Collagen IV

An immunoassay for the quantitative determination of murine collagen type IV

Dilute Standards and Samples

Wash Plates

Add Standards and Samples to plates

Add Anti Collagen IV Antibody

Incubate for 60 Minutes

Wash Plates

Add Conjugate

Incubate for 60 Minutes

Wash Plate

Develop for 5-10 Minutes

Add Color Stopper

Less than 3 hours to complete

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Collagen IV M: A quantitative assay for the determination of type IV collagen in biological samples of murine origin.

Technical Background: Collagen IV M is a structural component of basement membranes and other extracellular maticies, and is elaborated in cell culture by various cell types. Collagen IV M is designed to measure collagen IV in tissue or cell culture specimens of murine origin.

Collagen IV M is a competitive indirect ELISA. To complete the assay, samples containing collagen IV and rabbit anti-murine collagen IV are added to wells that are pre-coated with mouse 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 mouse collagen IV concentration of the sample.

An alternative procedure may be used to enhance assay sensitivity. Here, the sample and the anti-murine collagen IV antibody are placed in a microfuge tube and are allowed to react. Thus, an initial non-competitive reaction is allowed to occur. Subsequently, an aliquot is transferred to the plate, and allowed to react in the “competitive mode”. From this point, the assay follows the standard procedure.

A 1.5 hour pre-incubation with the Alternate Procedure typically increases sensitivity 10-20 fold. Longer pre-incubation periods, i.e. overnight, can further enhance sensitivity.

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.

Do not apply heat to the samples.

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

a. 1 Collagen IV M Test Plate
b. 2 EIA Diluent: 15 mL
c. 1 Murine Collagen IV Standard: 1 mL
d. 1 Rabbit Anti-murine Collagen IV Antibody: 15 mL
e. 1 Goat Anti-rabbit IgG HRP Conjugate: 12 mL
f. 1 Color Developer
g. 1 Color Stopper
h. Instruction manual

Murine Collagen IV Standard, EIA Diluent, Rabbit anti-murine 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. Save all unused reagents for future assays.

All kit reagents are supplied in ready to use liquid form. The collagen IV M plates are precoated and ready to use. A wash buffer must be supplied by the user. An 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) 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. A multi-channel pipettor capable of delivering 100 uL is recommended. Finally, a microplate reader equipped to determine absorbance at 450 nm is required.

1.5 mL microfuge tubes may be used for preparing serial dilutions of the standard, and for use as pre-incubation vessels in the enhanced sensitivity procedure.

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 7 microfuge tubes with 240 uL of EIA Diluent per tube.
2. Label the tubes C1, 1-6.
3. Transfer 240 uL of Murine Collagen IV Standard to tube 1.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 240 uL of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube 6.
8. Tubes 1-6 now contain dilutions of 10.0, 5.0, 2.5, 1.25, 0.625, and 0.313 µg/mL respectively.

Preparation of Sample Dilutions: Accurate determination of murine collagen IV concentration depends upon proper sample preparation. For initial studies,

examine experimental samples undilute and 1:2 in EIA Diluent in the assay. The results obtained from initial studies may be used to guide subsequent ones. An example of sample dilution is given below for clarity.

To analyze a tissue culture extract at 1:2, in duplicate, transfer 120 µL EIA Diluent to a microfuge tube. Add 120 µL of tissue culture extract to the tube. Mix contents by aspiration/expulsion as above. This sample has sufficient volume to remove 2 x 100 µL aliquots of 1:2 dilution for analysis.

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

1. 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.
2. Add 200 uL aliquots of EIA Diluent from the stock bottle to wells A1 and A2. These are the negative controls "C0" and will be used to standardize or "blank" the microplate reader.
3. Add 100 uL aliquots from C1 tube to wells B1 and B2. These are positive controls "C1" and are qualitative indicators of assay performance.
4. With a fresh tip, transfer 100 uL aliquots of Tube 6 dilution to wells H1 and H2.

