

Revised Protocol

---

Product Manual

# Hydroxyproline Assay Kit (Perchlorate-Free)

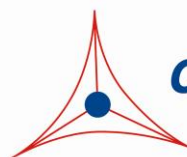
Catalog Number

STA-675

96 assays

**FOR RESEARCH USE ONLY**  
Not for use in diagnostic procedures

---



**CELL BIOLABS, INC.**  
*Creating Solutions for Life Science Research*

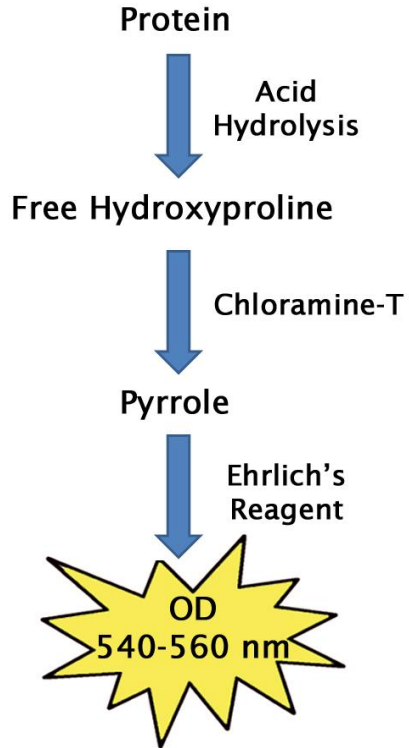
## **Introduction**

Hydroxyproline is an amino acid that is synthesized from the irreversible post-translational hydroxylation of proline by prolyl hydroxylase. Hydroxyproline is found almost exclusively in the protein collagen, in the Y position of the repeating tripeptide Gly-X-Y. By allowing sharp twisting of the collagen helix, hydroxyproline helps to stabilize the structure of collagen. In addition to collagen, hydroxylation of proline has been observed on the transcription factor Hypoxia Inducible Factor (HIF-1). Under normal oxygen conditions the protein EGLN1 hydroxylates HIF-1 alpha at proline 564, allowing ubiquitylation by the von Hippel-Lindau tumor suppressor (pVHL) and causing the targeting of HIF-1 for degradation by the proteasome.

Since hydroxyproline has been found on so few proteins other than collagen, measurement of hydroxyproline has been used as a marker to quantify levels of collagen and/or gelatin (partial hydrolysis of collagen resulting in a mixture of protein and peptides). It is estimated that hydroxyproline makes up 13.5% of collagen. In addition, hydroxyproline measurement has been used to identify certain diseases that involve breakdown of collagen. For example, increased levels of collagen have been measured in serum of Paget's Bone Disease. In addition, increased hydroxyproline levels have been correlated with prostatic carcinoma bone metastases, hepatic fibrosis, as well as melamine and cyanuric acid induced nephrotoxicity.

## **Assay Principle**

Cell Biolabs' Hydroxyproline Assay Kit provides a convenient colorimetric method for the detection of total hydroxyproline from tissue, plasma, serum, or urine acid-hydrolysates. First, the unknown samples or hydroxyproline standards are added to a 96 well plate. Then, a Chloramine T mixture is added to convert the hydroxyproline to a pyrrole. Finally, a 4-(Dimethylamino) benzaldehyde (DMAB) mixture (also known as Ehrlich's Reagent) is added to the well which reacts with the pyrrole to produce a chromophore (Figure 1) and the absorbance of the plate is read at 540-560 nm. The content of hydroxyproline in the unknown samples is determined by comparison with a predetermined hydroxyproline standard curve. The provided reagents are sufficient for the evaluation of 96 assays including standards and unknown samples.



**Figure 1. Assay principle.**

### **Related Products**

1. MET-5054: L-Amino Acid Assay Kit (Colorimetric)
2. MET-5151: S-Adenosylhomocysteine (SAH) ELISA Kit
3. MET-5152: S-Adenosylmethionine (SAM) ELISA Kit
4. STA-674: Glutamate Assay Kit

### **Kit Components**

1. Hydroxyproline Standard (Part No. 267501): One 100  $\mu$ L vial containing 1 mg/mL Hydroxyproline.
2. Assay Buffer (Part No. 267502): One 12 mL bottle.
3. Chloramine T Reagent (Part No. 267503): One 600  $\mu$ L vial.
4. 2X Ehrlich's Concentrate (Part No. 267504): One 5 mL bottle.
5. Ehrlich's Diluent (Part No. 267506): One 5 mL bottle.

