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Sunlong Medical™ Rat Hepcidin (HEPC) ELISA Kit

Cat No.:EL0306Ra

Type:	Sandwich ELISA	**Detection Range:**	0.156ng/mL-10ng/mL
Species:	Rat	**Storage Temp.:**	2-8°C
Format:	96T/48T	**Shelf Life:**	6 months
Intended Use: For quantitative in vitro detection of Rat HEPC in cultured cells, serum, plasma and other samples.			

Assay Principle

This kit employs double antibody sandwich ELISA technology: **Capture Antibody** is coated on the microplate to capture HEPC from samples and standards. After washing, biotin-labeled **Detection Antibody** is added and incubated, followed by washing to form a "**Capture Antibody-Antigen-Detection Antibody**" immune-complex. Subsequently, Streptavidin-Horseradish Peroxidase (**SA-HRP**) is added and incubated. After incubation and washing, **TMB Substrate** is added for color development. If the target analyte is present in the sample, a blue color develops. Stop Solution is then added to terminate the reaction. During the assay, unbound components are washed away. The **Optical Density** (OD) is measured at 450 nm using a microplate reader. The intensity of the color is proportional to the HEPC concentration in the sample, and the concentration is calculated by plotting a standard curve.

Precautions

1. **This kit is for research use only. Not for use in diagnostic procedures.**
2. After opening the sealed bag containing the pre-coated strips, any unused strips should be immediately resealed in a ziplock bag with desiccant. To minimize inter-assay variation, strips can be stored at 2-8°C if the next assay is within 48 hours. For longer intervals, store at -20°C and re-run the standard curve in the next assay.
3. The Concentrated Wash Buffer, Concentrated Biotinylated Antibody, and Concentrated HRP Conjugate must be diluted as instructed before use. Prepare these solutions as needed on the day of use.
4. It is recommended to mix Substrate Solution A and Substrate Solution B in a disposable tube first before adding to the wells. Prepare the mixed substrate solution immediately before use. The prepared substrate solution should remain colorless until added to the plate.

5. **The Stop Solution provided in this kit is a dilute sulfuric acid solution, which is corrosive. Handle with care. If contact with skin occurs, wipe off immediately and rinse thoroughly with water.**
6. **Operate strictly according to the instructions. If in doubt, please confirm with technical support before proceeding to avoid waste of samples and time.**
7. Do not mix reagents from different lot numbers. Do not use reagents from other sources or manufacturers.
8. Seal plates, absorbent paper, EP tubes, and pipette tips used during sample addition are for single use only. Do not reuse.

Kit Components

Component	96T Config	48T Config	Storage Condition
Pre-coated Plate	8 wells × 12 strips	8 wells × 6 strips	2-8℃
Standard	1 vial × 200μL	1 vial × 100μL	
100× Biotinylated Ab	1 vial × 100μL	1 vial × 50μL	
100× SA-HRP	1 vial × 100μL	1 vial × 50μL	
20× Diluent	1 bottle × 25mL	1 bottle × 15mL	
Substrate A	1 bottle × 6mL	1 bottle × 3mL	
Substrate B	1 bottle × 6mL	1 bottle × 3mL	
Stop Solution	1 bottle × 6mL	1 bottle × 3mL	
20× Wash Buffer	1 bottle × 25mL	1 bottle × 15mL	
Plate Sealer	4 sheets	4 sheets	RT
Instruction	1 copy	1 copy	

Materials Required but Not Provided:

Equipment & Instruments	Other Materials
Microplate reader with 450 nm filter	Absorbent paper or lab wipes
Pipettes of various volumes	Distilled or deionized water
Incubator capable of maintaining 37°C	Various pipette tips and EP tubes

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Sample Collection and Handling:

