

INSTRUCTIONS FOR USE

uPA ELISA

Item No. 06489892

The logo for WILEX, featuring the word "WILEX" in a bold, black, sans-serif font. A small red triangle is positioned above the letter "X".

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Manufactured by:

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Intended Use

The uPA ELISA is an enzyme-linked immunoassay used to quantitate pro-, high molecular weight (HMW)- and low molecular weight (LMW)-uPA (urokinase-type plasminogen activator) found in human serum or plasma.

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Method Principle	Solid Phase Sandwich ELISA
Analytical Range	0 pg/mL to 350 pg/mL
Specimen Types	Human serum or plasma
Sample Test Volume	100 microliters of sample preparation per well Specimen preparation varies
Sensitivity	25 pg/mL

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Background

uPA is a 52-kDa serine proteinase. It participates in the proteolytic process, which takes place in tissue remodeling, cell migration, and angiogenesis, as well as tumor cell invasion and metastasis. uPA is secreted by cells as an inactive single-chain precursor, pro-uPA. Enzymatic cleavage of pro-uPA at lysine 158 produces an active heterodimer, called high molecular weight-uPA (HMW-uPA), consisting of two subunits, A and B. When pro-uPA is secreted, it binds to a receptor (uPAR) on the cell surface through an EGF-like domain on the A chain. Subsequent binding of plasmin to uPA can convert pro-uPA into the proteolytically active heterodimer. In turn, active uPA rapidly converts the inactive plasmin precursor, plasminogen, to enzymatically active plasmin, which is directly involved in extracellular matrix degradation as well as in the activation of other procollagenases and latent growth factors [1–4]. Additional cleavage of uPA after lysine 135 releases the 17-kDa amino-terminal fragment (ATF), leaving the carboxy-terminal 33-kDa low molecular weight-uPA (LMW-uPA), which retains full catalytic activity [5].

The enzymatic activity of uPA is regulated by two plasminogen activator inhibitors called PAI-1 and PAI-2. These can bind to the catalytically active B chain of uPA. When active uPA is bound to its receptor, subsequent PAI-1 binding results in internalization and degradation of the complex [6].

Secreted uPA may originate from several cell types, including tumor tissue [7], adjacent stromal cells, or fibroblasts [8]. The significance of uPA expression and subsequent proteinase activity has been the subject of detailed studies in a variety of models. In 1992, Huber, et al. [9], examined plasma levels of uPA and alpha-fetoprotein (AFP) in liver cancer and showed that by combining the information from the two tumor markers, the accuracy of detection of liver cancer

was increased. In 1993, Huber, et al., showed similar results in colon cancer using plasma levels of uPA and CA 19-9 [10]. In an earlier study, it was determined that breast cancer patients had elevated plasma uPA levels in comparison to normal controls [11]. More recently, another report showed that elevated uPA levels can serve as a prognostic factor in metastatic breast cancer, and that metastatic breast cancer patients with elevated uPA levels have a shorter time to progression and shorter survival when treated with second line hormonal therapy [12]. Studies have also discovered elevated uPA levels in colon and pancreatic cancer patients; for example, one study reported that serum uPA levels were elevated in 28 of 80 (35%) female pancreatic cancer patients and 53 of 108 (49%) male pancreatic cancer patients compared to controls [13,14].

Although more work is needed, clearly plasma or serum as a sample can be useful for examining the potential clinical utility of uPA in a variety of cancers and at any time during cancer development. Serum or plasma samples can be analyzed for all forms of uPA using the uPA ELISA.

Principle of the Assay

The uPA ELISA is a sandwich enzyme immunoassay that utilizes two monoclonal antibodies to human uPA as the capture reagents. These Capture Antibodies have been immobilized on the interior surface of microtiter plate wells. To perform the test, an appropriate volume of specimen is incubated in the coated well to allow binding of the antigen by the Capture Antibody. The immobilized antigen is then reacted with the uPA detector rabbit antiserum. The amount of Detector Antibody bound to antigen is measured by binding it with a goat-anti-rabbit IgG-horseradish perox-

idase (HRP) Conjugate. Color development by incubation with OPD (o-phenylenediamine) Substrate enables quantitation of captured uPA. The colored reaction product is quantitated by spectrophotometry and reflects the amount of uPA analyte in the sample. The uPA ELISA is completely formulated and offers convenient testing format options.

