



User's Manual

Hydroxyproline ELISA Kit

REF DEIA3605

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

For the quantitative measurement of Hydroxyproline in serum, plasma, tissue homogenates, cell culture supernatants and other biological fluids.

General Description

Hydroxyproline is a major component of the protein collagen. Hydroxyproline and proline play key roles for collagen stability. They permit the sharp twisting of the collagen helix. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen and/or gelatin amount. Hydroxyproline is produced by hydroxylation of the amino acid proline by the enzyme prolyl hydroxylase following protein synthesis (as a post-translational modification). The enzyme catalysed reaction takes place in the lumen of the endoplasmic reticulum. Although it is not directly incorporated into proteins, hydroxyproline comprises roughly 4% of all amino acids found in animal tissue, more than seven amino acids which are directly incorporated.

Principles of Testing

Hydroxyproline ELISA Kit is based on a competitive enzyme immunoassay technique. The microtiter well-plate in this kit has been pre-coated with an anti-Hydroxyproline antibody. Sample or standards are added to the wells along with a fixed quantity of biotinylated Hydroxyproline and incubated. The Hydroxyproline found in the sample or standards competes with the biotinylated Hydroxyproline for limited binding sites on the immobilized anti-Hydroxyproline antibody. Excess unbound biotinylated Hydroxyproline and sample or standard Hydroxyproline is washed from the plate. Avidin-HRP conjugate is added, incubated and washed. An enzymatic reaction is then produced through the addition of TMB substrate which is catalyzed by the immobilized HRP to generate a blue color product that changes to yellow after adding acidic stop solution. The density of yellow coloration is measured by reading the absorbance at 450 nm which is quantitatively proportional to the amount of biotinylated Hydroxyproline captured in the well and inversely proportional to the amount of Hydroxyproline which was contained in the sample or standard.

Reagents And Materials Provided

1. Anti-Hydroxyproline Microplate 96 Wells, (12 x 8 Well strips)
2. Hydroxyproline Lyophilized Standard, 2 x 8,000 nmol
3. Sample Diluent, 1 x 20 mL
4. 100X Hydroxyproline-Biotin Complex, 1 x 60 µL
5. 100X Avidin-HRP Conjugate, 1 x 120 µL
6. Biotin Complex Diluent, 1 x 12 mL
7. Conjugate Diluent, 1 x 12 mL

Storage Conditions: -20°C for 6 months

8. 25X Wash Buffer, 1 x 30 mL

9. TMB Substrate, 1 x 10 mL
10. Stop Solution, 1 x 10 mL

Storage Conditions: 4°C for 6 months

Materials Required But Not Supplied

1. Microplate reader capable of reading absorbance at 450 nm.
2. Automated plate washer (optional).
3. Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
4. Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
5. New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
6. Absorbent paper or paper toweling.
7. Distilled or deionized ultrapure water.
8. 37°C Incubator (optional)

Storage

Open kit immediately upon receipt. Store components at -20°C or 4°C for 6 months or until expiration date. Avoid any freeze/thaw cycles.

Specimen Collection And Preparation

Sample Preparation and Storage

1. Store samples to be assayed at 4°C for 24 hours prior being assayed.
2. For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
3. Samples not indicated in the manual must be tested to determine if the kit is valid.
4. Prepare samples as follows:

Serum - Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1,000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. Rinse the tissue with 1X PBS to remove excess blood then homogenized in 20 mL of 1X PBS and store overnight at ≤ -20°C. Perform two freeze-thaw cycles to break the cell membranes then centrifuge homogenates 5 minutes at 5,000 x g. Remove the supernatant and assay immediately or aliquot and store at ≤ -20°C.

Cell Culture Supernatants and other biological fluids - Remove particulates by centrifugation for 15 minutes at 1,000 x g, 2- 8°C and assay immediately or aliquot and store samples at -20°C or -80°C.

Avoid repeated freeze-thaw cycles.

Sample Dilution

Target protein concentration must be estimated and appropriate sample dilution selected such that the final target protein concentration falls near the middle of the assay linear dynamic range.

1. Dilute samples using Sample Diluent.
2. Mix diluted samples gently and thoroughly.
3. Pipetting less than 2 μ L is not recommended for optimal assay accuracy.
4. Optimal dilution must be determined by the user according to their specific samples.