## **Materials Not Supplied**

1. 12 N HCl
2. Activated charcoal
3. Water bath or incubator capable of heating to 60°C
4. Oven or autoclave for acid hydrolysis of samples at 95°C
5. 96 well ELISA strips or 96 well microtiter plate
6. 0.6 mL or 1.5 mL microcentrifuge tubes
7. 0.5 mL or 2 mL screw-cap microcentrifuge tubes or Teflon capped, pressure tight vials
8. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
9. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
10. Microplate reader capable of reading at 540-560 nm

## **Storage**

Upon receipt, store the entire kit at 4°C.

## **Preparation of Reagents**

- Chloramine T Mixture: Incubate Chloramine T Reagent for 10-15 minutes at 37°C. Vortex if needed to dissolve completely. For each well to be measured, add 6 µL of Chloramine T Reagent to 94 µL of Assay Buffer. Mix well. Use this mixture within 3 hours of preparation and discard unused Chloramine T Mixture. Aliquot remainder of unused Chloramine T Reagent before returning to 4°C storage to avoid multiple heat/cold cycles.
- Ehrlich's Reagent: Warm the 2X Ehrlich's Concentrate to room temperature to liquefy. For each well to be measured, mix 50 µL of 2X Ehrlich's Concentrate with 50 µL of Ehrlich's Diluent. Mix well. Use within 3 hours of preparation and discard unused Ehrlich's Reagent.

## **Preparation of Standard Curve**

Prepare a dilution series of Hydroxyproline standards in the concentration range of 0 to 100 µg/mL by diluting the Hydroxyproline Standard in distilled water (Table 1).

<b>Standard Tubes</b>	<b>1 mg/mL Hydroxyproline Standard (µL)</b>	<b>Distilled Water (µL)</b>	<b>Hydroxyproline (µg/mL)</b>
1	10	90	100
2	50 of Tube #1	50	50
3	50 of Tube #2	50	25
4	50 of Tube #3	50	12.5
5	50 of Tube #4	50	6.25
6		50	0

**Table 1. Preparation of Hydroxyproline Standards.**

## **Preparation of Samples**

All samples must undergo acid hydrolysis before use in the assay. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

- Cells: Resuspend  $3-6 \times 10^6$  cells in distilled water and proceed to Acid Hydrolysis Protocol below.
- Tissue: Homogenize in 100  $\mu\text{L}$  of distilled water for every 10 mg of tissue. Proceed to Acid Hydrolysis Protocol below.
- Urine, plasma, or serum: Use directly in the Acid Hydrolysis Protocol below.

### **Acid Hydrolysis Protocol for all Samples**

1. Transfer 100  $\mu\text{L}$  of sample to one of the following:
  - a. A 0.5 mL or 2 mL Teflon capped, pressure tight vial; then add 100  $\mu\text{L}$  of 12 N hydrochloric acid and incubate for 3 hours at 120°C, OR
  - b. A 0.5 mL or 2 mL screw-cap microcentrifuge tube; then add 100  $\mu\text{L}$  of 12 N hydrochloric acid and incubate for 24 hours at 95°C.
2. Let cool briefly, then clarify samples by one of the following methods:
  - a. Pass the hydrolyzed sample through a 0.45  $\mu\text{m}$  PVDF syringe filter, OR
  - b. Add 5 mg of activated charcoal, mix well by vortexing, centrifuge at 10,000  $\times g$  for 5 minutes, recover the supernatant and transfer to a new tube.
3. Proceed directly to the assay or store hydrolyzed samples at 4°C.

