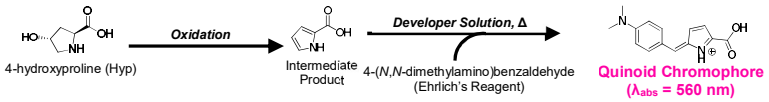
Hydroxyproline Assay Kit (Perchlorate-Free)

(Catalog #BN00499; 100 Reactions; Store at -20°C)

I. Introduction:

Hydroxyproline ((2*S*,4*R*)-4-hydroxyproline, Hyp) is a nonproteinogenic amino acid formed by the enzyme prolyl-4-hydroxylase, which catalizes the post-translational modification of polypeptide proline residues following protein synthesis. In animals, hydroxyproline is found almost entirely in collagen, which contains approximately 12-14% hydroxyproline by mass (the fibrilous protein elastin also contains small quantities of hydroxyproline). Hydroxyproline in tissue hydrolysates is thus a direct measure of the amount of collagen present. Collagen is the most abundant protein in mammals and many diseases are believed to affect collagen turnover, including tumor invasion and metastasis, rheumatoid arthritis, cardiopulmonary fibrosis and muscular dystrophy. The classical assay for determination of Hyp is based upon oxidation of Hyp to a pyrrole intermediate, followed by reaction with Ehrlich's reagent dissolved in concentrated perchloric acid ($HClO_4$). Perchloric acid is a hazardous material that is both toxic and highly reactive, requiring special handling and waste-disposal protocols. Assay Genie's Hydroxyproline Assay Kit (Perchlorate-Free) uses a proprietary acidic developer solution to accurately measure Hyp in tissue and protein/peptidase hydrolysates without the use of hazardous perchlorates. It is a quick and convenient protocol, yielding a brightly-colored chromophore with an absorbance peak at 560 nm. The assay can detect a minimum of 0.05 µg hydroxyproline per well in a 96-well format and generates colorimetric results that are virtually identical to the classical perchloric acid-based method.



II. Applications:

- Estimation of hydroxyproline concentration in various biological samples

III. Sample Type:

- Tissue homogenates (*i.e.* muscle, liver, lung, etc.)
- Human or animal biological fluids (serum, urine)

IV. Kit Contents:

Components	BN00499	Cap Code	Part Number
Oxidation Buffer	10 ml	WM	BN00499-1
Chloramine T Concentrate	600 µl	Red	BN00499-2
Developer Solution	5 ml	NM	BN00499-3
DMAB Concentrate (in DMSO)	5 ml	Amber	BN00499-4
Hydroxyproline Standard (1 mg/ml)	100 µl	Yellow	BN00499-5
Microplate Sealing Film	1 film	—	BN00499-5

V. User Supplied Reagents and Equipment:

- Microplate-based multiwall spectrophotomer
- Clear 96-well plates with flat bottom
- 10N sodium hydroxide (NaOH) and 10N hydrochloric acid (HCl)
- For hydrolysis: Polypropylene Vials (BV Cat. No. M1352) and Screw Caps (BV Cat. No. M1353)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Allow the Oxidation Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Chloramine T Concentrate:** Provided as a concentrated solution in dH_2O. Prior to use, warm to room temperature and vortex to ensure it is fully resuspended. Divide into aliquots and store at -20°C, protected from light. Limit aliquots to 3 freeze/thaw cycles.
- **Developer Solution:** Store at +4°C and allow solution to come to room temperature prior to use. After use, promptly retighten cap to minimize adsorption of airborne moisture.
- **DMAB Concentrate (in DMSO):** Provided as a ready-to-use solution in DMSO. Prior to use, warm to room temperature and mix by vortexing. After use, promptly retighten cap to minimize adsorption of airborne moisture. Store at -20°C, stable for 5 freeze/thaw cycles.
- **Hydroxyproline Standard:** Provided as a 1 mg/ml stock solution in dH_2O. Store at -20°C, stable for 5 freeze/thaw cycles.