Reagent Preparation

Equilibrate all materials to room temperature prior to use and use immediately.

Hydroxyproline Assay Standards

1. Prepare the Hydroxyproline standards no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.
2. Reconstitute one vial of the provided 8,000 nmol Lyophilized Hydroxyproline Standard for each experiment. Prepare the stock 4,000 nmol/L Standard by reconstituting one tube of Lyophilized Hydroxyproline Standard as follows:

Gently spin or tap the vial at 6,000 – 10,000 rpm for 30 seconds to collect all material at the bottom.

Add 2 mL of Sample Diluent to the vial.

Seal the vial then mix gently and thoroughly.

Leave the vial at ambient temperature for 15 minutes.

3. Prepare a set of seven serially diluted standards as follows:

Label tubes with numbers 2 – 8.

Use the reconstituted 4,000 nmol/L Hydroxyproline Standard as the high standard point (Tube #1).

Add 300 μ L of Sample Diluent to Tube #'s 2 – 8.

Prepare Standard #2 by adding 300 μ L of 4,000 pg/mL Hydroxyproline (Tube #1) to Tube #2. Mix gently and thoroughly.

Prepare Standard #3 by adding 300 μ L of Standard #2 from Tube #2 to Tube #3. Mix gently and thoroughly.

Prepare further serial dilutions through Tube #7. Reference the table below as a guide for serial dilution scheme.

Tube #8 is a blank standard (only Sample Diluent), which should be included with every experiment.

Standard Number (Tube)	Standard To Dilute	Volume Standard to Dilute (µL)	Volume Sample Diluent (µL)	Total Volume (µL)	Final Concentration
1	8,000 nmol/L Reconstituted Hydroxyproline Standard	NA	2,000	2,000	4,000 nmol/L
2	4,000 nmol/L	300	300	600	2,000 nmol/L
3	2,000 nmol/L	300	300	600	1,000 nmol/L
4	1,000 nmol/L	300	300	600	500 nmol/L
5	500 nmol/L	300	300	600	250 nmol/L
6	250 nmol/L	300	300	600	125 nmol/L
7	125 nmol/L	300	300	600	62.5 nmol/L
8	NA	0	300	300	0.0 (Blank)



1X Hydroxyproline-Biotin Complex

1. Prepare the 1X Hydroxyproline-Biotin Complex immediately prior to use by diluting the 100X Hydroxyproline-Biotin Complex 1:100 with Complex Diluent.
2. For each well strip to be used in the experiment (8-wells) prepare 500 µL by adding 5 µL of 100X Hydroxyproline-Biotin Complex to 495 µL Complex Diluent.
3. Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

1X Avidin-HRP Conjugate

1. Prepare the 1X Avidin-HRP Conjugate immediately prior to use by diluting the 100X Avidin-HRP Conjugate 1:100 with Conjugate Diluent as follows.
2. Briefly and gently mix the 100X Avidin-HRP Conjugate prior to pipetting.
3. For each well strip to be used in the experiment (8-wells) prepare 1,000 µL 1X Avidin-HRP Conjugate by adding 10 µL of 100X Avidin-HRP Conjugate to 990 µL Conjugate Diluent.
4. Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

Microplate Preparation

1. Micro-plates are provided ready to use and do not require rinsing or blocking.
2. Unused well strips should be returned to the original packaging, sealed and stored at 4°C.
3. Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

1X Wash Buffer

1. If crystals have formed in the 25X Wash Buffer concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.
2. Add the entire 30 mL contents of the 25X Wash Buffer bottle to 720 mL of ultra-pure water to a clean > 1,000 mL bottle or other vessel.

3. Seal and mix gently by inversion. Avoid foaming or bubbles.
4. Store the 1X Wash Buffer at room temperature until ready to use in the procedure. Store the prepared 1X Wash Buffer at 4°C for no longer than 1 week. Do not freeze.