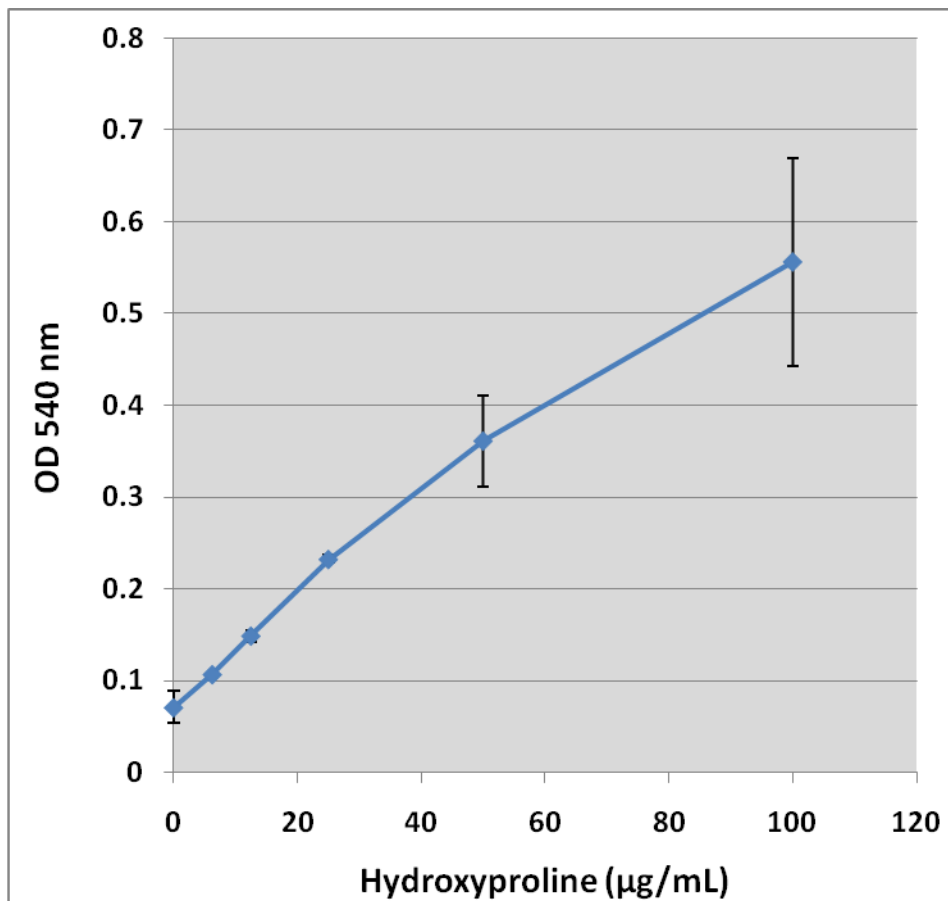
## **Assay Protocol**

1. Prepare and mix all reagents thoroughly before use. Each sample, unknown and standard should be assayed in duplicate.
2. Add 10  $\mu\text{L}$  of unknown acid hydrolyzed samples to separate microcentrifuge tubes.  
*Note: If needed, unknown samples may be diluted in water.*
3. Evaporate unknown acid-hydrolyzed samples under vacuum to dryness at 60-80°C for 30-45 minutes. If a vacuum source is not available, evaporation may be performed on a heat block or in an oven or waterbath.  
*Note: Unknown samples must be dried to remove any residual HCl that could inhibit the colorimetric assay reaction.*
4. Add 10  $\mu\text{L}$  of each hydroxyproline standard to separate tubes.
5. Add 100  $\mu\text{L}$  of the Chloramine T Mixture to each tube.
6. Incubate for 30 minutes at room temperature.
7. Add 100  $\mu\text{L}$  of Ehrlich's Reagent to each tube.
8. Incubate **45** minutes at 60°C.  
*Note: Precipitation may occur during this step or the next step.*
9. Transfer all tubes to 4°C and incubate for 5 minutes.
10. Centrifuge all tubes at 6000 $\times g$  for 15 minutes at room temperature.
11. Transfer 150  $\mu\text{L}$  of the supernatant to separate microplate wells.

