INTENDED USE

The IMUBIND® uPA ELISA is an enzyme-linked immunoassay for the quantitative determination of human urokinase-type plasminogen activator in tissue extracts, plasma and cell culture supernatants. Single chain uPA (sc-uPA, pro-uPA) and HMW-uPA forms of urokinase-type plasminogen activator are all recognized by the assay, as is receptor-bound uPA and uPA complexed with PAI-1 and PAI-2.

This assay is For Research Use Only. It is not intended for diagnostic or therapeutic procedures.

EXPLANATION OF THE TEST

Urokinase-type plasminogen activator, uPA, is a protease involved in the degradation of the extracellular tumor matrix and has been implicated in cell proliferation, invasion and metastasis.1,2 Secreted by tumor cells in an enzymatically inactive single-chain form, sc-uPA binds to receptors on the surface of tumor cells.3 Sc-uPA is converted into an enzymatically active two-chain molecule by serine proteases (plasmin, plasma kallikrein, trypsin), metalloproteases (thermolysin) or cysteine proteases (Cathepsin B and L).4-6 Studies have shown that elevated uPA content in breast cancer tumor tissue correlates with an increased risk of shortened relapse-free survival and overall survival times in breast cancer patients and is an independent prognostic indicator for breast cancer.7-14 In gastric cancer patients, uPA levels have been found to be associated with poor prognosis in clinically important subgroups. Preliminary studies show it to be a new prognostic factor for survival in completely resected gastric cancer patients.15

PRINCIPLE OF THE METHOD

The IMUBIND uPA ELISA employs a murine monoclonal antibody against human uPA as the capture antibody. Samples incubate in microwells coated with the monoclonal anti-human uPA and are detected with a biotinylated anti-human uPA antibody that recognizes the bound uPA 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. uPA levels are quantified by measuring solution absorbances at 450 nm and comparing the values with those of a standard curve.

REAGENTS

96 Anti-human uPA coated microwells (6 x 16 well strips in a frame) and cover sheet
6 vials uPA Standards, 0-1.0 ng/mL (single-chain, lyophilized)
2 vials Detection Antibody, biotinylated anti-human uPA (lyophilized)
1 vial Enzyme Conjugate, Streptavidin-horseradish peroxidase, 60 µL
1 vial Enzyme Conjugate Diluent (lyophilized)
1 vial Substrate, TMB, 11 mL
1 vial Detergent, 25% Triton X-100, 12 mL
2 packets PBS Buffer, pH 7.4
WARNINGS AND PRECAUTIONS
For Research Use only. Do not use kit components beyond the expiration date. Do not mix reagents from different kits. Do not smoke, eat or drink in areas where specimens or kit reagents are handled. Avoid microbial contamination of the kit components. Do not mouth pipette or ingest reagents.

<table>
<thead>
<tr>
<th>uPA Standards</th>
<th>Warning</th>
</tr>
</thead>
<tbody>
<tr>
<td>H319, P264, P280, P305+P351+P338, P337+P313</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Detection Antibody</th>
<th>Warning</th>
</tr>
</thead>
<tbody>
<tr>
<td>H319, P264, P280, P305+P351+P338, P337+P313</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme Conjugate Diluent</th>
<th>Danger</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>2-Methyl-4-isothiazol-3-one</td>
</tr>
<tr>
<td>H317, P261, P272, P280, P302+P352, P333+P313, P362</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme Conjugate Diluent</th>
<th>Danger</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>Polyethylene glycol octylphenol ether</td>
</tr>
<tr>
<td>H317, H318, H334, H412, P261, P280, P273, P305+P351+P338, P310</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Danger</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>Polyethylene glycol octylphenol ether</td>
</tr>
<tr>
<td>H318, H411, P280, P273, P305+P351+P338, P310</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PBS Packet</th>
<th>Warning</th>
</tr>
</thead>
<tbody>
<tr>
<td>H319, P264, P280, P305+P351+P338, P337+P313</td>
<td></td>
</tr>
</tbody>
</table>

Hazard Statements:
- H317 May cause an allergic skin reaction.
- H318 Causes serious eye damage.
- H319 Causes serious eye irritation.
- H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
- H411 Toxic to aquatic life with long lasting effects.
- H412 Harmful to aquatic life with long lasting effects.

