TECHNICAL MANUAL

Rat Hydroxyproline (HYP) ELISA Kit

- **SKU CODE:** RTEB1742
- **SIZE:** 96T/48T
- **DETECTION PRINCIPLE:** Competitive
- **RUO:** Research-Use-Only

Revised: 12/2025

Rat Hydroxyproline (HYP) ELISA Kit

Please read entire manual carefully before starting experiment. DO NOT mix reagents and use reagents from different kits or batches to prevent assay failure.

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1. Key Features

Detection Method:

Competitive, Coated with Antigen

Sample Type:

Plasma, Serum

Reactivity:

Universal

Range:

62.5-4000 nmol/L

Sensitivity:

< 31.25 nmol/L

Intra-Assay CV:

≤4.3%

Inter-Assay CV:

≤7.5%

Spike Average Recovery:

97%

2. Storage & Expiry

Assay Genie ELISA Kits are shipped on ice packs. Please store this ELISA Kit and/or components as described in section 4. Date of expiration is on the ELISA Box label.

3. Product Description

The Assay Genie Rat Hydroxyproline (HYP) ELISA Kit is a highly sensitive assay for the quantitative measurement of a Rat Hydroxyproline (HYP) in the following samples: Plasma, Serum.

This kit utilizes a competitive enzyme-linked immunosorbent assay (ELISA) format. The microtiter plate provided in this kit has been pre-coated with the analyte. During the reaction, the analyte in the sample or standard competes with a fixed amount of biotin-labeled analyte for sites on an antibody specific to the analyte. Excess conjugate and unbound sample or standard are washed from the plate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated.

Then a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of the analyte in the samples is then determined by comparing the O.D. of the samples to the standard curve.

This dual function kit includes validated Bradford Reagent to quantify total protein concentration for accurate sample normalization.

4. Kit Contents

Each kit contains reagents for either 48 or 96 assays, please store the reagents per conditions below.

No	Component Name	Size (48T)	Size (96T)	Storage
1	Microplate	1	1	Stored at -20°C
2	Standard	1 vial	2 vials	
3	Sample Diluent	1 x 10 mL	1 x 20 mL	
4	Assay Diluent A	1 x 5 mL	1 x 10 mL	
5	Assay Diluent B	1 x 5 mL	1 x 10 mL	
6	Detection Reagent A	1 x 60 µL	1 x 120 µL	
7	Detection Reagent B	1 x 60 µL	1 x 120 µL	
8	Wash Buffer (25 X concentrate)	1 x 15 mL	1 x 30 mL	Stored at 4°C
9	Substrate	1 x 5 mL	1 x 10 mL	
10	Stop Solution	1 x 5 mL	1 x 10 mL	
11	Plate Sealers	3 pieces	5 pieces	-
12	Bradford Reagent	1 vial	1 vial	Room Temperature