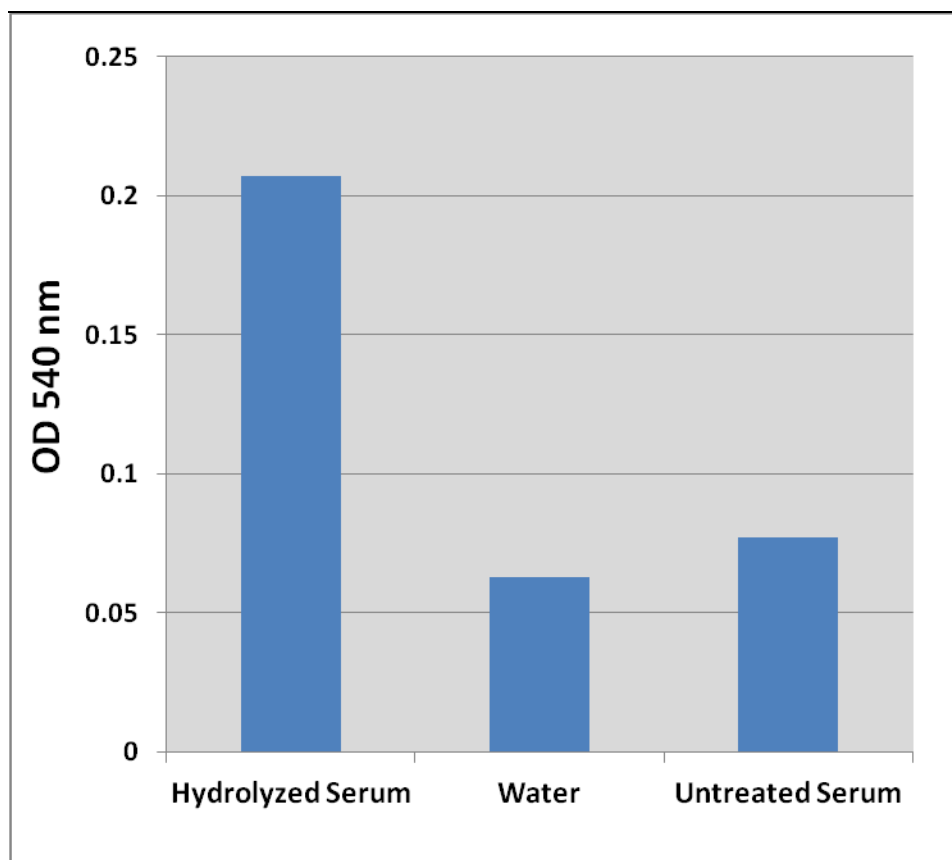
12. Read absorbance of each well on a microplate reader using 540-560 nm as the primary wave length.

### **Example of Results**

The following figures demonstrate typical Hydroxyproline Assay Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.



**Figure 2: Hydroxyproline Standard Curve.**



**Figure 3: Detection of Hydroxyproline in Human Serum.** Pooled human serum (or water as a negative control) was treated by acid hydrolysis according to the Preparation of Samples Section. Samples were tested according to the Assay Protocol.

### References

1. Gorres K.L and Raines RT. (2010) *Crit Rev Biochem Mol Biol.* **45**: 106-124
2. Gasser AB, Depierre D and Courvoiser B. (1979) *Br. J. Cancer.* **39**: 280-283
3. Rinsho K. and Aoyagi K. (1982) *Tohoku J. Exp. Med.* **137**:461-462
4. Schnackenberg L.K., Sun J., Pence L.M., Bhattacharyya S., Gamboa da Costa G., and Beger R.D. (2012) *Food Chem. Toxicol.* **50**:3978-3983.
5. Toyoki Y., Sasaki M., Narumi S., Yoshihara S., Morita T., and Konn M. (1998) *Hepatogastroent.* **45**: 2261-2264.
6. Bishop M.C. and Fellows G.J. (1977) *Br.J. Urol.* **49**:711-716.
7. Gilbertson T.J. (1983) *J. Clin. Chem. Clin. Biochem.* **21**:129-132.
8. Jaakkola P., Mole D.R., Tian Y.M., Wilson M.I., Gielbert J., Gaskell S.J., von Kriegsheim A., Hebestreit H.F., Mukherji M., Schofield C.J., Maxwell P.H., Pugh C.W., and Ratcliffe P.J. (2001) *Science* **20**:468-472.