For instructions, see the Detailed Protocol and Evaluation of Results sections of this booklet.

Summary of Procedure

Steps	Incubations
1. Add samples and Standards to wells	2 hours, 37°C
2. Wash	
3. Add Detector Antibody to wells	1.5 hours, RT*
4. Wash	
5. Add Conjugate to wells	30 minutes, RT*
6. Wash	
7. Add Substrate to wells	45 minutes, RT* (dark)
8. Add Stop Solution to wells	
9. Read plate at 490 nm	

*Room temperature (20–27°C)

Materials Provided

Samples and Standards should be assayed in duplicate. A standard curve must be included each time samples are analyzed. The following components are supplied:

Microtiter plate—Supplied one (1) precoated and ready to use, with 96 wells (12 strips of eight wells) in a zip-lock bag with a desiccant pack. Wells are coated with two unique monoclonal antibodies.

uPA Standards—Six (6) separate vials containing lyophilized pro-uPA. Standards levels have been calibrated in picograms of uPA per mL. Reconstitute Standard vials with 1 mL of Filtered RO Water included in this kit. Do NOT further dilute Standards.

Standard#	Concentration	Volume/Vial
6	350 pg/mL	1 mL
5	250 pg/mL	1 mL
4	150 pg/mL	1 mL
3	75 pg/mL	1 mL
2	25 pg/mL	1 mL
1	0 pg/mL	1 mL

Filtered RO Water—One (1) 7-mL bottle containing Filtered RO Water.

Sample Diluent—One (1) bottle containing 100 mL of PBS (phosphate-based buffer) with BSA and 0.1% sodium azide.

Detector Antibody—One (1) bottle supplied ready to use, containing 12 mL of rabbit anti-uPA antiserum in 0.01 M PBS (pH 7.4), a protein stabilizer, and 0.1% sodium azide.

Conjugate Diluent—One (1) bottle containing 12 mL of 0.01 M PBS (pH 7.4), BSA, and 0.01% chloroacetamide.

Conjugate Concentrate—One (1) vial containing 0.4 mL of 50X goat-anti-rabbit IgG-horseradish peroxidase in buffer. Must be diluted to 1X with Conjugate Diluent to make Working Conjugate. See **Table 1**, p. 15.

Substrate Diluent—Two (2) bottles, 9 mL each of 0.1 M citrate buffer (pH 5.0) and 0.01% H₂O₂ (hydrogen peroxide).

Substrate Tablets—One (1) vial containing four (4) OPD tablets. These must be dissolved in Substrate Diluent (1 tablet/4 mL) to make Working Substrate. See **Table 1**, p. 15.

Stop Solution—One (1) bottle supplied ready to use, containing 12 mL of 2.5 N H₂SO₄ (sulfuric acid).

Platewash Concentrate (20X)—One (1) 100-mL bottle of phosphate-based buffer. Dilute one (1) part concentrate in 19 parts high-quality deionized water prior to use.

Materials Required but Not Provided

- Pipettors: 2–20 μL , 20–200 μL , and 200–1000 μL precision pipettors with disposable tips
- Precision repeating or multichannel pipettor
- Wash bottle or multichannel dispenser for plate washing
- Microcentrifuge and tubes for sample preparation
- Vortex mixer
- Plate reader or spectrophotometer capable of measuring absorbance in 96-well plates at a wavelength of 490 nm
- 500- or 1000-mL graduated cylinder
- Reagent reservoirs
- Deionized water of high quality
- Plastic wrap or adhesive plate sealers
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels or laboratory lint-free wipes
- 37°C incubator

uPA ELISA Controls—Controls have been developed to provide customers with control material for quality monitoring of day-to-day assay performance. uPA ELISA Controls consisting of lyophilized uPA are sold separately. Refer to uPA ELISA Controls Item No. 06489906 when ordering. Controls are supplied in lyophilized form. Controls should be reconstituted with 1.0 mL of deionized water. Controls should be added to the uPA ELISA microtiter plate at 100 μ L/well. Store reconstituted uPA ELISA Controls at 2–8°C. Protect from light. Controls that have been reconstituted may be stored at 2–8°C, protected from light, and used for up to two weeks.