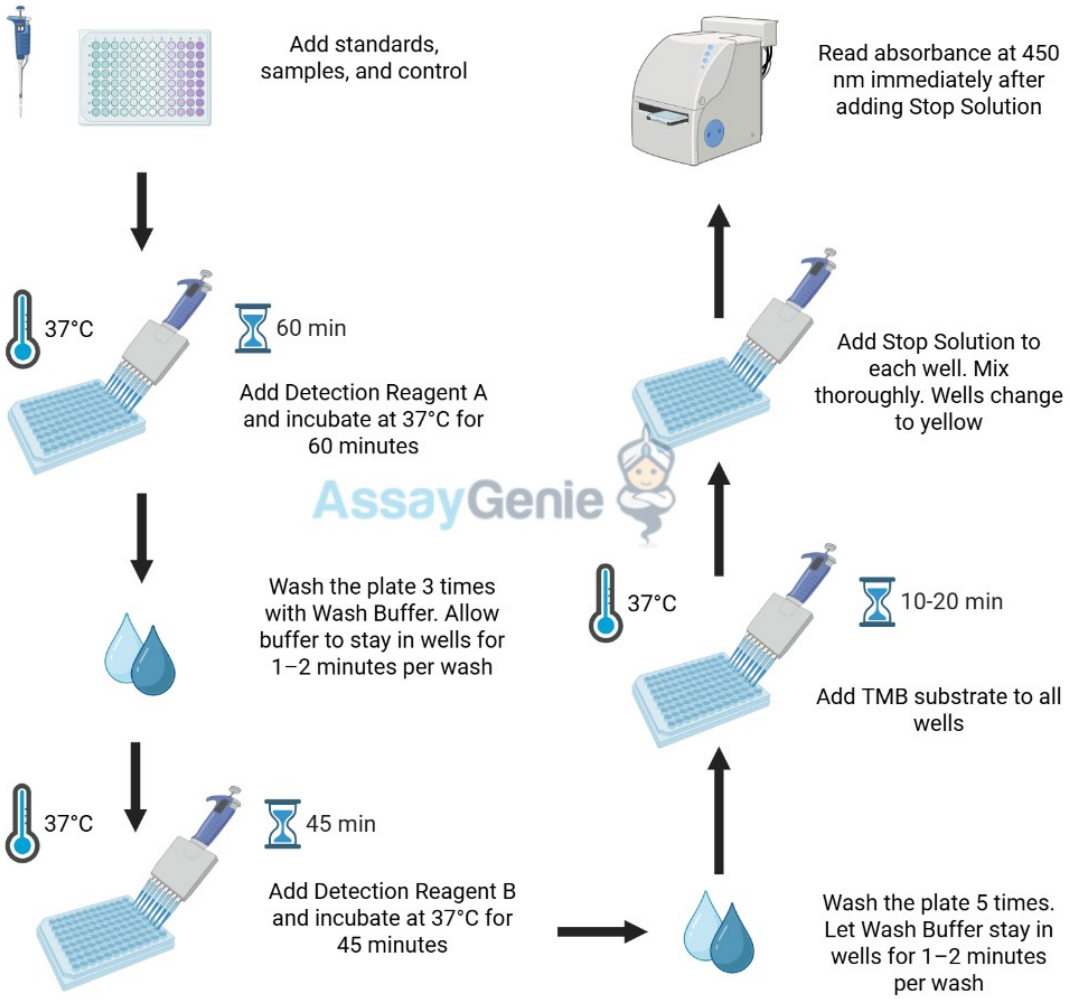
Additional materials required:

1. 37°C incubator.
2. Plate Reader with 450nm filter.
3. Precision pipettes and disposable pipette tips.
4. Distilled water.
5. Disposable tubes for sample dilution.
6. Absorbent paper.

5. Precautions

1. This kit is ideal for research purposes only and not for diagnostics or therapeutic uses.
2. Store all components as listed in this manual. Do not use the ELISA Kit after its expiration date.
3. Do not remove microtiter plate from the storage bag until needed.
4. Allow all reagents and samples to reach room temperature before use.
5. Ensure unopened and unused plate are kept dry to avoid contamination.
6. Before using the kit, centrifuge tubes to spin down standard and/or antibody.
7. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction and avoid foaming and mix gently until the crystals have completely dissolved. **The reconstituted Standards Detection Reagent A and B can be used only once.** This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.
8. Prepare all reagents, samples and standards as directed in this manual.
9. Duplicate wells are recommended for both standard and sample testing.
10. Do not let the microplate wells dry during the assay.
11. Maintain consistent incubation times and temperatures as variations can affect results.
12. Do not reuse tips and tubes to avoid cross contamination.
13. Avoid using the reagents from different batches together.

6. Assay Summary



7. Sample Preparation

The procedures outlined in this document are provided as general recommendations for sample preparation in ELISA assays. Due to the variability of biological samples and specific assay requirements, users are advised to optimize protocols based on their own experimental conditions.

Note: *Please note that not all of these samples were necessarily tested using this kit. For details regarding validation, kindly reach out to technical support Team at techsupport@assaygenie.com*.

General Considerations

To prevent denaturation or degradation of target proteins, it is recommended to process samples promptly and store them under appropriate conditions.

