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IMUBIND® Total uPAR ELISA Kit

Product # 893

for measuring total uPAR antigen in
human tissue extracts, plasma
and cell culture supernatants

INTENDED USE

The IMUBIND® Total uPAR ELISA kit is an enzyme-linked immunoassay for the measurement of human urokinase-type plasminogen activator receptor, uPAR, in tissue extracts, human plasma and cell culture supernatants. The lower detection limit of this assay is 0.1 ng total uPAR/mL of sample. Soluble, native and recombinant uPAR as well as uPAR/uPA and uPAR/uPA/PAI-1 complexes are all recognized by this assay. Antigen recognition was demonstrated by Western blot analysis of full length wild type uPAR secretions from a transfected osteosarcoma cell (2A2) and an RKO cell line, as well as recombinant uPAR from Baculovirus and CHO cell lines.

This assay is for research use only. It is not intended for diagnostic or therapeutic procedures.

EXPLANATION OF THE TEST

The urokinase-type plasminogen activator receptor, uPAR, is a GPI-linked single chain glycoprotein having a molecular weight between 50 kD and 60 kD. It also exists as a deglycosolated protein having a molecular weight of 35 kD. uPAR is composed of three domains: Domain I is involved with uPA binding, Domains II and III aid in orienting the uPAR molecule on the cell membrane. The presence of a cellular receptor for uPA was first demonstrated by Vassalli¹, who observed a saturable specific binding of uPA to the surface of monocytes. uPAR binds both the enzymatically inactive single chain pro-uPA and the enzymatically active two-chain HMW-uPA with high affinity (0.1-1.0 nM).² Binding occurs via the Growth Factor Domain in the Amino Terminal Fragment of uPA (amino acids 10-30). The enzymatically active two-chain LMW-uPA, which lacks the Amino Terminal Fragment, does not bind to uPAR. Recent studies have demonstrated inhibition of uPAR reduces the metastatic potential of human PC3 prostate carcinoma cells.³

PRINCIPLE OF THE METHOD

The IMUBIND Total uPAR ELISA employs a mouse monoclonal antibody against human uPAR as the capture antibody. Samples incubate in precoated microtest wells and a second, biotinylated antibody is used to recognize the bound uPAR molecules. Adding streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody-enzyme detection complex.

The addition of a perborate/3,3',5,5' - tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP creates a blue colored solution. Sensitivity is increased by addition of a sulfuric acid stop solution, yielding a yellow color. Total uPAR levels are quantified by measuring solution absorbances at 450 nm and comparing the values with those of a standard curve.

REAGENTS

- 6 x 16 well precoated microtest strips with holder and lid
- 6 vials uPAR Standards, 0-3 ng/mL
- 2 vials Detection Antibody, biotinylated anti-human uPAR (lyophilized)
- 1 vial Enzyme Conjugate, Streptavidin-horseradish peroxidase (60 μ L)
- 1 vial Enzyme Conjugate Diluent (lyophilized)
- 1 vial Substrate, TMB (11 mL)
- 1 vial Detergent, 25% Triton X-100 (10 mL)
- 2 packets PBS Buffer, pH 7.4

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- 0.22 μ m filtered deionized water H₂O
- 50-200 μ L eight channel multi-pipette
- 10-200 μ L single pipette
- Microtest plate reader at 450 nm
- 0.5N H₂SO₄
- Bovine Serum Albumin (BSA, e.g. Sigma A-7030)
- Tris Buffered Saline (TBS), pH 8.5

REAGENT PREPARATION

A. uPAR Standards

1. Add 1.0 mL filtered deionized H₂O to the 0.25, 0.75, 1.5, 2.0, and 3.0 ng/mL standard vials and 2.0 mL filtered deionized H₂O to the 0.0 ng/mL standard vial.
2. Agitate gently for 3 minutes. Do Not Shake!

B. Detection Antibody

Add 5.5 mL distilled H₂O per vial and agitate gently for 3 minutes.