VII. Hydroxyproline Assay Protocol:

1. Sample Preparation:

a. For tissue samples, add 100 µl of dH_2O for every 10 mg of tissue and thoroughly homogenize with a glass bead (Dounce) or ultrasonic probe homogenizer. Transfer 100 µl of sample homogenate to a pressure-tight, screw-capped polypropylene vial and add 100 µl of 10N concentrated NaOH (not provided). Ensure the cap is securely tightened and heat at 120°C for 1 hour (**See notes**). Following alkaline hydrolysis, place vial on ice, allow vial to cool briefly before opening cap and add 100 µl of 10N concentrated HCl (not provided) to neutralize residual NaOH. Vortex and centrifuge vials at 10,000 x *g* for 5 min to pellet any insoluble debris that may remain following hydrolysis.

b. In case of biological fluids (urine or serum), mix samples with equal volumes of 10N concentrated NaOH (*i.e.* 100 µl urine and 100 µl of 10N NaOH) in pressure-tight, screw-capped polypropylene vials and hydrolyze at 120°C for 1 hour. Cool vials on ice and neutralize hydrolysate by adding an equivalent volume (*i.e.* 100 µl) of 10N concentrated HCl. For urine samples, decolorize with activated charcoal by adding 4 mg of activated charcoal to the neutralized hydrolysate. Vortex and centrifuge at 10,000 x *g* for 5 min to remove precipitate & activated charcoal and transfer clarified supernatant to a new tube.

c. Transfer 10 µl of each neutralized sample hydrolysate to desired well(s) in a clear, flat-bottom 96-well plate.

Notes:

- Extremely tough samples (containing bone or exoskeletal tissue) may require heating for longer than 1 hour for complete hydrolysis.
- Hydrolysates of certain samples (*e.g.* fatty tissues) may contain lipid debris that is difficult to pellet by centrifugation. Take care when pipetting hydrolyzed samples to avoid transferring these insoluble globules to sample wells.
- For unknown samples, we recommend performing a pilot experiment to ensure readings are within the standard curve range and adjusting the volume of sample hydrolysate accordingly (2-20 µl of hydrolysate may be used) or diluting hydrolysate if necessary.
- For sample hydrolysis, polypropylene vials yield best results. We recommend Assay Genie's Polypropylene Vials and Caps Cat. No. M1353 and M1352).

2. **Standard Curve Preparation:** Prepare a 0.1 µg/µl solution of Hydroxyproline Standard by adding 20 µl of the 1 mg/ml Hydroxyproline Standard stock to 180 µl of dH_2O. Add 0, 2, 4, 6, 8, and 10 µl of the 0.1 µg/µl solution into a series of wells, generating 0, 0.2, 0.4, 0.6, 0.8 and 1 µg of hydroxyproline/well.
3. **Reaction Mix Preparation:**
   a. Evaporate the sample hydrolysate and standard curve wells to dryness by heating the plate at 65°C on a hot plate/dry heat block or microplate incubator. *To prevent warping/etching of the plastic, do not expose the microplate to extreme temperatures (>85°C).*
   b. For each reaction well to be analyzed (including standard curve wells), prepare 100 µl of Oxidation Reagent Mix by adding 6 µl of Chloramine T Concentrate to 94 µl of Oxidation Buffer. Make a sufficient amount of the Oxidation Reaction Mix for all of the assay wells. Add 100 µl of the Oxidation Reagent Mix to each well and incubate the plate at room temperature for 20 min.
   c. Add 50 µl of Developer Solution to each reaction well and incubate the plate at 37°C for 5 min.
   d. Add 50 µl of DMAB Concentrate solution to each reaction well and mix contents thoroughly. Seal the plate with the sealing film (provided) and incubate at 65°C on a hot plate/dry heat block or microplate incubator for 45 min.

