Porcine Heparin ELISA Kit
96T

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FIVEphoton Biochemicals

For research use only.
Not for diagnostics.

Part No. pHEP-ELISA 96T

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Store: 4 ^{o}C. Expiration: 12 months.
Safety: Stop solution contains acid. Avoid contact and inhalation. Wear eye protection and gloves.

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Porcine Heparin ELISA Kit

Part No. pHEP-ELISA
Standard Concentration: 2400 U/L
Assay Range: 62.5 U/L – 2000 U/L
Sensitivity: 10 U/L

For research only. Not for diagnostic applications.
Storage: 4°C, 12 months shelf life.
Safety: Stop solution contains acid. Avoid eye and skin contact

Overview: Familiarize yourself with this protocol prior to performing the assay. It is recommended that the researcher overviews the literature to identify detailed methods for sample preparation: The sample isolation methods discussed in this protocol serve only as a general guide. Standards and samples should be measured at least in duplicate to account for pipetting error.

This kit measures Porcine Heparin in sandwich ELISA format. The ELISA plate is provided pre-coated with an anti-Porcine Heparin monoclonal antibody. The experimenter mixes the samples with a provided polyclonal anti-Porcine Heparin antibody labeled with biotin and with the included Streptavidin-HRP solution. This sample-antibody-biotin-Streptavidin-HRP mixture is then added to the ELISA plate, which is incubated for 60 min at 37°C. Unbound biotin-linked antibody is washed away and color reagents are added that change the color of the solution to blue. The blue solution then turns yellow after stop solution addition. The absorbance of the resulting yellow solution at 450nm corresponds to the concentration of Porcine Heparin in the samples.

Sample Preparation: The below discussion is provided as a general guide. Prior to performing the assay, the researcher should identify optimal sample preparation methods for their experiment.

1. Note that samples containing NaN_3 may inhibit Horse Radish Peroxidase (HRP) and the color reaction.
2. If possible, assay the sample immediately after isolation. Otherwise store samples at -20°C or -80°C without freeze-thawing cycles.
3. **Serum**: Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant carefully. If particulates are detected after storage, centrifuge again and collect the supernatant for assays. A significant dilution (for example, 200-1000X fold) of serum in a sample diluent solution (1% BSA in PBS pH 7.4 – made by experimenter, not provided with the kit) may be required to maintain assay specificity.
4. **Blood plasma**: In accordance with the requirements of sample collection, EDTA or sodium citrate should be used as the anti-coagulant. Add EDTA or sodium citrate and mix 10-20 minutes. Centrifuge at 2000-3000 RPM for approximately 20 minutes. Collect the supernatants carefully. If particulates are detected after storage, centrifuge again

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and collect the supernatant for assays. A significant dilution (for example, 200-1000X fold) of plasma in a sample diluent solution (1% IgG-free BSA in PBS pH 7.4 – made by experimenter, not provided with the kit) may be required to maintain assay specificity.

5. **Urine**: Collect by sterile tube. Centrifuge at 2000-3000 RPM for approximately 20 minutes. Collect the supernatants carefully. When particulates appear due to storage, centrifuge again and collect the supernatant to assay.

6. **Cell culture supernatant**: Collect in sterile tubes when examining secreted components. Centrifuge at 2000-3000 RPM for approximately 20 minutes to sediment cells. Collect the supernatants carefully.

7. **Intracellular and membrane bound components**. To assay intracellular components, use a non-denaturing cell lysis buffer to lyze membranes and solubilize cytoplasmic and membrane bound components. Add ice cold lysis buffer with protease inhibitors to cells. Keep the tube in ice for 30 min. Tap the tube several times during this period to mechanically shear membranes. Centrifuge cellular debris in a microcentrifuge at 18000 rpm for 15 min. Collect the supernatant to assay.

8. **Tissue sample**: Use a homogenization protocol in a non-denaturing buffer to release protein components. Add protease inhibitors and keep the solution ice cold during the homogenization steps. Centrifuge debris and assay the supernatant.

Do not use denaturing cell lysis buffers such as RIPA for protein extraction.

9. Samples can be aliquoted and stored at -80^{o}C for later use.

Table 1. Materials supplied. Store all materials at 4^{o}C. ***If particulates are observed in any of the reagents, centrifuge and use the clarified supernatant for the assay.***

1	Standard (2400U/L)	0.5ml	7	Chromogen Solution A	6ml
2	Standard diluents (1% IgG-free BSA in PBS pH 7.4)	3 ml	8	Chromogen Solution B	6ml
3	Anti-pHEP monoclonal antibody coated ELISA plate (96T)	12 well× 8 strips	9	Stop Solution	6ml
4	Streptavidin-HRP	6 ml	10	Instruction Manual	1
5	30×wash solution	20 ml	11	Closure Membrane	2
6	Anti-porcine heparin polyclonal antibody labeled with biotin (detection antibody)	1ml	12	Sealed bags	1

Materials required but not supplied

1. Sample dilution buffer (1% IgG-free BSA in PBS pH 7.4)
2. 37^{o}C incubator
3. Standard absorbance plate reader
4. Precision pipettes and disposable pipette tips
5. De-ionized water
6. Disposable tubes for sample dilution
7. Absorbent paper