1. **Serum:** Collect whole blood into serum separation tubes. Allow clotting at room temperature for 30 minutes to 2 hours. Centrifuge at 2-8°C, 2500-3500 × g for 20 minutes. Carefully collect the supernatant.
2. **Plasma:** Use EDTA or citrate as anticoagulant as required. Centrifuge samples within 30 minutes of collection at 2-8°C, 3000 × g for 15 minutes. Carefully collect the supernatant. If precipitation occurs during storage, re-centrifuge.
3. **Urine:** Collect using sterile tubes. Centrifuge at 2-8°C, 2500-3500 × g for 10 minutes. Carefully collect the supernatant. If precipitation occurs during storage, re-centrifuge. Handle pleural fluid, ascitic fluid, and cerebrospinal fluid similarly.
4. **Cell Culture Supernate:** Collect the fluid and centrifuge at 2-8°C, 2500-3500 × g for 20 minutes to remove impurities and cell debris. Collect the supernatant for assay.
5. **Cell Lysate:** Wash adherent cells gently with ice-cold PBS (0.01M, pH=7.4), then digest with trypsin. Collect cells by centrifugation at 2-8°C, 1000 × g for 5 minutes. Suspension cells can be collected directly by centrifugation. Wash collected cells 3 times with cold PBS. Resuspend 1×10^6 cells in 150-200μL PBS. Lyse cells by repeated freeze-thaw cycles or ultrasonication (it is recommended to add protease inhibitors to PBS; if the target concentration is expected to be low, reduce the PBS volume). Centrifuge the lysate at 2-8°C, 10000 × g for 10 minutes. Collect the supernatant for assay.
6. **Tissue Samples:** Rinse the tissue with ice-cold PBS (0.01M, pH=7.4) to remove residual blood. Weigh and mince the tissue. Add the minced tissue to an appropriate volume of PBS (generally a 1:9 weight/volume ratio, e.g., 1g tissue to 9mL PBS; the volume can be adjusted according to experimental needs and should be recorded. It is recommended to add protease inhibitors to PBS) in a homogenizer. Grind thoroughly on ice. To further lyse cells, subject the homogenate to repeated freeze-thaw cycles or ultrasonication. Finally, centrifuge the homogenate at 2-8°C, 10000 × g for 5-10 minutes. Collect the supernatant for assay.
7. **Other Biological Samples:** Centrifuge at 2-8°C, 2500-3500 × g for 20 minutes. Carefully collect the supernatant.

Sample Appearance: Samples should be clear and transparent. Suspended matter should be removed by centrifugation.

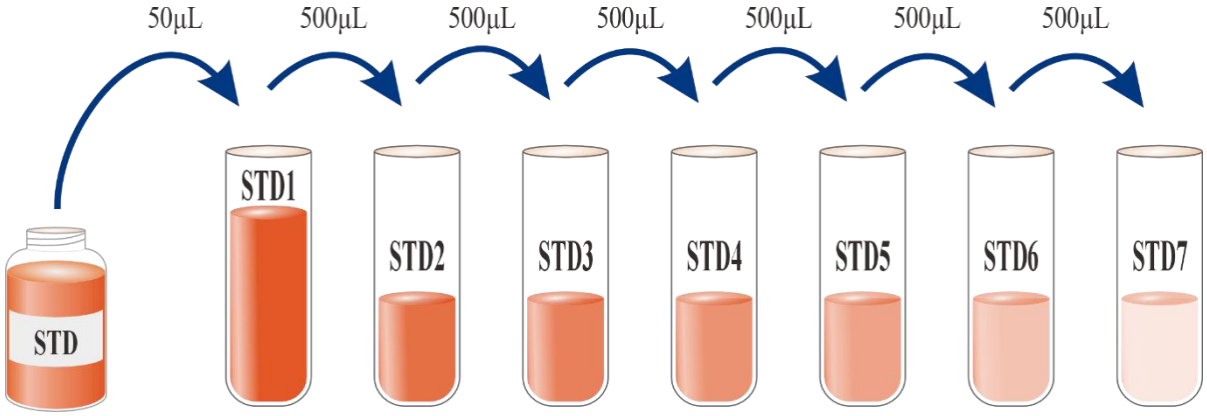
Sample Storage: Assay samples as soon as possible after collection. If testing cannot be performed immediately, aliquot the samples and store at -20°C (test within 1 month) or -80°C (test within 6 months). Avoid repeated freeze-thaw cycles.

Reagent Preparation

Allow all reagents to equilibrate to room temperature for about 30 minutes before use.