   Notes:
   - Always prepare fresh Oxidation Reagent Mix as necessary for the number of samples and standards to be quantified. Once diluted to working concentration and exposed to light and air, Chloramine T is only stable for ~1-2 hrs.
   - Following evaporation of the sample hydrolysates, a crystalline residue will be left in the well. Gentle shaking will help dissolve the crystals in Oxidation Reagent Mix more quickly. Hydrolysates from certain samples may impart a faint yellow tint to the Oxidation Reagent Mix. This slight colorization usually dissipates upon addition of Developer Solution and does not interfere with the assay.
4. **Measurement:** Remove the plate from the heat source and measure the absorbance of all sample and standard curve wells at 560 nm (OD_{560}) in endpoint mode. *For maximum signal intensity, measure absorbance within 20 min of removing plate from the heat source.*
5. **Calculations:** For the hydroxyproline standard curve, subtract the zero standard (0 µg/well reagent blank) reading from all standard and test sample readings, plot the background-subtracted values and calculate the slope of the standard curve. For test samples, apply the background-subtracted OD_{560} values to the standard curve to get *B* µg of hydroxyproline in the well.

$$\textbf{Hydrolyzed Sample Hydroxyproline Concentration} = \frac{B}{V} \times D = \text{µg/µl}$$

Where: ***B*** is the amount of hydroxyproline, calculated from the standard curve (in µg)
V is the volume of sample hydrolysate added to the well (in µl)
D is the *post-hydrolysis* sample dilution factor (if applicable, *D*=1 for undiluted samples)

Note: The calculation above gives the hydroxyproline concentration in the sample hydrolysate. The dilution factor *D* is only needed if the sample is diluted *after* the hydrolysis step. When calculating the amount of hydroxyproline in the original sample homogenate, remember to account for the 3-fold dilution that occurs during generation of the hydrolysate.

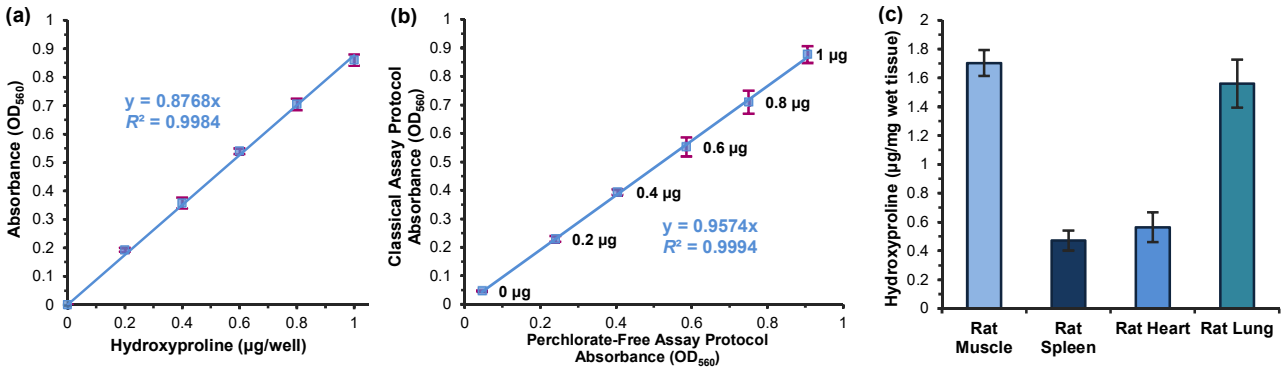


Figure: (a) Hydroxyproline Standard curve. **(b)** Correlation between hydroxyproline standard curve absorbance values obtained using the classical perchloric acid-based assay kit (Cat #BN00785) and the perchlorate-free assay. The two assay methods show excellent correlation (R^2 > 0.999). A Bland-Altman plot indicated a high degree of agreement between the two assays, with a mean difference of only 3.06 ± 0.33% between absorbance values generated by the respective methods. **(c)** Estimation of total hydroxyproline content in rat tissues. Rat leg muscle, spleen, heart and lung samples were homogenized with dH_2O, hydrolyzed with 10N NaOH for 1 hour at 120°C and neutralized with 10N HCl. For each sample, 10 µl of the final neutralized hydrolysate was assayed. Hydroxyproline levels (calculated as µg hydroxyproline/mg wet tissue) for the samples were: 1.71 ± 0.09 µg/mg for muscle, 0.47 ± 0.07 µg/mg for spleen, 0.56 ± 0.10 µg/mg for heart and 1.56 ± 0.17 µg/mg for lung. Data are mean ± SEM of 3-4 replicates, assayed according to the kit protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.