- **Storage Conditions**:
    - **Short-term**: 2-8 °C for up to 5 days.
    - **Medium-term**: -20 °C for up to 6 months.
    - **Long-term**: -80 °C or cryopreservation in liquid nitrogen.
- **Thawing Protocol**: Frozen samples should be thawed rapidly in a 15-25 °C water bath to minimize ice crystal-induced damage. Thawed samples can be analyzed immediately or stored temporarily at 2-8 °C.
- **Freeze-Thaw Cycles**: Repeated freeze-thaw cycles should be strictly avoided due to their detrimental effect on protein stability.

A. Blood-Derived Samples

- **Serum:** Allow whole blood to coagulate at room temperature (2 h) or 2-8 °C overnight. Centrifuge at 1000 × g for 20 min and collect the supernatant. Store or use immediately.

- **Plasma:** Collect in anticoagulant tubes (EDTA, citrate, or heparin), mix gently, and centrifuge within 30 min at 1000 × g, 2-8 °C for 15 min. Store or assay as needed.

B. Tissue Homogenates

Tissue samples should be homogenized prior to use. Avoid buffers containing NP-40, Triton X-100, or DTT, as these strongly inhibit the assay. We recommend using 50 mM Tris + 0.9% NaCl + 0.1% SDS, pH 7.3.

The recommended protocol is as follows:

- **Sample Collection and Washing**
    - Place the target tissue on ice.
    - Rinse the tissue with pre-cooled PBS buffer (0.01 M, pH 7.4) to remove residual blood.
    - Weigh the tissue for further processing.
- **Homogenization**
    - Grind the tissue on ice using an appropriate lysate.
    - The lysate volume should correspond to the tissue weight; typically, 9 mL PBS is used per 1 g of tissue. It is recommended to add protease inhibitors to the PBS (e.g., 1 mM PMSF). ***Note:*** *PBS buffer or mild RIPA lysis buffer can be used for homogenization. When using RIPA, adjust pH to 7.3.*
- **Cell Disruption**
    - Further disrupt the tissue using ultrasonic homogenization or freeze–thaw cycles.
        - Ultrasonic homogenization: Keep samples on an ice bath during sonication to avoid overheating.
        - Freeze–thaw cycles*:* Repeat twice for effective lysis.
- **Centrifugation and Storage**
    - Centrifuge the homogenate at 5000 × g for 5 minutes.
    - Collect the supernatant for immediate analysis, or aliquot and store at –20°C or –80°C for future assays.

- **Protein Concentration Measurement**
    - Determine total protein concentration using the Bradford Reagent included in this kit.
    - For ELISA assays, the total protein concentration should generally be 1–3 mg/mL.
    - Tissues with high endogenous peroxidase levels (e.g., liver, kidney, pancreas) may react with TMB substrate, causing false positives. If this occurs, treat samples with 1% H_2O_2 for 15 minutes before repeating the assay.

Note: *Liver, kidney, and pancreas samples often contain high levels of endogenous peroxidase, which may react with the chromogenic substrate at elevated sample concentrations, potentially resulting in false positive signals.*

If analysis of these tissues is required, a gradient dilution assay is recommended. A proportional decrease in signal with increasing dilution typically indicates minimal interference and supports the accuracy of the results.

To further minimise potential interference, samples can be pre-treated with 1% hydrogen peroxide (H_2O_2) for 15 minutes prior to testing. To prepare the treatment solution, add 1 µl of pure H_2O_2 to 100 µl of sample (1% v/v).

C. Cell Culture Supernatant

Centrifuge the sample at 2500 rpm for 5 minutes at 2–8°C. Carefully collect the clarified cell culture supernatant for immediate analysis, or aliquot and store it at –80°C for future assays.

D. Cell Lysates

- **Suspension Cell Lysate:** Centrifuge the cell suspension at 2500 rpm for 5 minutes at 2–8°C and collect the cell pellet. Wash the pellet with pre-cooled PBS (0.01 M, pH 7.4) and mix gently. Repeat centrifugation and discard the supernatant. Add 0.5–1 mL of cell lysis buffer containing an appropriate protease

inhibitor (e.g., PMSF, final concentration: 1 mM). Lyse the cells on ice for 30–60 minutes or disrupt them using ultrasonic homogenization.