Wash Buffer/Diluent Working Solution Preparation: If crystals have formed in the concentrated (20×) Wash Buffer/Diluent, warm it at 37°C until all crystals are completely dissolved. Dilute 1:20 with distilled water (e.g., add 1 mL of concentrated Wash Buffer to 19 mL of distilled water).

Standard Preparation: Take out the standard from the kit. Prepare 7 tubes. First, dilute the 200ng/mL standard (200uL) as needed with 1× Diluent to a concentration of 10ng/mL (e.g., 50uL of standard stock + 950uL of 1× Diluent to prepare 1000μL of 10ng/mL standard). Then, add 500μL of 1× Diluent to each of 6 separate tubes. Perform a serial 2-fold dilution of the 10ng/mL standard in these 6 tubes to create 6 gradients, resulting in a total of 7 standard concentrations: 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.3125ng/mL, and 0.15625ng/mL. Transfer 500μL from the highest concentration standard to the next tube, mix gently by pipetting, and so on for the serial dilution. Use 1× Diluent as the zero standard (0ng/mL).



Concentrations after dilution are as follows (Unit: ng/mL)

STD (Stock)	STD1	STD2	STD3	STD4	STD5	STD6	STD7
200ng/mL	10	5	2.5	1.25	0.625	0.3125	0.15625

Biotinylated Ab Working Solution Preparation: 10 minutes before use, centrifuge the 100× Biotinylated Antibody at 1000 × g for 1 minute. Then dilute the 100× Biotinylated Antibody with 1× Diluent to prepare the 1× Biotinylated Antibody working solution. Prepare this working solution on the day of use according to the required volume.

SA-HRP Working Solution Preparation: 10 minutes before use, centrifuge the 100× SA-HRP solution at 1000 × g for 1 minute. Then dilute the 100× SA-HRP with 1× Diluent to

prepare the 1× SA-HRP working solution. Prepare this working solution on the day of use according to the required volume.

Note: If the HEPC concentration in the sample is higher than the highest standard point, dilute the sample with an appropriate dilution factor as needed and re-assay. Multiply the result by the dilution factor.

Assay Procedure

All standards and samples are recommended to be assayed in duplicate.

1. **Microplate Preparation:** Determine the number of strips required for the assay. Remove other unused strips and immediately return them to the sealed bag containing desiccant.
2. **Sample Incubation:** Add 100µL of different concentration standards and pre-treated samples to the appropriate wells (it is recommended to dilute samples at least 1-fold with the general diluent before adding to reduce matrix effects,**and add 1× Diluent buffer to the blank well.**). Cover with the plate sealer. Incubate at 37°C protected from light for 1 hour. After incubation, aspirate or decant the liquid from each well. Add 300µL of 1× Wash Buffer to each well, gently swirl for 30 seconds, then discard the contents. Blot the plate dry by tapping it firmly onto absorbent paper. Repeat this wash step 3 times.
3. **Antibody Incubation:** Add 100µL of Biotinylated Antibody working solution to each well. Mix gently. Cover with a new plate sealer. Incubate at 37°C protected from light for 1 hour. After incubation, repeat the wash procedure as in step 2, 4 times.
4. **Enzyme Conjugate Incubation:** Add 100µL of 1× SA-HRP working solution to each well. Cover with a new plate sealer. Incubate at 37°C protected from light for 30 minutes. After incubation, repeat the wash procedure as in step 2, 4 times. **Blot the plate dry on fresh absorbent paper.**
5. **Substrate Development:** Mix Substrate Solution A and Substrate Solution B in a 1:1 ratio to prepare the substrate working solution (prepare as needed; it should be colorless and transparent). Add 100µL of the prepared substrate working solution to each well. Cover with a new plate sealer. Incubate at 37°C protected from light for 15 minutes.
6. **Stop Reaction:** After the color development, add 50µL of Stop Solution to each well. Gently tap the plate to mix. Measure the Optical Density (OD) at 450 nm using a pre-warmed microplate reader within 5 minutes.

