Human Ubiquitin (UB) ELISA Kit – Biotin Detection Antibody Format 96T

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FIVEphoton Biochemicals

For research use only.
Not for diagnostics.

Part No. hUB-ELISA

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Store: 2-8^{o}C. Expiration: 12 months after arrival.
Safety: Stop solution contains acid. Avoid contact and inhalation. Wear eye protection.

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Standard Peptide: 4800 pg/ml

Assay Range: 10 pg/ml - 4000 pg/ml

Overview: Please read this protocol completely prior to using the product. The kit measures Human Ubiquitin in sandwich ELISA format. The ELISA plate is provided pre-coated with an anti-ubiquitin monoclonal antibody. The experimenter mixes the samples with a polyclonal detection anti-Ubiquitin antibody (that is provided biotin labeled) with the provided streptavidin-HRP solution *prior* to the addition of the solutions to the ELISA plate. The sample-antibody-biotin-streptavidin-HRP mixture is then added to the ELISA plate, which is incubated for 60 min at 37^{o}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 addition of the stop solution. The absorbance of the resulting yellow solution at 450nm corresponds to the concentration of Human UB in the samples.

Sample Preparation - The below is provided as a generic overview. Please review the literature to identify the optimal method to isolate your target protein.

All solutions should be free of particulates. Centrifuge or filter all samples to remove particulates. The experimenter should consider adding protease inhibitors and EDTA to their samples to limit degradation.

1. **Serum**: Coagulate at room temperature for 10-20 min. Centrifuge for 20 min at 2000-3000 rpm. Collect supernatant for assay. If precipitation appears, centrifuge again. Assay the supernatant fraction.
2. **Plasma:** Use suitable EDTA or heparin as an anticoagulant. Mix for 10-20 min using a stir bar. Centrifuge for 20 min at 2000-3000 rpm. Collect supernatant. If precipitation appears, centrifuge again and collect the supernatant.
3. **Urine**: Collect in a sterile container. Centrifuge 20-min at 2000-3000 rpm. Collect supernatant, If precipitation appears, centrifuge again. Collect supernatant for assay.
4. **Cell culture supernatant:** Detection of extracellular components: Centrifuge culture media for 20 min at 2000-3000 rpm. Collect supernatant for assay.
5. **Membrane fraction:** Lyse cells with a non-denaturing detergent buffer (such as FIVEphoton Biochemicals part no. ELSP-1). Sediment debris and assay the clarified supernatant fraction.
5. Samples can be store at -80^{o}C. Avoid repeated freeze-thaw cycles. You may aliquot samples for later ELISA assays.
6. **Avoid denaturing cell lysis buffers that contain SDS such as RIPA buffer.**
7. Samples can be diluted in a sample dilution solution with the formula: 1% BSA in PBS pH 7.4. A 1:5 dilution is recommended. i.e. 10 µl sample, 40 µl sample dilution solution.

Table 1. Materials supplied. Store all materials at 4°C

1	Standard peptide: 4800 pg/ml	0.5ml	7	Chromogen Solution A	6 ml
2	Standard diluents (1% BSA in PBS pH 7.4)	3 ml	8	Chromogen Solution B	6 ml
3	Anti-Ub Coated ELISA plate (96T)	12 well×8 strips	9	Stop Solution	6 ml
4	Streptavidin-HRP	6 ml	10	Instruction Manual	1
5	30×wash solution	20 ml	11	Closure Membrane	2
6	Anti-Ub antibody labeled with biotin (detection antibody)	1ml	12	Sealed bags	1

Materials required but not supplied

1. Sample dilution buffer ((1% BSA (IgG, protease free) in PBS pH 7.4))
2. 37°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% BSA (IgG, protease free) in PBS pH 7.4 for dilution of samples (i.e."sample dilution solution").
2. Equilibrate the ELISA plate strips to room temperature for at least 30 minutes. The plate strips should be kept in a zip lock bag to prevent drying of the wells. Store unused sections of the ELISA plate in a sealed bag at 4°C.
3. Use new pipettes tips at each step to prevent contamination.
4. Do not use reagents from other ELISA kits..
5. Substrate B is light sensitive. Limit light exposure.
6. **Manual Wash Method:** Gently aspirate off the liquid in each well. Turn over the plate and pat-dry on absorbent paper. Add 100μl wash solution and repeat 5 times. Therefore 600ul wash solution is needed per well. An automatic washer can also be employed to wash the ELISA wells.

