suPARnostic®

Enzyme immunoassay for quantitative determination of soluble urokinase plasminogen activator receptor in human plasma and serum

Code no. E001 suPARnostic® AUTO Flex ELISA

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Supporting Patient Triage

Please check www.virogates.com for the most recent updates on all suPARnostic® products, assay instructions, and other tools.

ViroGates

CE IVD

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INTENDED PURPOSE

For *In Vitro* diagnostic use.

The suPARnostic® AUTO Flex ELISA is used for the quantitative determination of soluble urokinase Plasminogen Activator Receptor (suPAR) in human plasma and serum.
Interpretation of results must be made considering the patient's clinical history and if available, results of other diagnostic tests.

The suPARnostic® AUTO Flex ELISA with 8 x 12 break-apart wells is allowing flexibility in the number of samples tested (up to 91 samples in singlets) and/or multiple assays to be performed from one set of reagents.

suPAR IS A MARKER OF DISEASE PROGRESSION

suPAR is the soluble form of urokinase Plasminogen Activator Receptor (uPAR). The amount of suPAR is a measure of immune activation and inflammation.[1] suPAR is a biomarker which is increased by the presence and severity of disease. suPAR has a high negative predictive value for ruling out disease progression. This means that patients with a low (<3 ng/mL) suPAR level have a good prognosis and a low risk of re-admission and mortality[3], supporting the decision of discharge of the patient. A high suPAR level (>6 ng/ml) is a strong measure of chronic inflammation and the underlying risk of negative outcomes, including short term mortality (in hospital, 30 days, or 90 days)[2] supporting the decision of further examination of the patient.

PRINCIPLES OF ASSAY PROCEDURE

suPARnostic® is a CE/IVD marked product range applied for determination of soluble urokinase Plasminogen Activator Receptor (suPAR) in human EDTA-, heparin-plasma or serum.
The suPARnostic® AUTO Flex ELISA is a simplified double monoclonal antibody sandwich assay where samples and peroxidase-conjugated anti-suPAR are mixed in the included mixing plate prior to incubation in the anti-suPAR precoated optically clear microwells. The assay utilizes monoclonal mouse and rat antibodies against human suPAR. The suPAR standard is calibrated against an internal Golden Standard. All values are calculated back to this standard to ensure samples from different labs and/or different assay lots can be directly compared when the suPARnostic® AUTO Flex ELISA is used. suPAR concentrations determined using the suPARnostic® AUTO Flex ELISA are expressed in ng/mL.

In the assay the suPAR standards, curve control, and patient specimens are mixed with peroxidase-conjugated anti-suPAR in the included white microwell mixing plate. This solution is then transferred from the white to the optically clear microwell plate which is pre-coated with anti-suPAR antibody. During a one-hour incubation period, a sandwich is formed consisting of solid-phase antibody, suPAR, and the peroxidase-conjugated antibody. Following a washing step, where unbound material is removed, a chromogenic substrate is added to the wells. The more suPAR a sample contains, the more intense is the blue color which develops. After 20 minutes incubation in the dark, the color development is stopped by the addition of sulphuric acid which changes the color in the wells to yellow. The absorbance at 450 nm is measured using a microtiter plate reader. A calibration curve is prepared from the suPAR standard, and the concentration of suPAR in the patient specimen is determined by interpolation.

REAGENTS

A. Materials supplied in suPARnostic® AUTO Flex ELISA (Code No. E001)

This kit contains reagents sufficient to perform 96 tests - up to 91 patient samples in singlets. The 'break-apart' wells give the flexibility to perform as many or few tests as required. Minimum 3 Standards, 1 Blank, and 1 Curve Control must be included in each assay run. If not, all wells are used the remainder should be returned to the storage pouch with desiccant and stored at 4°C until required for the next analysis.

1. **White Microtiter Plate** with 96 wells for mixing samples, standards, control, and samples with peroxidase-conjugate, in a clear plastic pouch. Quantity: 1 plate. Preparation: Ready to use.

2. **Clear Microtiter Plate** with break-apart wells precoated with anti-suPAR antibody. There are 96 test wells per plate, in an aluminum storage pouch with desiccant sachet. Quantity: 8 wells x 12 strips. Preparation: Ready to use.

3. **Standards:** Recombinant suPAR, in PBS buffer with proprietary additives and 0.05% Bronidox® as preservative. Quantity: 5 Standards, each vial contains 400 µL of standard containing protein stabilizer. For suPAR concentration in this kit lot, please see separate Analytical Value Sheet.