Precautionary Statements:
- P261 Avoid breathing dust.
- P264 Wash thoroughly after handling.
- P272 Contaminated work clothing must not be allowed out of the workplace.
- P273 Avoid release to the environment.
- P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
- P302 + P352 IF ON SKIN: Wash with plenty of water.
- P305 + P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P310 Immediately call a POISON CENTER/docto.
- P333 + P313 IF skin irritation or rash occurs: Get medical advice/attention.
- P337 + P313 IF eye irritation persists: Get medical advice/attention.
- P362 Take off contaminated clothing and wash before reuse.

REAGENT PREPARATION AND STORAGE
A. uPA Standards
1. Add 1.0 mL filtered deionized water to the 0.10, 0.25, 0.50, 0.75 and 1.0 ng/mL standard vials and 2.0 mL filtered deionized water to the 0.0 ng/mL standard vial.
2. Agitate gently for 3 minutes. Do Not Shake!

B. Detection Antibody
Add 5.5 mL filtered deionized water per vial and agitate gently for 3 minutes.

C. Enzyme Conjugate Diluent
Add 20 mL filtered deionized water to the vial and mix well.

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

Store unused antibody coated microwells, liquid reagents and lyophilized reagents at 2°-8°C until the expiration dates indicated on labels. Reconstituted reagents may be stored at 2°-8°C for up to 2 weeks.

SPECIMEN COLLECTION AND PREPARATION
A. Detergent Extraction of Homogenized Tissue Samples
1. Suspender powder from homogenized 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 16 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 µL portions.
6a. For storage, freeze at -80°C or colder.
6b. For immediate use in the ELISA, dilute the tissue extract 1:20 in Sample Buffer.

2
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 1:20 in Sample Buffer.

PROCEDURE

Materials Provided – See Reagents
Materials Required But Not Provided
0.22 µm filtered deionized water
50-200 µL eight channel multi-pipette
10-200 µL single pipette
Microwell plate reader at 450 nm
0.5 M H₂SO₄ (Note: Use caution when handling sulfuric acid)
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)
TRIS Buffered Saline (TBS), pH 8.5

Assay Procedure

Day ONE
1. Remove the necessary number of antibody coated microwells from the foil pouch. Return unused microwells to the pouch and reseal it with the desiccant inside and store at 2°-8°C.
2. Add 100 µL of uPA Standard, control or diluted sample to the microwell, cover and incubate 16-20 hours at 4°C. Perform measurements in duplicate.

Day TWO
3. Wash all microwells 4 times with Wash Buffer.
4. Add 100 µL of Detection Antibody to each microwell, cover and incubate for 1 hour at room temperature.
5. Wash all microwells 4 times with Wash Buffer.
6. For using all 96 microwells 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 using less then 96 wells). Add 100 µL of diluted enzyme conjugate to each microwell, cover and incubate for 1 hour at room temperature.
7. Wash all microwells 4 times with Wash Buffer.
8. Add 100 µL of Substrate solution to each microwell, cover and incubate for 20 minutes at room temperature. A blue color will develop.
9. Stop the enzymatic reaction by adding 50 µL of 0.5 M H₂SO₄. Tap the sides of the microwells to ensure even distribution of the H₂SO₄. The solution color will turn yellow. Read the absorbances on a microwell plate reader at a wavelength of 450 nm within 10 minutes.

RESULTS

Representative Standard Curve
Construct a standard curve by plotting the mean absorbance value calculated for each uPA standard versus its corresponding uPA concentration. Interpolate the uPA concentrations for the diluted samples directly from the standard curve. A standard curve should be generated each time the assay is performed. The following curve is for demonstration purposes only, plotted using a 2nd order polynomial regression analysis.

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

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

B. Plasma and Tissue Culture Supernatant Samples
Multiply the sample value by the dilution factor to determine the uPA concentration of the plasma/tissue culture supernatant.