- **Adherent Cell Lysate:** Remove the supernatant and wash the cells three times with pre-cooled PBS. Add 0.5–1 mL of cell lysis buffer supplemented with an appropriate protease inhibitor (e.g., PMSF at a final concentration of 1 mmol/L). Scrape the adherent cells using a cell scraper and transfer the cell suspension to a centrifuge tube. Lyse the cells on ice for 30–60 minutes, or disrupt the cells by ultrasonic treatment.

Follow next steps for protein extraction and supernatant collection:

- **Protein Release and DNA Disruption**
    - During lysis, pipette gently or intermittently shake the tube to enhance protein extraction.
    - Mucilaginous material formed during lysis is DNA, which can be broken down by ultrasonic disruption (3–5 mm probe, 150–300 W, 3–5 seconds per cycle, with 30-second intervals for 1–2 minutes total).
- **Supernatant collection**
    - After lysis or ultrasonic treatment, centrifuge the lysate at 10,000 rpm for 10 minutes at 2–8°C. Collect the supernatant for immediate use or aliquot and store at –80°C for future assays.

Notes: *Refer to the "Tissue Sample Notes" for additional buffer and inhibitor recommendations.*

E. Other Sample Types

For more information about how to process other sample types, (e.g., body fluids, breast milk & more), please contact our Tech Support Team at techsupport@assaygenie.com.

7.1. Protein Quantification (Optional)

To quantify total protein levels, use the Bradford Reagent included in this kit. Visit Bradford Protein Assay Protocol to view the full protocol.

8. Standard and Reagent Preparation

Washing Procedure

A. Automated Washer:

Add 400 µL wash buffer into each well, the interval between injection and suction should be set about 60s.

B. Manual wash:

Add 400 µL Wash Buffer into each well, soak it for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean absorbent paper on a hard surface.

Reagent Preparation

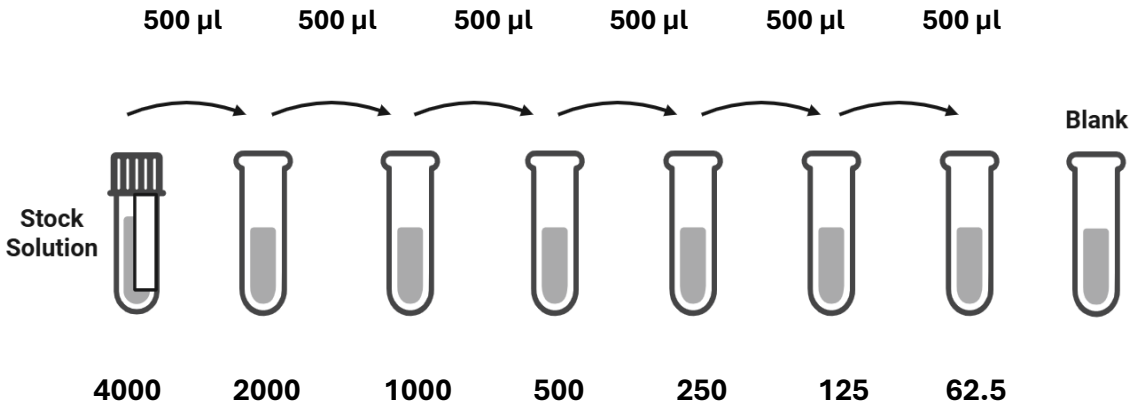
Bring all reagents and samples to room temperature 20 minutes before use (18 - 25°C). For repeated assays, please use only strips and standards required and store remaining reagents at the appropriate temperatures.