4. **Curve Control:** Recombinant suPAR, in PBS buffer with proprietary additives and 0.05% Bronidox®. Quantity: 1 vial with 400 µL.

5. **Peroxidase Conjugate** (200 x concentrated): Peroxidase-conjugated mouse anti-human suPAR in buffer (containing 50% Ethylene-glycol) with proprietary additives and antimicrobial agent. Quantity: 1 brown vial with 120 µL. Preparation: Depending on the number of wells to be used, prepare the re- quired amount in a suitable container, and use within 30 minutes.
   Precaution: Light sensitive, avoid unnecessary exposure to light. Ensure that conjugate mix is used within 30 minutes of preparation.

6. **Dilution Buffer:** PBS buffer, pH 7.4, with proprietary additives and 0.05% Bronidox® as preservative. Quantity: 1 x 30 mL. Preparation: Ready to use.

7. **TMB Solution:** 3,3',5,5'-tetramethylbenzidine (TMB).
   Precaution: Light sensitive, avoid unnecessary exposure to light. Quantity: 1 x 15 mL. Ready to use. Remove only the amount required for each assay (with 10% extra for pipetting margin).

8. **Wash Buffer:** 10 x concentration of PBS buffer with proprietary additives and 0.05% Bronidox® as preservative. Quantity: 1 bottle containing 100 mL. Preparation: Dilute one plus nine (1:10) with distilled or deionized water.

9. **Stop Solution:** 0.45 M sulfuric acid (H_2SO_4). Quantity: 16 mL. Preparation: Ready to use.

10. **Sealing Tape:** Quantity 4 Sheets of adhesive tape.

11. **Empty Plastic Bottle:** For preparation of conjugate solution, if required. Quantity: 1 bottle. Ready to use.

B. Materials required but not provided

- Adjustable pipette with tips, 10 µL – 100 µL, 100 µL – 1000 µL
- Multi-channel precision pipette, 50 - 300 µL adjustable
- Reagent reservoirs and/or small tubes for preparing dilutions
- Timer
- Deionized or distilled water
- Microtiter plate reader capable of reading absorbency at 450 nm and an additional reference filter 650 nm for dual wavelength reading (450 – 650 nm)
- ELISA microplate washer, squeeze bottle or suitable container to wash wells
- Absorbent paper or cloth
- Refrigerator

C. Reagent storage and stability

Store kit components at 2-8°C. Expiry is indicated on the labels.

REAGENT PRECAUTIONS AND RECOMMENDATIONS

- For professional use.
- Do not use kit components beyond the indicated kit expiration date.
- Do not mix reagents from different kit lots.
- Stop Solution – component number 9 – contains 0.45 M sulfuric acid; avoid contact with skin and eyes.
- Do not expose reagents to excessive light.
- Do not freeze any of the kit components.
- Use only the microtiter wells provided with the kit.
- Do not mouth pipette 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.
- Do not mix samples from different patients or from different blood samplings of the same patient.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves.

SAMPLE COLLECTION AND STORAGE

To prepare plasma samples, whole blood is drawn into a centrifuge tube containing EDTA or heparin anti-coagulant. Centrifuge the blood at 3,000 x g for 10 minutes. Serum samples are prepared according to the recommendation from the manufacturer of the blood collection tubes.
Transfer and store samples in separate marked tubes. Date and identify each sample. For long-term storage, keep at -20°C. Avoid freeze/thaw cycles.

Grossly hemolyzed, lipemic or microbiologically contaminated samples should not be used. Samples with abnormally elevated levels of hemoglobin or bilirubin may interfere with assay performance and sensitivity.

Be aware of possible dilution of suPAR in the case of transfusion, infusion, or similar.

Sample Type	*Sample Requirement*
Plasma or Serum	15 µL for one replicate

ASSAY PROCEDURE - Strip Method (Singlets)

suPARnostic® AUTO Flex ELISA (Code No. E001)

Equilibrate all reagents to room temperature (18 - 26°C) for 1 hour prior to use. Determine the required number of wells from both the White mixing plate and the Clear coated plate. Take the White mixing plate from the zip-lock bag. After use, dry and return the White mixing plate to the zip-lock bag for future use. Carefully cut the end of the foil pouch and remove the Clear coated plate from the pouch; return unused wells to the foil pouch and return to 4°C storage for future use.