Calculation of Results

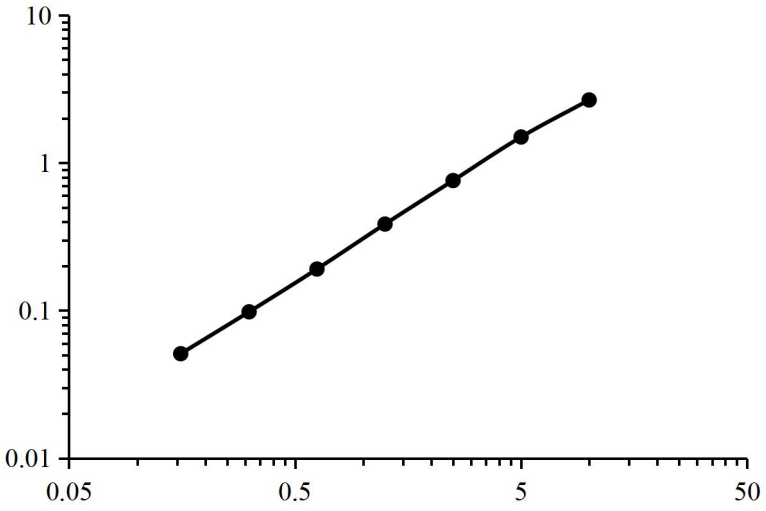
Calculate the average OD value for each standard and sample duplicate, then subtract the average OD value of the zero standard (blank) well to obtain the corrected OD value. Plot the corrected OD values (y-axis) against the corresponding standard concentrations (x-axis) on graph paper using a four-parameter logistic (4-PL) curve fit (omit the zero standard value when plotting). Alternatively, use computer software capable of generating a four-parameter logistic (4-PL) curve fit to create the standard curve.

If the OD value of a sample is higher than the highest standard point, the sample should be appropriately diluted and re-assayed. Multiply the concentration obtained from the standard curve by the dilution factor.

Typical Data

The following data and curve are for reference only. The experimenter must establish a standard curve based on their own experimental data.

Standard Conc. (ng/mL)	10	5	2.5	1.25	0.625	0.3125	0.15625	0
OD Value	2.703	1.534	0.792	0.424	0.227	0.131	0.086	0.04
Corrected OD Value	2.663	1.494	0.752	0.384	0.187	0.091	0.046	0



The standard curve shown in this figure is for example purposes only. Use the standard curve generated from the standards in the same assay to calculate sample results.

Precision

Intra-assay and inter-assay Coefficients of Variation (CV) are both <10%.

- **Intra-assay Precision:** Three known concentration samples were assayed 20 times on one plate. The CV of the concentrations was calculated.
- **Inter-assay Precision:** Three known concentration samples were assayed in 20 replicates across three different plates. The CV of the concentrations was calculated.

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	0.31	0.63	1.25	0.31	0.63	1.25
Standard Deviation	0	0.01	0.04	0.01	0.02	0.04
CV (%)	1.57	2.09	2.99	2.44	2.7	3.49

Recovery

Recovery was tested by spiking known concentrations of Rat HEPC into different sample matrices. The recovery range and average recovery are shown below.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=8)	88-102	95
Plasma (n=8)	94-102	98
Cultured Cells (n=8)	100-119	103

Sensitivity

The minimum detectable concentration (sensitivity) of Rat HEPC, determined by testing samples, is 0.075ng/mL.

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Linearity

High concentration Rat HEPC was spiked into samples and then serially diluted 2-fold, 4-fold, 8-fold, and 16-fold within the range of the standard curve to assess linearity. Recovery rates and average recovery are shown below.

Dilution		Serum (n=4)	Cultured Cells (n=4)
1:2	Recovery Range (%)	94-113	94-107
	Avg. Recovery (%)	99	97
1:4	Recovery Range (%)	100-115	97-111
	Avg. Recovery (%)	101	104
1:8	Recovery Range (%)	102-119	100-117
	Avg. Recovery (%)	107	113
1:16	Recovery Range (%)	107-121	104-121
	Avg. Recovery (%)	110	118

Specificity

This kit specifically detects recombinant Rat HEPC. Other related proteins were prepared at 50 ng/mL in the dilution buffer and tested for cross-reactivity. No significant cross-reactivity was observed.