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Activity Assay for
Human uPA
Product No. PL 91
For Research Use Only

INTRODUCTION

Urokinase-type Plasminogen Actvator (uPA) is a glycosylated serine protease that is synthesized in endothelial and kidney epithelial cells. There are two forms of uPA, the low molecular weight (LMW ~31 kDa) and high molecular weight (HMW ~55 kDa). The HMW form undergoes autoproteolysis resulting in the LMW plus and 18.5 kDa amino terminal fragment (ATF). It is this ATF that has been shown to inhibit proliferation and invasion of cancer cells by binding to uPA receptors (1).

PRINCIPLES OF PROCEDURE

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of active uPA levels in biological fluid. This test kit operates on the basis of sandwich ELISA where free active uPA enzyme complexes with PAI-1 and is quantitated with the use of an HRP labeled secondary antibody.

First, the biotinylated PAI-1 binds to the avidin coated wells. Next, active uPA present in the standard or unknown, complexes with PAI-1. Inactive or complexed uPA is removed in a subsequent wash step. A primary antibody specific for uPA is then added to each well followed by the HRP conjugated secondary antibody. The bound conjugated secondary antibody is detected by the addition of substrate, which generates an optimal color after 10 minutes. Quantitative test results may be obtained by the measure and comparison of the sample and standard absorbance readings when read with a microplate reader at 450 nm.

MATERIALS PROVIDED

Component Description	Volume	Storage
Biotinylated PAI-1	1 lyopholized vial	4°C
Human uPA Activity Standard	1 lyopholized vial	4°C
TMB Substrate	10 mL	4°C
Anti-Human uPA Primary Antibody	1 lyopholized vial	4°C
HRP Secondary Antibody	1 vial	4°C
10x Wash Buffer	50 mL	4°C
Coated Plate		4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Precision pipettes with a range of 5 µL to 1,000 µL with disposable tips
2. Multichannel pipette
3. 96-well plate reader for measurement of absorbance at 450 nm

4. Deionized water
5. 1 N Sulfuric Acid (H_2SO_4)
6. Blocking Buffer (See Reagent Preparation)
7. TBS Buffer (See Reagent Preparation)
8. Microplate Shaker

STORAGE

Store this kit and all of its components at 4°C at all times.

WARNINGS AND PRECAUTIONS

1. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents is not recommended as it may be detrimental to the assay.
2. Exercise universal precautions during the performance or handling of this kit or any component contained therein.
3. All specimens should be considered potentially infectious. Exercise proper handling precautions.

PROCEDURAL NOTES

1. This assay should be performed at room temperature.
2. If not using the entire plate at once, prepare only the appropriate amount of primary antibody, biotinylated PAI-1 and uPA standard. The remaining stock solutions should then be frozen and stored at –70°C. Viable for up to two weeks when stored at –70°C. All other components should remain refrigerated.
3. Use aseptic technique when opening and removing reagents from vials and bottles.
4. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION AND STORAGE

Samples should be collected using trisodium citrate, acidified citrate or Stabilite^{tm} (DiaPharma) collection media. Collection should be in accordance with the collection vials manufacturers instructions or in a 1:10 ratio of collection media to blood.

Immediately, upon collection of blood, the samples should be centrifuged at 3000 x g. This should ensure the removal of platelets as they can release PAI-1 that in turn complexes with uPA. The plasma can be transferred to a clean plastic tube and stored frozen for up to one month. Samples are stable for approximately 5 hours when stored at 4°C with the Stabilyte^{tm} media. Store at -70°C for extended storage up to 5 months.

If you expect high concentrations of uPA from your samples they can be diluted with 3% BSA Blocking Buffer (see Reagent Preparation below).

Note: Detergents such as Triton X cause interference with the assay. If using detergent extracted samples, it is necessary to dialyze the samples overnight to remove the detergent.

REAGENT PREPARATION

The following solutions should be prepared fresh before starting the assay.

1. **TBS Buffer:** 0.10 M TRIS, 0.15 M NaCl, pH 7.4

2. **Blocking Buffer:** 3% BSA in TBS Buffer
3. **Wash Buffer:** Dilute to 1x with deionized water

STANDARD CURVE PREPARATION

See attached Dilution Table.

ASSAY PROCEDURE

Note: This assay should be performed at room temperature.