C. Enzyme Conjugate Diluent

Add 20 mL filtered deionized H₂O to the vial and mix well.

D. Wash Buffer

1. Dissolve contents of 1 PBS packet in 900 mL of filtered deionized H₂O.
2. Add 4 mL of 25% Triton X-100.
3. Dilute to a final volume of 1 Liter with filtered deionized H₂O.
4. Mix well.

E. Sample Buffer

Prepare an appropriate amount of Sample Buffer by adding BSA to Wash Buffer to a final concentration of 1 % w/v (1 gm BSA/100 mL Wash Buffer).

F. 10% Triton X-100

Add 4 mL of 25% Triton X-100 to 6 mL of filtered deionized H₂O.

REAGENT STORAGE AND STABILITY

Store unused microtest strips, liquid reagents and lyophilized reagents at +2° - +8°C until the expiration dates indicated on their respective labels. Reconstituted reagents may be stored at +2° - +8°C for up to one month.

SAMPLE PREPARATION

A. Detergent Extraction of Homogenized Tissue Samples

1. Suspend powder from pulverized frozen tissue samples (100-300 mg wet weight) in 1.8 mL TBS, pH 8.5.
2. Add 0.2 mL 10% Triton X-100 in TBS, pH 8.5, to the tissue suspension to yield a 1% Triton X-100 final preparation.
3. Stir for 12 hours at 4°C.
4. Centrifuge the suspension at 100,000 x g for 60 minutes at 4°C to separate cell debris.
5. Decant the supernatant/tissue extract and measure the total protein content of the extract using a BCA protein assay. If necessary, adjust the total protein content to 2-3 mg/mL with TBS, pH 8.5. Aliquot the extract into 100 μ L portions.
- 6a. For storage, freeze at -80°C or in liquid nitrogen.
- 6b. For immediate use in the ELISA, dilute the tissue extract 1:20 in Sample Buffer.

B. Tissue Culture Supernatant Samples

Dilute samples 1:5 (recommended initial dilution) in Sample Buffer. Note: some cell systems may require a higher dilution factor (up to 1:500).

C. Plasma Samples

Dilute samples 1:5 in Sample Buffer.

ASSAY PROCEDURE

DAY ONE

1. Remove the necessary number of precoated microtest strips from the foil pouch and place them in the plate holder. Tightly reseal the foil pouch with the desiccant inside and store at +2° - +8°C.
2. Add 100 μ L of total uPAR Standard, reference control, or diluted sample, to microtest wells, cover with lid and incubate overnight at +4°C. Perform measurements in duplicate.

DAY TWO

3. Wash wells 4 times with **Wash Buffer**.
4. Add 100 μL of **Detection Antibody** to each well, cover with lid and incubate for 1 hour at room temperature.
5. Wash wells 4 times with **Wash Buffer**.
6. For running all 96 wells at one time, add 12 μL of **Enzyme Conjugate** to 12 mL of **Enzyme Conjugate Diluent** (add 2 μL conjugate to 2 mL of diluent for each 16 well strip when running less than 96 wells). Add 100 μL of diluted enzyme conjugate to each well, cover with lid and incubate for 1 hour at room temperature.
7. Wash wells 4 times with **Wash Buffer**.
8. Add 100 μL of **Substrate** solution to each well, cover with lid and incubate for 20 minutes at room temperature. A blue color will develop.
9. Stop the enzymatic reaction by adding 50 μL of 0.5N H_2SO_4 . Tap the sides of the strip-wells to ensure even distribution of the H_2SO_4 . The solution color will turn yellow. Read the absorbances on a microtest plate reader at a wavelength of 450 nm within 30 minutes. Deduct the background average of the blanks from the standards and sample readings.

CALCULATION OF RESULTS

Using the mean absorbance value for each diluted sample, determine the corresponding total uPAR concentration in ng/mL obtained from the standard curve.