Precautions and Recommendations

- Store components at 2–8°C. Do not expose reagents to excessive light. Do not freeze any of the kit components.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain either sodium azide or chloroacetamide as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipet or ingest any of the reagents.

- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves and dispose of biological samples properly.
- Do not handle the Substrate Tablets with fingers or permit contact with skin, metal, or oxidizing agents. Dispose of OPD-containing solutions in compliance with local regulations.
- Wear disposable gloves and eye protection when handling Stop Solution (2.5 N sulfuric acid).

Sample Preparation

SERUM AND PLASMA

Remove all flocculent material from specimens by centrifugation in a tabletop microcentrifuge. The initial concentration of the serum or plasma specimen to be examined should not exceed a concentration of 10% (a 1:10 dilution of specimen in Sample Diluent).

Standards and Controls Preparation

Since the uPA Standards are prepared as lyophilized (freeze-dried) material, the operator must reconstitute each Standard with 1 mL of Filtered RO Water, supplied. Prior to opening each vial, ensure that the dried material is at the bottom of each vial. Gently tap the vials, if necessary, to reposition the material. Use a calibrated pipettor and clean tip to dispense 1 mL of Filtered RO Water

into each vial. Replace the rubber stopper, gently swirl to mix, and allow the vials to sit for 5 minutes. Gently invert the vials to ensure that all of the dried material is dissolved. A similar procedure is used for Controls. Refer to the uPA ELISA Controls IFU. Store Reconstituted uPA ELISA Standards at $\leq -18^{\circ}\text{C}$ after use.

Detailed Protocol

RECOMMENDED PROCEDURES

IMPORTANT: Warm all samples, Standards, and other kit reagents to room temperature before addition to assay plate wells.

1. Addition of reagents must be in the order specified.
2. All six Reconstituted Standards, three Controls, and the prediluted test specimens should be run in duplicate. Change tips during this process. Avoid carryover of one Standard into another. The transfer of samples, Standards, and Controls from tubes to the microtiter wells can be greatly simplified by using semi-automation. The hand-held expandable and programmable 8-place pipet, Impact EXP[®] (Matrix Technologies Corp., Hudson, NH), is ideal for this purpose.
3. **CAUTION:** When inverting the microtiter plate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. Fill the open portion of the frame with uncoated or used strips when 96-port mechanical washers are used.

4. Preparation of Platewash
 - a. If the Platewash Concentrate is cold, allow it to reach room temperature (20–27°C) before use (approximately 45 minutes). Make sure all crystals are dissolved. If necessary, warm at 37°C and mix or shake.
 - b. Dilute one (1) volume of Platewash Concentrate with 19 volumes of distilled or deionized water. Mix well. This solution is Platewash. The total volume required will depend on the washing method/instrument used. Approximately 1 L of this solution is required to prime an automated washer and run one microtiter plate; about 700 mL is required for each microtiter plate when manual washing is performed.
 - c. Platewash must be freshly prepared each day. Do not store Platewash.
5. Microtiter plate washing may be automated, semiautomated, or manual but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned, and maintained. Whichever method is used, the solution used to wash plates must be Platewash.
 - a. Automatic Microtiter Plate Washer—Set the fill volume to 300 μL /well. Prime the instrument with Platewash. Use two 3-cycle washes. After the initial 3-cycle wash, rotate the plate 180 degrees and repeat.
 - b. Manual Microtiter Plate Washer—Wash six times, using 300 μL per well per wash. Fill the entire plate, then aspirate in the same order.

- c. Hand-Held Syringe—Wash six times, using 300 μL per well per wash. Blot the plate upside-down between washes.
6. After the final wash, invert the microtiter plate and firmly strike it on an absorbent surface. Wells should not be completely dry. Some residual liquid protects bound reagents from the damages of dessication. Visually check that all wells are empty.