Assay Procedure

Technical Application Tips

1. Do not mix or substitute components from other kits.
2. To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
3. Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
4. Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.
5. Replicate wells are recommended for standards and samples.
6. Cover microplate while incubating to prevent evaporation.
7. Do not allow the microplate wells dry at any point during the assay procedure.
8. Do not reuse tips or tube to prevent cross contamination.
9. Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
10. Completely remove of all liquids when washing to prevent cross contamination.
11. Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
12. Equilibrate all materials to ambient room temperature prior to use (standards exception).
13. For optimal results in inter- intra-assay consistency, equilibrate all materials to room temperature prior to performing assay (standards exception) and perform all incubations at 37°C.
14. Pipetting less than 1 µL is not recommended for optimal assay accuracy.
15. Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
16. Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
17. Samples containing bilirubin, precipitates or fibrin strands or are hemolytic or lipemic might cause inaccurate results due to interfering factors.
18. TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.

Procedure

Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.

Optimal results for intra- and inter-assay reproducibility will be obtained when performing incubation steps at 37°C as indicated below.

1. Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.
2. Retain at least one well as an absolute Blank without any samples or reagents.
3. Add 50 µL of serially titrated standards, diluted samples or blank into wells of the Anti-Hydroxyproline Microplate. At least two replicates of each standard, sample or blank is recommended.

4. Immediately add 50 µL of 1X Hydroxyproline-Biotin Complex to each well (excluding absolute Blank).
5. Cover the plate with the plate sealer and incubate at 37°C for 60 minutes.
6. Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
7. Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
8. Wash plate three times with 1X Wash Buffer as follows:
 - a. Add 200 µL of 1X Wash Buffer to each assay well.
 - b. Incubate for 2 minutes.
 - c. Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
 - d. Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
 - e. Repeat steps 8a through 8d two more times.
9. Add 100 µL of 1X Avidin-HRP Conjugate to each well.
10. Cover the plate with the plate sealer and incubate at 37°C for 45 minutes.
11. Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
12. Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
13. Repeat wash as in step 8.
14. Add 90 µL of TMB Substrate to each well, cover with plate sealer, and incubate at 37°C in the dark for 15-30 minutes. Wells should change to gradations of blue. If the color is too deep based on the standard, adjust incubation times. (**Note:** optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the bottom four standard wells, while the remaining standards still appear clearer.)
15. Add 50µL of Stop Solution to each well. Well color should change to gradations of yellow immediately. Add the Stop Solution in the same well order as done for the TMB Substrate.
16. Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 15. If wavelength correction is available, set to 540 nm or 570 nm.

Calculation

For analysis of the assay results, calculate the Relative OD450 for each test or standard well as follows:

$$\text{(Relative OD450)} = \text{(Well OD450)} - \text{(Mean Blank Well OD450)}$$

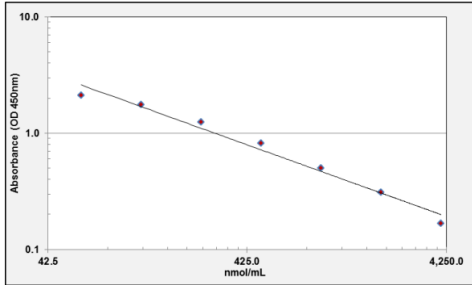
The standard curve is generated by plotting the mean replicate Relative OD450 of each standard serial dilution point vs. the respective standard concentration. The Hydroxyproline concentration contained in the samples can be interpolated by using linear regression of each mean sample Relative OD450 against the standard curve. This is best achieved using curve fitting software.

Note: If wavelength correction readings are available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: If the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

Typical Standard Curve

This standard curve is for demonstration purposes only. An assay specific standard curve should be performed with each assay.



nmol/mL	Absorbance (OD 450nm)
4,000	0.168
2000	0.311
1000	0.499
500	0.821
250.0	1.243
125.0	1.758
62.5	2.116

Detection Range

62.5 - 4,000 nmol/L

Detection Limit

< 31.25 nmol/L (Derived by linear regression of OD450 of the Mean Blank + 2xSD)

Reproducibility

Three sample's concentrations were measured in replicate within an assay plate and across replicate assays to assess Intra- and Inter-Assay precision.

Mean Intra-Assay Precision: %CV < 4.3%, n=20

Mean Inter-Assay Precision: %CV < 7.5%, n=20

Precautions

1. Read instructions fully prior to beginning use of the assay kit.
2. Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.
3. Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.
4. For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).
5. Kit cannot be used beyond the expiration date on the label.