KAMIYA BIOMEDICAL COMPANY

Mouse Plasminogen Activator, Urokinase Receptor (uPAR) ELISA

For the quantitative determination of mouse uPAR in serum, plasma, tissue homogenates and other biological fluids

Cat. No. KT-25373

For Research Use Only.

Product Information

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INTENDED USE

The kit is a sandwich enzyme immunoassay for the *in vitro* quantitative measurement of mouse uPAR in serum, plasma, tissue homogenates and other biological fluids. For research use only.

COMPONENTS

Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1
Calibrator	2
Calibrator Diluent	1 × 20 mL
Detection Reagent A	1 × 120 µL
Detection Reagent B	1 × 120 µL
Assay Diluent A	1 × 12 mL
Assay Diluent B	1 × 12 mL
TMB Substrate	1 × 9 mL
Stop Solution	1 × 6 mL
Wash Buffer (30X concentrate)	1 × 20 mL
Plate sealer for 96 wells	4

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Single or multi-channel pipettes with high precision and disposable tips.
3. Microcentrifuge Tubes.
4. De-ionized or distilled water.
5. Absorbent paper for blotting the microplate.
6. Container for Wash Solution.
7. 0.01 mol/L (or 1x) Phosphate Buffered Saline (PBS), pH 7.0-7.2.

STORAGE

All the reagents should be kept according to the labels on vials. The **Calibrator**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20°C upon receipt while the others should be at 4℃. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Opened test kits will remain stable for 1 month, provided it is stored as described above.

TEST PRINCIPLE

The microplate provided in this kit has been pre-coated with an antibody specific to uPAR. Calibrators or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to uPAR. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and

incubated. After TMB substrate solution is added, only those wells that contain uPAR, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of uPAR in the samples is then determined by comparing the O.D. of the samples to the calibration curve.

SAMPLE COLLECTION AND STORAGE

Serum

Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4℃ before centrifugation for 20 minutes at approximately 1,000 × g. Assay freshly prepared serum immediately or store samples in aliquot at -20℃ or -80℃ for later use. Avoid repeated freeze/thaw cycles.

Plasma

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1,000 × g at 4℃ within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20℃ or -80℃ for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates

The preparation of tissue homogenates will vary depending upon tissue type.
1. Tissues were rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization.
2. Minced the tissues to small pieces and homogenized them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular location of the target protein) (w:v = 1:20-1:50, e.g. 1 mL lysis buffer is added in 20-50 mg tissue sample) with a glass homogenizer on ice (Micro Tissue Grinders works, too).
3. The resulting suspension was sonicated with an ultrasonic cell disrupter till the solution is clarified.
4. Then, the homogenates were centrifuged for 5 minutes at 10,000 × g. Collect the supernates and assay immediately or aliquot and store at ≤-20℃.

Other biological fluids

Centrifuge samples for 20 minutes at 1,000 x g. Collect the supernates and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

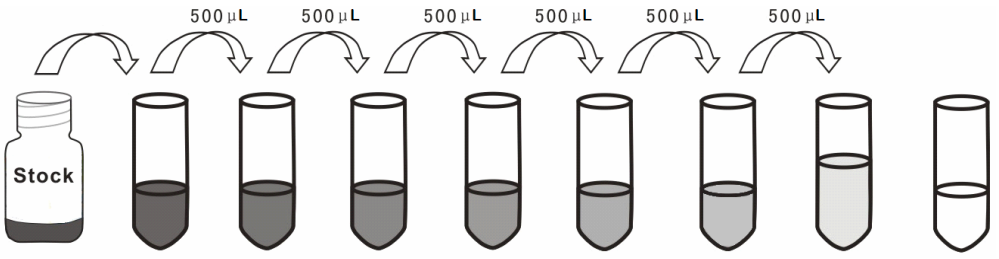
1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. When performing the assay, bring samples to room temperature.
3. Sample hemolysis will influence the result, so hemolytic specimen should not be used.
4. It is highly recommended to use serum instead of plasma for the detection based on quantity of our in-house data.

REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.

Calibrator

Reconstitute the **Calibrator** with 1.0 mL of **Calibrator Diluent**, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the calibrator in the stock solution is 10,000 pg/mL. Please firstly dilute the stock solution to 5,000 pg/mL and the diluted calibrator serves as the highest calibrator (5,000 pg/mL). Then prepare 7 tubes containing 0.5 mL Calibrator Diluent and use the diluted calibrator to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted calibrator such as 5,000 pg/mL, 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL, 312 pg/mL, 156 pg/mL, 78 pg/mL, and the last EP tubes with **Calibrator Diluent** is the blank as 0 pg/mL.