Assay Procedure

1. Remove the microtiter plate from the bag. From the number of specimens to be tested, calculate the number of strips required. (Remember that each specimen dilution or Standard requires two wells, and two wells are needed for Substrate blank determination. The standard curve requires 12 wells.) Store unused strips in the zip-lock bag with desiccant at 2–8°C (see Reagent Stability and Storage).
2. Dilute specimens in Sample Diluent (See Sample Preparation. Do NOT dilute Standards).
3. Briefly, without foaming, mix the Standard or specimen dilution and add 100 μL each to duplicate wells. Set up four wells with the 0 pg/mL Standard, two to measure the background absorbance and two for use as the Substrate blank wells.
4. Cover the microtiter plate with a piece of plastic wrap or adhesive sealer, and **incubate for two hours at 37°C**.
5. Remove the plastic wrap and wash the microtiter plate with Platewash as in Step 6 of the Detailed Protocol. Tap dry on a stack of paper towels or laboratory lint-free wipes.

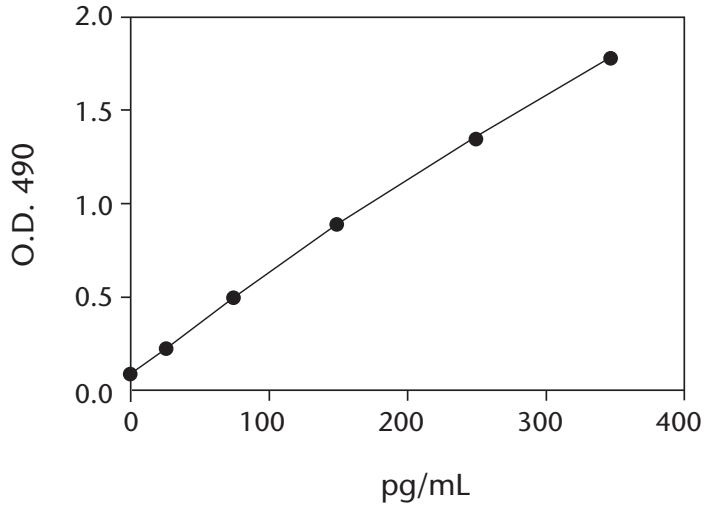
6. Add 100 μL of Detector Antibody to all wells **except the Substrate blank well. Incubate at room temperature (20–27°C) for 1.5 hours.**
7. During the incubation with Detector Antibody, prepare Working Conjugate by diluting the Conjugate Concentrate at 1:50 in Conjugate Diluent, and dispense into a clean reagent reservoir. See **Table 1**, p. 15, for the quantities to use for the number of strips being run.
8. Wash the microtiter plate with Platewash. Tap dry on a stack of paper towels or laboratory lint-free wipes.
9. Add 100 μL of Working Conjugate from Step 7 to all wells **except the Substrate blank well. Incubate at room temperature (20–27°C) for 30 minutes.**
10. During the incubation with Working Conjugate, prepare Substrate by dissolving Substrate Tablets in Substrate Diluent. See **Table 1**, p. 15, for the quantities to use for the number of strips being run. Vortex vigorously to assure complete solubilization. Once prepared, Substrate should be used within 30 minutes. Avoid exposure to light.
11. Wash the microtiter plate with Platewash. Tap dry on a stack of paper towels or laboratory lint-free wipes.
12. Including the Substrate blank wells, add 100 μL of Substrate to all wells. **Incubate the microtiter plate in the dark at room temperature (20–27°C) for 45 minutes.**
13. Add 100 μL of Stop Solution to each well to stop the reaction.
14. Read the absorbance at 490 nm within 30 minutes.

TABLE 1. uPA ELISA—PREPARATION OF ASSAY REAGENTS

# Strips Used	Conj. Concentrate	Conj. Diluent	Substrate Tablets	Substrate Diluent
1	20 μ L	0.98 mL	1	4 mL
2	40 μ L	1.96 mL	1	4 mL
3	60 μ L	2.94 mL	1	4 mL
4	80 μ L	3.92 mL	1	4 mL
5	100 μ L	4.90 mL	2	8 mL
6	120 μ L	5.88 mL	2	8 mL
7	140 μ L	6.86 mL	2	8 mL
8	160 μ L	7.84 mL	2	8 mL
9	180 μ L	8.82 mL	3	12 mL
10	200 μ L	9.80 mL	3	12 mL
11	220 μ L	10.78 mL	3	12 mL
12	240 μ L	11.76 mL	3	12 mL