## **Recent Product Citations**

1. Ha, K.B. et al. (2023). Beneficial Effects of a Curcumin Derivative and Transforming Growth Factor- $\beta$  Receptor I Inhibitor Combination on Nonalcoholic Steatohepatitis. *Diabetes Metab J*. doi: 10.4093/dmj.2022.0110.
2. Kundu, A. et al. (2023). Dendropanoxide Alleviates Thioacetamide-induced Hepatic Fibrosis via Inhibition of ROS Production and Inflammation in BALB/C Mice. *Int J Biol Sci*. **19**(9):2630–2647. doi: 10.7150/ijbs.80743.
3. Xu, C. et al. (2022). Hepatic neddylation deficiency triggers fatal liver injury via inducing NF- $\kappa$ B-inducing kinase in mice. *Nat Commun*. **13**(1):7782. doi: 10.1038/s41467-022-35525-6.
4. Kundu, A. et al. (2022). Tenovin-1 Ameliorates Renal Fibrosis in High-Fat-Diet-Induced Diabetic Nephropathy via Antioxidant and Anti-Inflammatory Pathways. *Antioxidants (Basel)*. **11**(9):1812. doi: 10.3390/antiox11091812.
5. Cassim Bawa, F.N. et al. (2022). Hepatic retinoic acid receptor alpha mediates all-trans retinoic acid's effect on diet-induced hepatosteatosis. *Hepatol Commun*. doi: 10.1002/hep4.2049.
6. Son, J.H. et al. (2021). Establishment of optimal decellularization conditions using porcine placenta. *J Anim Reprod Biotechnol*. **36**(4): 253–260. doi: 10.12750/JARB.36.4.253.
7. Bhat, N. et al. (2021). Dyrk1b promotes hepatic lipogenesis by bypassing canonical insulin signaling and directly activating mTORC2 in mice. *J Clin Invest*. doi: 10.1172/JCI153724.
8. Kawami, M. et al. (2021). Evaluation on epithelial-mesenchymal state and microRNAs focusing on isolated alveolar epithelial cells from bleomycin injured rat lung. *Toxicology*. **461**:152903. doi: 10.1016/j.tox.2021.152903.
9. Mavropalias, G. et al. (2021). Changes in plasma hydroxyproline and plasma cell-free DNA concentrations after higher- versus lower-intensity eccentric cycling. *Eur J Appl Physiol*. doi: 10.1007/s00421-020-04593-1.
10. Grondin, M.M. et al. (2021). Bi-Component T2 Mapping Correlates with Articular Cartilage Material Properties. *J Biomech*. doi: 10.1016/j.jbiomech.2020.110215.
11. Jiang, Y. et al. (2021). Histone H3K27 methyltransferase EZH2 and demethylase JMJD3 regulate hepatic stellate cells activation and liver fibrosis. *Theranostics*. **11**(1):361–378. doi: 10.7150/thno.46360.
12. Schappell, L.E. et al. (2020). A Microfluidic System to Measure Neonatal Lung Compliance Over Late Stage Development as a Functional Measure of Lung Tissue Mechanics. *J Biomech Eng*. doi: 10.1115/1.4047133.
13. Xu, Y. et al. (2020). Hepatocyte-specific Expression of Human Carboxylesterase 2 Attenuates Non-alcoholic Steatohepatitis in Mice. *Am J Physiol Gastrointest Liver Physiol*. doi: 10.1152/ajpgi.00315.2020.
14. Chiba, N. et al. (2020). Overexpression of hydroxyproline via EGLN/HIF1A is associated with distant metastasis in pancreatic cancer. *Am J Cancer Res*. **10**(8):2570–2581.
15. Kundu, A. et al. (2020). EX-527 Prevents the Progression of High-Fat Diet-Induced Hepatic Steatosis and Fibrosis by Upregulating SIRT4 in Zucker Rats. *Cells*. **9**(5). pii: E1101. doi: 10.3390/cells9051101.
16. Xu, Y. et al. (2020). Hepatocyte-Specific Expression of Human Carboxylesterase 1 Attenuates Diet-Induced Steatohepatitis and Hyperlipidemia in Mice. *Hepatol Commun*. doi: 10.1002/hep4.1487.
17. Sachan, R. et al. (2020). Dendropanax morbifera Protects against Renal Fibrosis in Streptozotocin-Induced Diabetic Rats. *Antioxidants (Basel)*. **9**(1). pii: E84. doi: 10.3390/antiox9010084.