Assay procedures

Blanks, standard and sample preparation. Prepare the blanks, standards and samples in a separate 96-well dish and transfer simultaneously to the ELISA plate. Do not prepare samples in the ELISA plate.

Note that the sample – detection antibody – streptavidin - HRP complex should be prepared by the experimenter prior to addition of the mixture to the ELISA plate wells:

*The experimenter may have to modify the protocol noted below to optimize conditions for blanking the reaction depending on the sample solutions and diluents. The experimenter should adjust the gain in the plate reader detector to maximize signal relative to background. Additionally, the experimenter should consider adding protease inhibitors and EDTA to their samples. Excessive background can generally be corrected by blanking against the correct solution and

by adjusting the gain of the plate reader UV detector.

Assay procedure

1. Standard dilutions. Use Table 2 as a guide for dilutions of standards in the provided standard diluent solution. Make dilutions in separate tubes or multi-well dishes, not in the ELISA dish. Use 2 replicates for the blank, standard and sample wells.

Table 2. Standard Dilutions

Standard Concentration	Standard Number	Dilution Instructions
2400 pg/ml	7	120µl original standard + 120µl standard diluents solution, mix
1200 pg/ml	6	120µl standard No. 7 + 120µl standard diluents solution, mix
600 pg/ml	5	120µl standard No. 6 + 120µl standard diluents solution, mix
300 pg/ml	4	120µl standard No. 5 + 120µl standard diluents solution, mix
150 pg/ml	3	120µl standard No. 4 + 120µl standard diluents solution, mix
75 pg/ml	2	120µl standard No .3 + 120µl standard diluents solution, mix
37.5 pg/ml	1	120µl standard No. 2 + 120µl standard diluents solution, mix

2. Blank, standard and sample preparation prior to addition to the ELISA wells:

a) Blank wells: Set aside 2 blank wells. Only add chromogen solutions A and B and then stop solution later during the procedural steps while developing the ELISA. Alternatively, use the "sample dilution solution" with sample vehicle at the same ratio used in the samples at a total volume of 40 µl, and perform all reactions in the protocol for the blank as with the samples (this method is preferred for serum samples).

b) Standard solution preparation: Add 50µl standard solution (that was previously diluted) and 50µl streptavidin-HRP solution to each standard. Detection antibody labeled with biotin has been added in advance to the standards, therefore anti-Ub detection antibody is not added to prepare the standards solutions.

c) Sample wells: Dilute 10ul sample in 30µl sample diluent solution (1% BSA, IgG and protease free, in PBS pH 7.4 – sample diluent is made by the experimenter), then add 10µl anti-Ub antibody labeled with biotin (detection antibody) and 50µl streptavidin-HRP. Mix gently; do not vortex.

d) Transfer the standard and sample solutions that you have prepared simultaneously to the ELISA dish. Seal the wells with a ziplock bag and rotate the plate gently for 60 minutes at 37^{o}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 in a manner that does not cross-contaminate the wells with liquid. Aspirate off the liquid. Fill each well with 100µl 1X wash solution. After 3 minutes, aspirate off the wash solution. Then repeat the wash procedure five times for 1 minute each time. Quickly blot dry the plate.

5. Color development: *Chromogen Solution B is light sensitive; protect from light.* Simultaneously transfer 50µl of chromogen solution A to each well and then simultaneously transfer 50µl of chromogen solution B to each well. *Protect the plate from li*ght. Incubate the plate protected from light for 10 minutes at 37^{o}C.

6. Stop solution: Add 50μl Stop Solution to each well to stop the reaction: Set the blank well as zero, and measure the absorbance (OD) of each well at 450nm within 15 minutes after adding the stop solution.

Data Analysis

1. Compile a standard curve using the blank 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 of UB concentration. Alternative methods of data analysis to calculate concentrations of your samples can also be employed.

Flow chart of the procedures

Separately prepare standards, blank and samples with detection antibody and HRP-streptavidin as required

Incubate with the ELISA plate for 1 hr at 37^{o}C.

Wash the plate five times.

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 10 min