Note: Remember to retain the White mixing plate and the plate frame for future use!

Preparation of buffers prior to use:

Wash Buffer, working solution

1. Dilute the required amount of stock solution one plus nine parts (1:10) with distilled or deionized water. For the entire plate, the whole bottle should be added to 900 mL distilled or deionized water. For each strip to be used a minimum of 10 mL Wash Buffer should be prepared. More washing solution will be required if an automatic plate washer is used. The working solution may be stored at 2 – 8°C for up to 6 months.

Peroxidase Conjugate, working solution

2. Prepare the required amount of Conjugate in a clean plastic container of suitable size as described in the table below (using Dilution Buffer, Com-ponent 6). For less than three strips it is recommended that the Conjugate to be added using a single channel pipette. For three or more strips the Conjugate may be added using a multi-channel pipette. The Conjugate should be used within 30 minutes of preparation. Unused Conjugate stock should be protected from light and returned to 4°C storage as soon as possible.

Number of strips	Volume of Conjugate stock (µL)	Volume of Dilution Buffer (mL)
1	15	3
2	20	4
3	25	5
4	30	6
5	35	7
6	40	8
7	45	9
8	50	10
9	55	11
10	60	12
11	65	13
12	70	14

Table 1. Volumes of Dilution Buffer and Conjugate stock solution needed to prepare Conjugate mix depending on number of used strips.

PROCEDURE

1. Decide on the number of Standards to be used, 3-5 Standards.
   Note: This procedure is based on 5 Standards.

2. Calculate the number of wells required for the assay and mark them on the White mixing plate; consider covering wells not to be used to avoid sample contamination. After use, dry the White mixing plate and return it to the zip-lock bag for future use.

3. Calculate the number of strips required for the assay. Carefully cut open the foil pouch and take out the plate. Remove extra strips from the Clear coated plate and return unused strips to the foil pouch with desiccant, seal, and return to 4°C storage for future use.
   Note: On completion of the assay retain both the plate and frames for future use, if less than the full plate has been used.

4. Transfer 15 µL of the Standards (clear cap, 3a, 3b, 3c, 3d, and 3e) to well A1 – E1 in the White mixing plate. It is recommended to add standards in duplicates.

5. Transfer 15 µL of Dilution Buffer (Component 6) to well F1 in the White mixing plate (Blank). It is recommended to measure Blank in duplicates.

6. Transfer 15µL of the Curve Control (blue cap) to well G1 in the White mixing plate. It is recommended to measure Curve Control in duplicates.

7. Transfer 15 µL of each sample to subsequent wells in the White mixing plate as required. It is recommended to measure samples in duplicates. **Note:** Standards, Blank, and Curve Control must be included with each run.

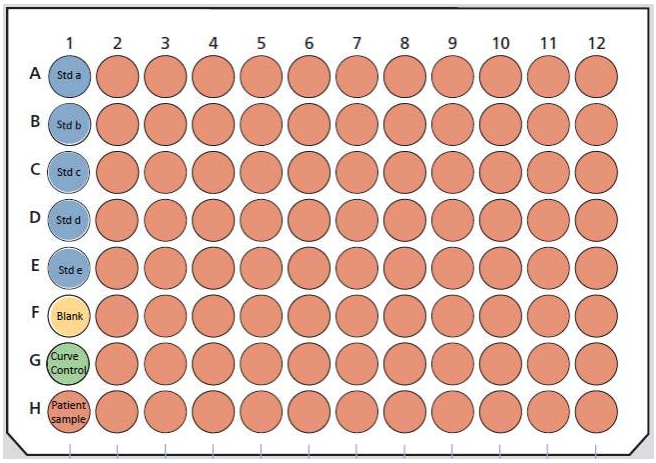


Figure 1 Typical distribution of Standards, Curve Control, Blank and samples on a plate – all singlets.

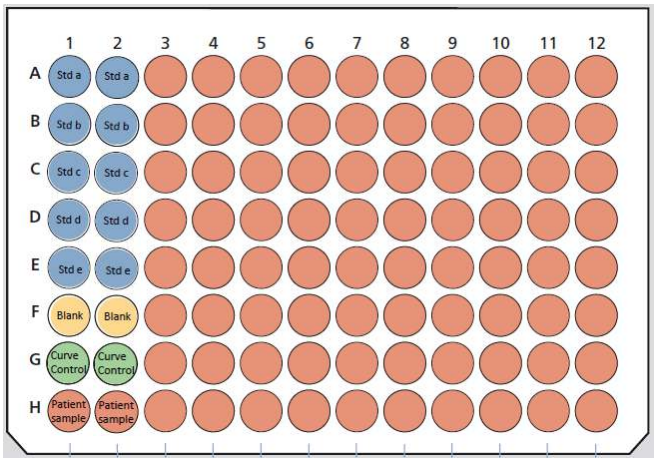


Figure 2 Typical distribution of Standards, Curve Control and Blank in duplicates. Samples in singlets or duplicates.