18. Arow, M. et al. (2020). Sodium-glucose cotransporter 2 inhibitor Dapagliflozin attenuates diabetic cardiomyopathy. *Cardiovasc Diabetol.* **19**(1):7. doi: 10.1186/s12933-019-0980-4.
19. Chang, H.H. et al. (2019). Intrarenal Transplantation of Hypoxic Preconditioned Mesenchymal Stem Cells Improves Glomerulonephritis through Anti-Oxidation, Anti-ER Stress, Anti-Inflammation, Anti-Apoptosis, and Anti-Autophagy. *Antioxidants (Basel).* **9**(1). pii: E2. doi: 10.3390/antiox9010002.
20. Henry, J.J.D. et al. (2019). Development of Injectable Amniotic Membrane Matrix for Postmyocardial Infarction Tissue Repair. *Adv Healthc Mater.* doi: 10.1002/adhm.201900544.
21. Huang, M. et al. (2019). Systemic Sclerosis Dermal Fibroblasts Induce Cutaneous Fibrosis Through LOXL4: New Evidence from 3D Skin-like Tissues. *Arthritis Rheumatol.* doi: 10.1002/art.41163.
22. Park, H.J. et al. (2019). Empagliflozin and Dulaglutide are Effective against Obesity-induced Airway Hyperresponsiveness and Fibrosis in A Murine Model. *Sci Rep.* **9**(1):15601. doi: 10.1038/s41598-019-51648-1.
23. Park, Y.H. et al. (2019). Insulin resistance mediates high-fat diet-induced pulmonary fibrosis and airway hyperresponsiveness through the TGF- $\beta$ 1 pathway. *Exp Mol Med.* **51**(5):59. doi: 10.1038/s12276-019-0258-7.
24. Pankova, D. et al. (2019). RASSF1A controls tissue stiffness and cancer stem-like cells in lung adenocarcinoma. *EMBO J.* pii: e100532. doi: 10.15252/embj.2018100532.
25. Xu, Y. et al. (2019). Lipocalin-2 Protects Against Diet-Induced Nonalcoholic Fatty Liver Disease by Targeting Hepatocytes. *Hepatology Communications.* doi:10.1002/hep4.1341.
26. MacDonald, J.A. et al. (2019). Extracellular matrix signaling activates differentiation of adult ovary-derived oogonial stem cells in a species-specific manner. *Fertil Steril.* **111**(4):794-805. doi: 10.1016/j.fertnstert.2018.12.015.
27. Lindsey, A.S. et al. (2019). Analysis of pulmonary vascular injury and repair during Pseudomonas aeruginosa infection-induced pneumonia and acute respiratory distress syndrome. *Pulm Circ.* **9**(1):2045894019826941. doi: 10.1177/2045894019826941.
28. Meng, L. et al. (2019). Yangyin Yiqi Mixture Ameliorates Bleomycin-Induced Pulmonary Fibrosis in Rats through Inhibiting TGF- $\beta$ 1/Smad Pathway and Epithelial to Mesenchymal Transition. *Evidence-Based Complementary and Alternative Medicine.* 1-13. doi:10.1155/2019/2710509.
29. Huang, M. et al. (2019). Lysyl oxidase enzymes mediate TGF- $\beta$ 1-induced fibrotic phenotypes in human skin-like tissues. *Lab Invest.* **99**(4):514-527. doi: 10.1038/s41374-018-0159-8.
30. Chuang, H.M. et al. (2018). Non-Canonical Regulation of Type I Collagen through Promoter Binding of SOX2 and Its Contribution to Ameliorating Pulmonary Fibrosis by Butylidenephthalide. *Int J Mol Sci.* **19**(10). pii: E3024. doi: 10.3390/ijms19103024.

## **Warranty**

These products are warranted to perform as described in their labeling and in Cell Biolabs literature when used in accordance with their instructions. THERE ARE NO WARRANTIES THAT EXTEND BEYOND THIS EXPRESSED WARRANTY AND CELL BIOLABS DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR WARRANTY OF FITNESS FOR PARTICULAR PURPOSE. CELL BIOLABS' sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of CELL BIOLABS, to repair or replace the products. In no event shall CELL BIOLABS be liable for any proximate, incidental or consequential damages in connection with the products.

## **Contact Information**

Cell Biolabs, Inc.  
7758 Arjons Drive  
San Diego, CA 92126  
Worldwide: +1 858-271-6500  
USA Toll-Free: 1-888-CBL-0505  
E-mail: [tech@cellbiolabs.com](mailto:tech@cellbiolabs.com)  
[www.cellbiolabs.com](http://www.cellbiolabs.com)

©2015-2023: Cell Biolabs, Inc. - All rights reserved. No part of these works may be reproduced in any form without permissions in writing.