Figure 1

Sample Standard Curve



Evaluation of Results

CONCENTRATION OF STANDARDS

uPA Standards have been calibrated in mass units (pg/mL) using an immunoaffinity purified pro-uPA preparation. These Standards therefore will quantitate pro-uPA as well as HMW-uPA (both 52 kDa). LMW-uPA is also detected by this assay but measurement of this form using the Standards will overestimate the quantity present. This should not adversely affect most measurements of uPA, but in cases where LMW-uPA is known to be present at a significant level, this point should be considered.

CONCENTRATION OF UNKNOWNNS

1. Average the absorbance values for each Standard and specimen dilutions to obtain the mean absorbance.
2. Determine the concentration of uPA for each specimen dilution by interpolation from the standard curve. Software packages are available (such as SoftMax Pro™, Molecular Devices, Sunnyvale, CA and KC4™, Bio-Tek Instruments, Inc., Winooski, VT) that can simplify this process. Use a quadratic (second order polynomial) curve fitting algorithm.

NOTE: Do not assign “blank” wells using software. This will subtract the average blank readings from all other wells. It is useful for quality control and troubleshooting purposes to be able to inspect the absorbance values reported for all wells without adjustments applied to the raw data.

- Results for samples and Controls can be expressed as pg/mL in the original sample by correcting the value obtained from the standard curve by the dilution factor used in the assay.

Sample Values

The levels listed should be used as a guideline only. The determination of normal ranges should be carried out by each laboratory using appropriate samples.

SAMPLE GROUP: HEALTHY MALES AND FEMALES (N=118)

Mean recovery of uPA	Recovery range
1192 pg/mL serum	459–1924 pg/mL serum

Assay Characteristics

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SENSITIVITY

The uPA ELISA will detect 25 pg/mL of uPA in the sample tested. The signal of the 25 pg/mL Standard is greater than two times the zero (background) signal.

SPECIFICITY

The uPA ELISA has been tested for specificity against tPA and cathepsin D and shows no cross-reactivity at high-challenge doses. Also, the assay has been analyzed for parallelism of tumor sample and plasma sample response with excellent agreement of replicate sample dilutions.

RECOVERY

Serum—A preparation of pro-uPA was spiked into neat serum and Sample Diluent. After two-fold serial dilution in Sample Diluent, starting at 1:6, dilutions were tested in the uPA ELISA, and 80% of spiked uPA was recovered in serum as compared to the level recovered from the Sample Diluent preparation.

Troubleshooting

A. ASSAY DOES NOT DEVELOP COLOR OR ODs ARE LOW

- Plate allowed to dry out after Conjugate step.
- Step(s) omitted or in wrong sequence.
- Room temperature during incubations fell below 20°C.
- Substrate was not warmed to room temperature (20–27°C) prior to addition to wells.

B. HIGH BACKGROUND SIGNAL

- Insufficient washing between steps.

- The Substrate blank well should read ≤ 0.08 absorbance units. Zero Standard should read ≤ 0.10 absorbance units. Higher readings indicate deterioration of Substrate or exposure of Substrate to light before or during the incubation step.
- Be certain the plate is read at the correct wavelength.

C. POOR DUPLICATES

- Insufficient washing, especially when accompanied by high background. Take special care when washing plates by hand, or have automatic washer serviced.
- Sporadic high signals may indicate contamination of Substrate by Conjugate. Be sure to use a fresh piece of plastic wrap or adhesive plate sealer for this step. Residual droplets of Conjugate on re-used plastic wrap or adhesive plate sealer may lead to false positive signals.
- Generating bubbles in wells on addition of reagents. Use care in pipetting.
- Splashing of reagents between wells will lead to erroneous results. Avoid jarring the plate.

Reagent Stability and Storage

All of the reagents included in the uPA ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date. Kit reagents should be stored at 2–8°C with the exception of Stop Solution and Platewash Concentrate, which may be stored at room temperature (20–27°C). Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack. Reconstituted Standards should be stored at $\leq -18^{\circ}\text{C}$ for up to 30 days.

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Notes:

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