8. Pipette 135 µL of the Peroxidase Conjugate solution (prepared above) to each used well. It is recommended to use 8-channel multi-pipette and suitable reagent reservoir.

9. Mix gently by slowly pipetting the contents of each well a few times up and down in the pipette tip, then transfer 100 µL to the corresponding well in the Clear coated plate. This should be done using a multi-channel pipette. Ensure the pipette tips are changed between each addition.

10. Cover the Clear coated plate with Sealing Tape (this may be cut to the correct size prior to use) to prevent evaporation and incubate for 1 hour at room temperature (18 - 26°C) in the dark.

11. Gently remove the Sealing Tape and decant the contents from the wells.

12. Wash wells 3 times with 250 µl per well of prepared Wash Buffer. This may be done using a multi- or single channel pipette. After emptying the contents of the wells, pipette 250 µL 1X Wash Buffer into the wells and repeat the process a further 2 times.
    Alternatively, the Wash Buffer may be gently poured into the wells or added using a squeeze bottle. Tap plate gently onto absorbent paper between each wash step. Carefully blot plate after the final wash and ensure no bubbles remain in the wells.
    Note: Improper washing will give erroneous results. This step must be carried out carefully. Do not allow the wells to dry out between incubations.

13. Add 100 µL TMB Solution to each well, cover the Clear coated plate with Sealing Tape and incubate for 20 minutes at room temperature (18 - 26°C) in the dark.
    Note: TMB Solution is easily contaminated. Only remove the required amount for the assay from the bottle (including 10% extra for pipetting margin). Discard unused TMB Solution. Do not return to bottle.

14. Remove the Sealing Tape and stop the reaction by adding 100 µL Stop Solution to each well. The color should change from blue to yellow due to the pH change.

15. Read the absorbance at 450 nm within 30 minutes of stopping the reaction.
    Note: For dual wavelength readers use a reference filter at approximately 650 nm. Ensure there are no air bubbles in any of the wells.

SUMMARY OF ASSAY PROCEDURE – STRIP METHOD (SINGLETS)

The White mixing plate is used in all the following steps:

Add Standards, Curve Control, Blank and, samples	15 µL/well
All wells: Add Conjugation working solution* and mix	135 µL/well
All wells: Transfer diluted samples from White mixing plate to Clear coated plate *	100 µL/well

The Clear coated wells are used in all the following steps:

Incubate at room temperature (18 - 26°C) in the dark	1 hour
Empty the plate and wash (250µL/well)	3 cycles
Add TMB Solution *	100 µL/well
Incubate at room temperature (18 – 26°C) in the dark	20 minutes
Add Stop Solution *	100 µL/well
Read absorbance	450 nm
Reference filter, optional	650 nm

** Use an 8-channel pipette if more than 3 strips are used*

CALCULATION OF RESULTS

ViroGates has developed a program for the calculation of suPAR values from ABS 450nm values. The program is free and can be downloaded at www.virogates.com or requested by e-mail (info@virogates.com).

Alternatively, calculate the corrected absorbance by subtracting the absorbance of the Blank (0 ng/mL) from all wells in the assay. Construct a standard curve in a linear system of co-ordinates by plotting the corrected absorbance of the Standard (ordinate, y-axis) against the corresponding suPAR concentration (abscissa, x-axis). Draw the best fitting line (see Figure 2)

Determine the suPAR concentration from the Curve Control and of each patient specimen by interpolation on the curve. It is not recommended to dilute the patient samples.

Curve control

The suPAR concentration determined for the Curve Control should fall within the range stated in the separate Analytical Value Sheet. If not, further investigation should be made to ascertain whether the cause is imprecise technique, improper handling, or reagent deterioration.