5. With the same tip, transfer 100 uL aliquots from tube 5 to wells G1 and G2.
6. Continue adding standard dilution in this fashion.
7. In a similar manner, add 100 uL aliquots of diluted sample to wells A3 and A4.
8. Continue adding diluted samples to the plate.
9. The plate now contains controls and standard dilutions in wells A-H, 1,2, and diluted experimental samples in duplicate over the balance of the plate.

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

1. Add 100 uL of rabbit Anti-murine Collagen IV Antibody to Wells A3-A12, and B-H 1-12.
2. Incubate the plate covered for 60 minutes at room temperature.

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 as before 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.

Alternate Assay Procedure:

The "Alternate Assay Procedure" increases the sensitivity of the assay. Table 1 provides examples of the range of sensitivities that

can be achieved with the pre-incubation procedure.

Table 1

Pre-Incubation	Dose-Response
None (Standard Method)	0.156-10 ug/mL
1.5 Hours	0.039-2.5 ug/mL
Overnight (21 Hours)	0.0024-0.156 ug/mL

This table is included as a general guide for the construction of a dose-response curve, actual results may vary.

An example of the alternate procedure using a 1.5 hour pre-incubation is included for clarity. The general design will follow that for the normal method, i. e. standards and samples are treated for analysis in duplicate wells.

1. Complete dilutions of standards as follows:
   a. Prepare nine centrifuge tubes as summarized in Table 2

Table 2

Tube Label	EIA Diluent Volume
C0	480 uL
C1	240 uL
2.5 ug/mL	420 uL
1.25 ug/mL	420 uL
0.625 ug/mL	"
0.313 ug/mL	"
0.156 ug/mL	"
0.078 ug/mL	"
0.039 ug/mL	"

   b. Transfer 60 uL of murine collagen IV standard (20 ug/mL) to the "2.5" tube. Vortex briefly. This tube now contains 2.5 ug collagen IV / mL
   c. Using a fresh pipet tip, transfer 240 uL from the "2.5" tube to the "1.25" tube, and mix thoroughly.
   d. Continue serial dilution through the last tube (0.039ug/mL).
   e. After preparing the last dilution, remove 240 uL so that all tubes C1, 0.039-2.5 ug/mL contain the same volume, 240 uL. The negative control has 480 uL.
2. Prepare sample tubes with 240 uL volumes of sample
3. Add 240 uL volumes of rabbit anti-murine collagen IV to tubes C1, 2.5-0.039, and all sample tubes. C0 is a negative control and receives no primary antibody.
4. Incubate the tubes at room temperature for 1.5 hours.
5. Wash the plate as described.
6. Transfer 200 uL volumes from each tube to respective wells on the plate, thus:
   a. 200 uL volumes from C0 to each well A1 and A2.
   b. 200 uL from C1 to each well B1 and B2
   c. And so on.
7. Incubate the plate for 1 hour.
8. Wash the plate as described.
9. Add 100 uL of goat anti-rabbit Ig G HRP conjugate/well.
10. Incubate for 1 hour.
11. Wash Plate

12. Add 100 uL TMB/well, and allow color to develop for 5-10 minutes.

13. Stop development by adding 100 uL Acid Stopper/well.

14. Determine absorbance at 450 nm after “blanking” the plate reader against well A1.

Analysis:

1. Determine the Absorbance of experimental wells at 450 nm blanked against well A1.
2. Calculate the average absorbances and enter the values in a photocopy of the enclosed worksheet.
3. Prepare a semi-logarithmic plot of standard dilutions with the log [Murine collagen IV] vs absorbance.
4. The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.
5. Subject these data to semi-logarithmic analysis to yield a mathematical model, of the form

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

6. Determine the estimated [Murine collagen IV] for each experimental sample.
7. Multiply appropriately for the dilution.

Quality Control:

Record 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. The tip should be used dry, hence not pre-wetted by sample, and washed out in the EIA Diluent by repeated aspiration and expellation.

Limitations:

1. 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.

2. 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 8ºC. 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.

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