Colorimetric Assay for
Total Mouse uPA
Product No. PL 95

For Research Use Only

Please read all instructions carefully before beginning this assay.
Store kit and its components at 4° C.

DESCRIPTION

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).

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of total uPA levels in biological fluid. This test kit operates on the basis of sandwich ELISA where active, latent and complexed 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, latent or complexed uPA present in the standard or unknown, complexes with PAI-1. A primary antibody specific for the various uPA forms is then added to each well followed by the HRP conjugated secondary antibody. The 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

1. **Mouse uPA Standard:** 1 vial.
2. **Substrate:** 10 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle
3. **Anti-Mouse uPA Primary Antibody:** 1 lyophilized vial of anti-mouse uPA antibody.
4. **HRP Secondary Antibody:** 1 vial of HRP conjugated secondary antibody.
5. **10X Wash Buffer:** 50 mL of 10x Wash Buffer. Bring to 1x prior to use.
6. **Coated Plate:** A 96 well microplate with capture antibody precoated on each well. The plate is ready for use as is. **DO NOT WASH!**

MATERIALS NEEDED BUT NOT PROVIDED

1. 1 N H_2SO_4.
2. Blocking buffer (see Reagent Preparation).
3. TBS buffer (see Reagent Preparation)
4. DI water.
5. Microplate reader with 450 nm filter.
6. Microplate shaker with uniform horizontal circular movement up to 300 rpm.
7. Beakers, flasks, cylinders, etc. required for preparation of reagents.
8. Precision pipettes that range from 1 µL-1000 µL and disposable tips.
9. Plastic film or plate cover to cover plate during incubation.

WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. **DO NOT** pipette reagents by mouth.
4. Always pour substrate out of the bottle into a clean test tube - **DO NOT** pipette out of the bottle (if your tip is unclean you could contaminate your substrate).
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be stored as instructed when not in use.

PROCEDURAL NOTES

1. Always use new pipette tips for the buffer, conjugate, standards, samples etc.
2. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
3. When pipetting into the wells, DO NOT allow the pipette tip to touch the inside of the well, or any of the reagents already in the well - this can cause cross contamination.
4. Standards and samples should be assayed in duplicate.
5. To quantitate, always run a standard curve when testing samples.
6. Gently mix specimens and reagents before use. Avoid vigorous agitation.
7. Before taking an absorbance reading, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
8. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Seal with a heat sealer. If a heat sealer is not available, thoroughly close the open end with tape. Remove excess air before sealing.
9. If not using the entire plate at once, prepare only the appropriate amount of primary antibody 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.

SAMPLE PREPARATION

Samples should be collected using trisodium citrate, acidified citrate or Stabilite™ (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 24 hours when stored at 4° C with the Sabilyte™ media.

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.

-TBS Buffer: 0.10 M TRIS, 0.15 M NaCl, pH 7.4
-Blocking Buffer: 3% BSA in TBS buffer (indicated above or as provided)

TEST PROCEDURES

Note: This assay should be performed at room temperature.

1. Remove microplate from the bag.
2. Prepare standards as indicated in the provided dilution table.
   Note: The standards should be applied to the plate immediately upon preparation.
3. Add 100 µL of the prepared standards and samples to each designated well. See **Scheme 1** for a suggested plate layout.
4. Shake the plate at 300 rpm on a plate shaker for 30 minutes.
5. 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. Repeat procedure 2 more times then proceed to step “f”.
   f. Remove contents of the plate by inversion into an appropriate disposal device.
   g. Tap out the remaining contents of the plate onto a lint free paper towel then 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.

6. Make a working concentration of Primary Antibody by reconstitution according to the amount indicated on the vial using 3% BSA blocking buffer. Slightly agitate the vial contents until completely dissolved.
7. Add 100 µL of the BSA/ Anti-Mouse uPA Primary Antibody to each well.
8. Shake plate at 300 rpm on the plate shaker for 30 minutes.
9. Wash wells according to step 5 located above in this section.
10. Make a working concentration of Secondary Antibody by combining the indicated amounts of secondary antibody and 3% BSA blocking buffer. Slightly agitate or mix by inversion.
11. Add 100 µL of the BSA/ secondary antibody solution to each well.
12. 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 allow to incubate for 10 minutes.
15. Quench reaction with 50 µL per well of 1 N H_2SO_4 and read plate at 450 nm.
16. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.
    NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

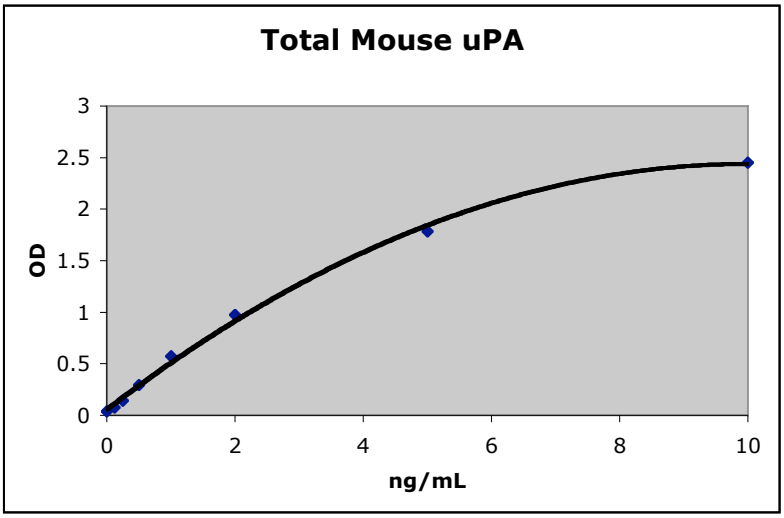
Scheme I

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	U_1	U_2	U_3	U_4
B	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	U_1	U_2	U_3	U_4
C	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U_{16}
D	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U_{16}
E	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}	U_{27}	U_{28}
F	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}	U_{27}	U_{28}
G	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}	U_{39}	BLK
H	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}	U_{39}	BLK

CALCULATIONS

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.

TYPICAL STANDARD CURVE



TYPICAL DATA

Note: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life or due to lot variance.

Typical Data:

Standard	Standard Concentration (ng/mL)	Optical Density (Absorbance Value)
S_0	0	0.040
S_1	0.125	0.075
S_2	0.25	0.142
S_3	0.5	0.295
S_4	1	0.573
S_5	2	0.975
S_6	5	1.784
S_7	10	2.451

PERFORMANCE CHARACTERISTICS

Assay Range: 0.125- 10 ng/mL

Samples with uA levels higher than 10 mg/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

TECHNICAL SUPPORT

If you need technical information or assistance with assay procedures, call our Technical Support Department at 1-800-692-4633 or 1-248-852-8815. Our staff will be happy to answer your questions about this or any other product in the Oxford Biomedical line.

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