Important notes and preparation for the assay

1. Make a solution that is 1% IgG-free BSA in PBS pH 7.4 for dilution of samples (i.e. ”sample dilution solution”).

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2. Equilibrate the ELISA plate strips and reagents, to room temperature for at least 30 minutes. Chromogen Solution B should be kept in the dark. The plate strips should be stored in a zip lock bag at 4°C. to prevent drying of the wells.
3. Use new pipettes tips at each step to prevent contamination.
4. Do not use reagents from other ELISA kits.
5. Chromogen Solution B is light sensitive. Limit light exposure.
6. **Manual Wash Method:** 600ul 1X wash solution in total per well is needed for all six wash steps. Gently aspirate off the liquid in each well. Turn over the plate and pat-dry on absorbent paper. Add 100µl wash solution and let percolate in the wells for 3 minutes, then aspirate. Repeat the wash steps with 100ul wash solution at each wash step, for five more times with 30 seconds per wash. An automatic washer can also be employed to wash the ELISA wells.
7. **Very Important**: Perform preliminary tests to determine the appropriate dilution of your samples to match the linear assay range of the ELISA kit. For example, remove a 8-well strip, prepare a standard well at the most concentrated and another well at the most diluted concentration of the assay range, set one well as the blank and perform a dilution series of a representative sample at 10X increments, then perform the test assay. Identify the dilution factor that provides a measurement at approximately midpoint of the assay range for most of your samples.

Assay procedures

Standard and sample preparation: Prepare the standards and samples in a separate 96-well dish and transfer the solutions simultaneously to the ELISA plate. Do not prepare samples in the ELISA plate.

Assay procedure

1. Standard dilutions. Use Table 2 as a guide for dilutions of standards in the provided standard diluents solution. Make dilutions in separate tubes or multi-well dishes, but not in the ELISA dish.

Table 2. Standard Dilutions

Standard Concentration	Standard Number	Dilution Instructions
1200 U/L	7	120µl undiluted standard + 120µl standard diluents solution, mix gently
600 U/L	6	120µl standard No.7 + 120µl standard diluents solution, mix gently
300 U/L	5	120µl standard No.6 + 120µl standard diluents solution, mix gently
150 U/L	4	120µl standard No.5 + 120µl standard diluents solution, mix gently
75 U/L	3	120µl standard No.4 + 120µl standard diluents solution, mix gently
37.5 U/L	2	120µl standard No 3 + 120µl standard diluents solution, mix gently

2. Standard and sample preparation: (Pre-mix solutions in a separate multiwell dish and transfer the solutions simultaneously to the ELISA dish. Do not pre-mix solutions in the ELISA dish).

a) **Standard solution wells**: Add 50µl of diluted standard and 50µl Streptavidin-HRP solution to each standard well. Note: Detection antibody labeled with biotin has been added in advance to the standards, therefore

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detection antibody is not added to the standards.

b) Sample wells: For each sample well, prepare 10ul sample (that may have been diluted previously to meet the assay range) in 30µl sample diluent solution (1% IgG-free BSA in PBS pH 7.4), then add 10µl anti- porcine heparin detection antibody and 50µl Streptavidin-HRP solution. Mix gently; do not vortex.

Accurately transfer 100«l of the premixed sample solutions to the ELISA dish. (You may opt to prepare excess volumes, for example 110µl, using these reagent ratios to assure 100µl of solution is accurately transferred to each well). Seal the wells with a ziplock bag and gently rotate the plate for 60 minutes at 37°C.

3. Preparation of wash solution: Dilute the 30X wash solution to 1X with dH_20. Prepare 600µl 1X wash solution per well.
4. Wash: Carefully remove the seal plate membrane or zip lock bag that protects the wells from drying: Do not cross-contaminate liquid. Gently aspirate off the liquid in each well. Turn over the plate and pat-dry on absorbent paper. Add 100µl wash solution and let percolate in the wells for 3 minutes prior to aspirating. Repeat the wash steps five times, percolating 100ul wash solution for 30 seconds, then aspirating. An automatic washer can also be employed to wash the ELISA wells. Blot dry the plate, but do not allow the wells to dry.
5. Color development: First add 50µl chromogen solution A to each well and then add 50µl chromogen solution B to each well. Gently mix solutions A and B. Incubate **protected from light** for 10 minutes at 37°C.
6. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow).
7. Read samples at 450nm within 15 min of adding the stop solution: Set the blank wells as zero, and measure the absorbance (OD) of each well at 450nm.

Data Analysis

1. Compile a standard curve using the standard solutions and the corresponding OD values. You may wish to calculate a linear regression equation from the standard curve to determine the concentration of your samples. Take into account dilution factors in your calculation. Alternative methods of data analysis to calculate concentrations of your samples can also be employed.

Flow chart of the procedures

Separately prepare standards, and samples

Incubate with the ELISA plate for 1 hr at 37°C.

Wash the plate

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Add Chromogen solutions A and B. Incubate for 10 min at 37^{o}C for color development.

Add stop solution

Measure OD value at 450nm within 15 min

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