1. Add 10 mL of the 3% BSA Blocking Buffer solution directly to the Biotinylated PAI-1 vial and slightly agitate until completely dissolved.
2. Add 100 µL of the BSA/Biotinylated PAI-1 solution to the both standard and test wells.
3. Shake the plate at 300 rpm on a plate shaker for 30 minutes.
4. Wash wells according to the following wash procedure:
   a. Remove contents of the plate by inversion into an appropriate disposal device.
   b. Tap out remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Remove contents of the plate by inversion into an appropriate disposal device.
   f. Repeat procedure 2 more times, then proceed to step "g".
   g. Tap out the remaining contents of the plate onto a lint-free paper towel and proceed to step 6.

   Note: The decanted wells should be void of visible moisture before proceeding. If moisture is still visible then follow step "g" until satisfactory results are obtained.
5. Prepare the standards as indicated in the provided dilution table.
   Note: The standards should be applied to the plate immediately upon preparation.
6. Add 100 µL of the standards and samples to each well. See **Scheme I** for a suggested plate layout. Shake plate at 300 rpm on the plate shaker for 30 minutes.
7. Wash wells according to step 5 located above in this section.
8. Make a working concentration of Primary Antibody by adding the indicated amount of 3% BSA Blocking Buffer directly to the lyophilized Primary Antibody vial. Shake lightly or mix by inversion.
9. Add 100 µL of the BSA/ Anti-Human uPA Primary Antibody to each well. Shake plate at 300 rpm on the plate shaker for 30 minutes.
10. Wash wells according to step 5 located above in this section.
11. Combine the indicated amount of Secondary Antibody and 3% BSA Blocking Buffer.
12. Add 100 µL of the BSA/ Secondary Antibody solution to each well. Shake plate at 300 rpm on the plate shaker for 30 minutes.
13. Wash wells according to step 5 located above in this section.
14. Add 100 µL of TMB Substrate to each well and incubate for 10 minutes.
15. Stop the reaction with 50 µL per well of 1 N H_2SO_4 and read the plate at 450 nm.
16. If accounting for substrate background, use 2 wells as blanks with only substrate in the wells (150 µL/well).

Scheme I:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_2
B	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_2
C	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}
D	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}

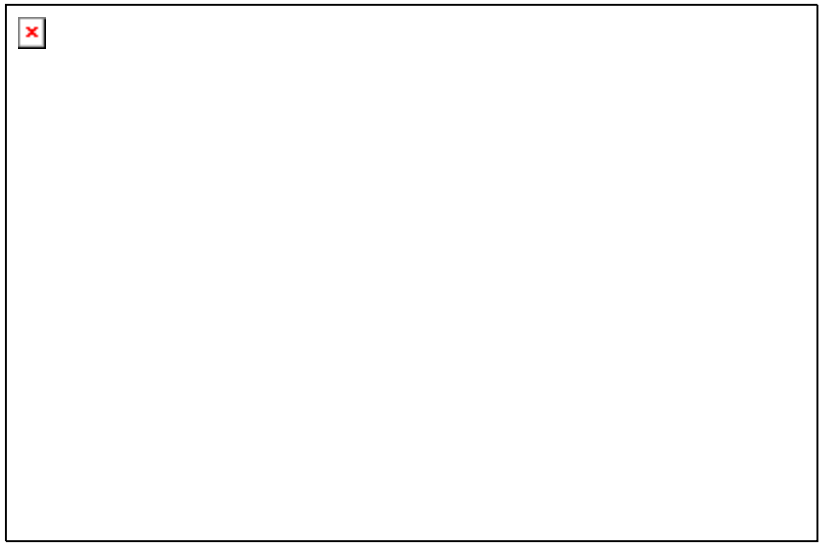
E	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
F	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
G	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}
H	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}

CALCULATIONS

Many microplate readers can be programmed to do these calculations automatically when reading the plate. Consult your instrument manual.

1. Subtract the average O.D. value of the blank wells (BLK) from all other pairs of wells.
2. Average the O.D. values for each pair of duplicate wells.
3. Plot a standard curve using the average O.D. value for each standard value versus the concentration of standard.
4. Determine the concentration of each unknown by interpolation from the standard curve.

Figure 2: Typical Standard Curve



PERFORMANCE CHARACTERISTICS

Assay Range: 0.1- 50 ng/mL

Samples with uPA levels higher than 50 ng/mL should be diluted in similar media devoid of active uPA or 3% BSA Blocking Buffer.

REFERENCES

1. Luparello, C., *et al*. (1996) *Eu J Cancer* A: 702-707

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