A. Wash Buffer:

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

B. Standard Dilution:

1. Prepare standard 15 minutes before use. Centrifuge the standard tube for 1 min at 10,000 x g.
2. Reconstitute the Standard with 1.0 mL of Sample Diluent to produce a stock solution (4000 nmol/L). Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. *Make serial dilutions as needed*. (***Note:*** *DO NOT perform serial dilutions directly onto the wells) . The undiluted stock serves as the standard with the highest concentration* (4000 nmol/L). *The Sample Diluent serves as the zero blank (0 ng/mL).*



C. Detection Reagent A and B:

Dilute to the appropriate working concentrations using Assay Diluent A and Assay Diluent B at a 1:100 ratio, respectively.

9. Assay Procedure

1. **Plate Setup:** Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate.
2. **Standard, Sample and Control (Blank) Loading:** Add 50 µL of Standard, Blank, or Sample per well.
3. **Detection Reagent A**: Immediately add 50 µL of Detection Reagent A working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C. ***Note:*** *If Detection Reagent A appears cloudy warm to room temperature until solution is uniform*.
4. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 400µL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, completely remove remaining Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick, clean absorbent paper.
5. **Detection Reagent B**: Add 100 µL of Detection Reagent B working solution to each well. Cover with the Plate sealer. Incubate for 45 minutes at 37°C.
6. **Wash**: Remove the cover, and wash plate 5 times as conducted in step 4.
7. **Substrate Addition**: Add 90 µL of Substrate Solution to each well. Cover with a new Plate sealer and incubate for 10-20 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual colour change, but this should not exceed more than 30 minutes. When apparent gradient appears in standard wells, user should terminate the reaction.
8. **Stop Reaction**: Add 50 µl of Stop solution into each well and mix thoroughly. The colour changes into yellow immediately.
9. **OD Measurement**: Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

10. Data Analysis

This assay uses a competitive inhibition enzyme immunoassay format; therefore, the assay signal intensity is inversely proportional to the concentration of Rat Hydroxyproline (HYP) in the sample.

Average the duplicate absorbance readings for each standard, control, and sample. Generate a standard curve by plotting Rat Hydroxyproline (HYP) concentration on the y-axis against absorbance on the x-axis. Determine the best-fit line through the standard points using regression analysis.

For diluted samples, multiply the concentration obtained from the standard curve by the corresponding dilution factor to calculate the final concentration. Curve fitting and data analysis may be performed using appropriate software (e.g., CurveExpert).

Note: *If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution*.

1.

11. ELISA Troubleshooting

Problem	Possible Causes	Solutions
Standard curve without signal	Incorrect reagent order; Mixed components from different kits; Missing reagents.	Ensure correct reagent order and use components from the same kit. Verify all reagents are added.
Overflow OD	Mixed components from different kits; Over-concentrated working solution.	Use correct components and prepare solutions at recommended concentrations.
Poor standard curve	Incorrect curve fitting model.	Try alternative curve fitting models.
Samples without signal	Sample concentration too low; Incompatible buffer; Incorrect preparation; Sample degradation or excessive freeze-thaw.	Reduce dilution or concentrate sample. Check buffer compatibility and follow proper preparation and storage.
High CV%	Precipitate formation; Unclean plate; Foaming; Uneven washing; Incomplete reagent mixing; Pipetting inconsistency.	Dilute samples if needed, avoid foaming, ensure uniform washing, mix reagents thoroughly, and use calibrated pipettes.
Low standard signal	Improperly reconstituted standards; Degraded standards; Incorrect pipetting; Expired kit; Improper storage; Over-dried wells.	Reconstitute standards properly, use fresh kits, follow storage recommendations, and prevent wells from drying.
Slow colour development	TMB not equilibrated; Incorrect microplate reader wavelength; Over-washing.	Pre-warm TMB (30 min at 37°C), confirm correct wavelength (450 nm), and follow recommended washing times.
High background	Insufficient washing; Contaminated wash buffer; Excess detection reagents; Delayed reading; TMB exposed to light.	Wash adequately, prepare fresh wash buffer, use correct reagent amounts, read results promptly, and incubate TMB in the dark.

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Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Manufacturers Statement: This final kit system is assembled and quality-released by Assay Genie Limited.