes development, contact Exocell.

3. Color in wells is too dark: Decrease the development time. If a 5 minute development is still too dark, repeat the assay and reduce the secondary incubation to 30 minutes.

   If color is dark and the standard dilutions fail to show the appropriate dose-response, Color Developer may have been contaminated with conjugate or the plate was poorly washed. Repeat the assay and take care in the pipetting and in the washing operations.

4. Color in sample well(s) is darker or lighter than lowest or highest concentrations of the standard curve. Change sample dilution protocol appropriately.

5. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.

6. Microplate ELISAs may be prone to edge effects wherein the outer rows and columns show a darker response than the inner ones. This effect may be minimized by incubating the plate in a closed humid container. A plastic food storage container with a tight fitting lid and a water moistened paper towel work well in this respect. Place the moistened towel in the bottom of the container, and place the plate upon it. Position the cover and incubate as described.