A. Tissue Samples

1. Multiply the sample value by 20 to obtain the total uPAR concentration of the tissue extract as the extract was diluted 20-fold in the assay.
2. Divide the tissue extract total uPAR concentration by the protein concentration (mg/mL) of the tissue extract to convert ng total uPAR/mL of sample to ng total uPAR/mg protein.

B. Plasma and Tissue Culture Supernatant Samples

Multiply the sample value by the dilution factor to determine the total uPAR concentration of the plasma/tissue culture supernatant.

EXPECTED VALUES

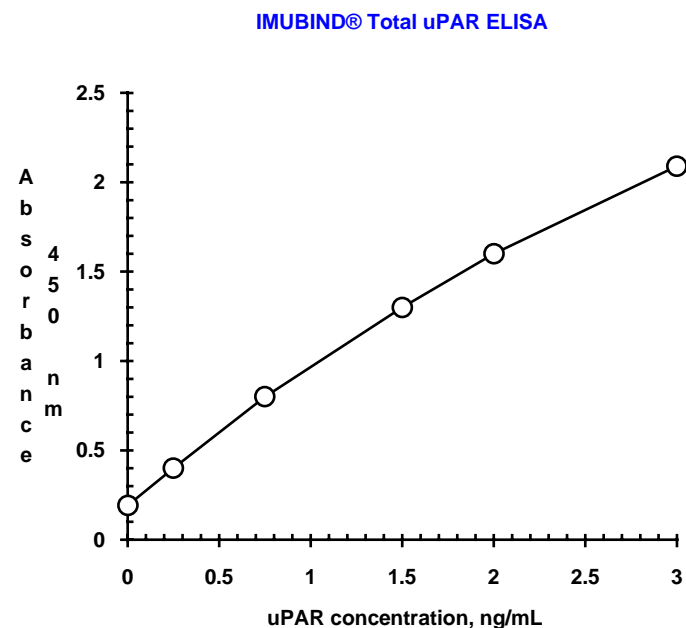
Total uPAR levels in normal human plasma have not yet been established. Initial testing suggests, if present, uPAR levels are less than 0.1 ng/mL, the lower detection limit of this assay.

INTERPRETATION OF RESULTS

To date, while uPAR has been found to be overexpressed in invasive breast⁴, colorectal⁶ and ovarian⁷ cancers, and has been localized in sections of solid tumors of various human cancers, few assessments of its prognostic significance for disease-free survival or overall survival have been established. A recent study has shown high levels of uPAR in colorectal tumors (> 4.1 ng uPAR/mg total protein) is an independent prognostic factor for 5-year overall survival and in comparing patients with Dukes' stage C vs. B and stage C2 vs. C1.⁵

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value calculated for each total uPAR standard versus the corresponding total uPAR concentration. A standard curve should be generated each time the assay is performed.



REFERENCES

1. Vassalli, J., *et al.* A cellular binding site for the M_r 55,000 form of human urokinase plasminogen activator. *Journal of Cellular Biology* 1985; **100**: 86-92.
2. Blasi, F. Surface receptors for urokinase plasminogen activator. *Fibrinolysis* 1988; **2**: 73-83.
3. Crowley, C., *et al.* Prevention of metastasis by inhibition of the urokinase receptor. *Proceedings of the National Academy of Science* 1993; **90**: 5021-5025.
4. Bianchi, E., *et al.* The urokinase receptor is expressed in invasive breast cancer but not in normal tissue. *Cancer Research* 1994; **54**: 861-866.
5. Ganesh, S., *et al.* Urokinase receptor and colorectal cancer survival. *The Lancet* 1994; **344**: 401-402.
6. Wang, H., *et al.* Transcriptional Activation of the Urokinase Receptor Gene in Invasive Colon Cancer. *International Journal of Cancer* 1994; **58**: 650-657.
7. Schmalfeldt, B., *et al.* Primary Tumor and Metastasis in Ovarian Cancer Differ in Their Content of Urokinase-type Plasminogen Activator, Its Receptor, and Inhibitors Types 1 and 2. *Cancer Research* 1995; **55**: 3958-3963.