Tube	1	2	3	4	5	6	7	8	9
pg/mL	10,000	5,000	2,500	1,250	625	312	156	78	0

Detection Reagent A and Detection Reagent B

Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute them to the working concentration 100-fold with **Assay Diluent A** and **B,** respectively.

Wash Solution

Dilute 20 mL of Wash Solution concentrate (30X) with 580 mL of de-ionized or distilled water to prepare 600 mL of Wash Solution (1X).

TMB substrate

Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Prepare calibrators within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
2. Making serial dilution in the wells directly is not permitted.
3. Please carefully reconstitute Calibrators or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10 µL for one pipetting.
4. The reconstituted Calibrators, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30X), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

SAMPLE PREPARATION

1. We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

2. Please predict the concentration before assaying. If values for these are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.

3. Serum/plasma samples require about a 2 fold dilution. A suggested 2-fold dilution is 100 µL Sample + 100 µL PBS. Sample should be diluted by 0.01 mol/L PBS (PH=7.0-7.2).

4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.

6. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.

7. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernates may not be detected by the kit.

8. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

ASSAY PROCEDURE

1. Determine wells for diluted calibrator, blank and sample. Prepare 7 wells for calibrator, 1 well for blank. Add 100 µL each of dilutions of calibrator (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 hour at 37°C.

2. Remove the liquid of each well, don't wash.

3. Add 100 µL of **Detection Reagent A** working solution to each well, cover the wells with the plate sealer and incubate for 1 hour at 37°C.

4. Aspirate the solution and wash with 350 µL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 µL of **Detection Reagent B** working solution to each well, cover the wells with the plate sealer and incubate for 30 minutes at 37℃.

6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.

7. Add 90 µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Add 50 µL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20℃.
2. **Samples or reagents addition: Please use the freshly prepared Calibrator.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of calibrators, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.

4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

CALCULATION OF RESULTS

Average the duplicate readings for each calibrator, control, and samples and subtract the average zero calibrator optical density. Construct a calibration curve by plotting the mean O.D. and concentration for each calibrator and draw a best fit curve through the points on the graph or create a calibration curve on log-log graph paper with uPAR concentration on the y-axis and absorbance on the x-axis. Using some plot software is also recommended. If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

PERFORMANCE

Detection Range

78 - 5,000 pg/mL.

The calibration curve concentrations used for the ELISA's were 5,000 pg/mL, 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL, 312 pg/mL, 156 pg/mL, 78 pg/mL.

Sensitivity

The minimum detectable dose of uPAR is typically less than 28 pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero calibrator replicates and calculating the corresponding concentration.

Specificity

This assay has high sensitivity and excellent specificity for detection of uPAR.

No significant cross-reactivity or interference between uPAR and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between uPAR and all the analogues, therefore, cross reaction may still exist.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and calibrators;
2. Add 100 µL calibrator or sample to each well. Incubate 1 hour at 37℃;
3. Aspirate and add 100 µL prepared Detection Reagent A. Incubate 1 hour at 37℃;
4. Aspirate and wash 3 times;
5. Add 100 µL prepared Detection Reagent B. Incubate 30 minutes at 37℃;
6. Aspirate and wash 5 times;
7. Add 90 µL Substrate Solution. Incubate 10-20 minutes at 37℃;
8. Add 50 µL Stop Solution. Read at 450 nm immediately.

IMPORTANT NOTE

1. The final experimental results will be closely related to validity of the products, so the kit should be used prior to the expiration date. And please store the kits exactly according to the instruction.

2. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for reference.
3. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
4. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism. TMB Substrate should remain colorless till it is reacted with the enzyme which binds to the microplate.
5. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microplate from the storage bag until needed.
6. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. at 450 ± 10 nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment.
7. Variation in sample preparation and each step of experimental operation may cause different results. In order to get better reproducible results, the operation of each step in the assay should be controlled.
8. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
9. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
10. The calibrator of the kit and immunogen used for antibody preparation are commonly recombinant proteins, as different fragments, expression systems, purification methods might be used in recombinant protein preparation, we can not guarantee the kit could detect recombinant protein from other companies. So, it is not recommended to use the kit for the detection of recombinant protein.
11. Please predict the concentration of target molecules in samples, or arrange a preliminary experiment, it is a good way to solve specific problem, e.g. the concentration of samples are beyond the detection range of the kit.
12. The kit might not be suitable for detection of samples from some special experiment, for instance, knock-out experiments, due to their uncertainty of effectiveness.
13. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

FOR RESEARCH USE ONLY.

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