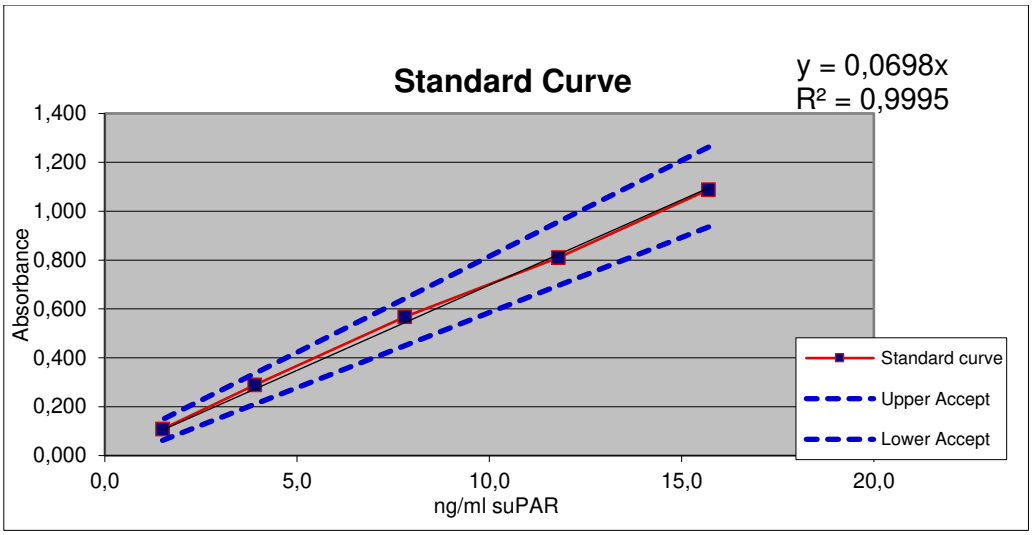


Figure 1 Typical Standard Curve

PERFORMANCE CHARACTERISTICS

A. Imprecision

For each of five plasma samples the imprecision was calculated from the means of up to eight duplicate determinations for up to five separate runs. Standard deviations and coefficients of variation (CV) are listed in the table below. These results were done using one Standard.

Sample (ng/mL)	Within days CV	Between days CV	Total CV	n
2.3	3.5%	5.1%	6.0%	40
2.4	4.7%	3.5%	5.6%	40
3.7	1.3%	2.3%	2.4%	14
5.4	2.1%	2.2%	2.9%	16
7.2	1.7%	1.7%	2.3%	16

B. LOB, LOD, LOQ

Limit of Blank (LOB) shows the variation of blank sample. In this case it was pooled human plasma diluted with dilution buffer at 1+15 ratio.

LOB = 0.1 ng/mL

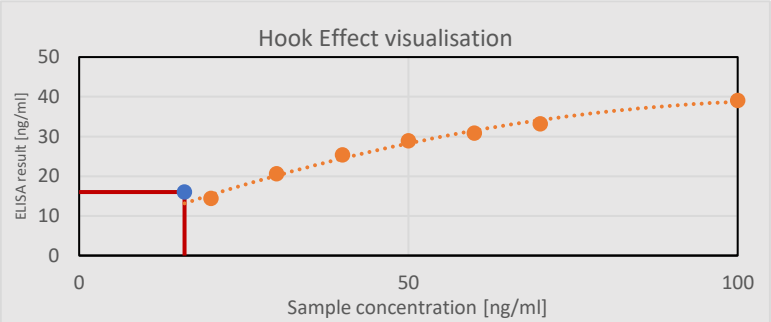
Limit of detection (LOD) is the lowest concentration of suPAR possible to detect that is not a blank sample.

LOD = 0.4 ng/mL

Limit of Quantification (LOQ) is the lowest suPAR concentration that can not only be reliably detected but at which some predefined goals for bias and imprecision are met. LOQ may be the same as LOD or higher.

LOQ = LOD

C. Hook effect



Observation: There is no observed hook effect up to 100 ng/mL suPAR concentration in spiked human plasma.

Note: suPAR concentrations ranging from 0-500ng/mL was measured. Depicted above is the range from 0-100ng/mL.

Results for 200 and 500 ng/mL were stable at maximum level of about 40 ng/mL. This is the limitation of ELISA and plate reader performance because maximum OD for samples after TMB and STOP solution steps is 3.0 (1.0 is established for 15ng/ml).

WASTE HANDLING

Discard unused reagents and waste in accordance with country, federal, state, and local regulations.

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3. Rasmussen LJ, et al. Soluble urokinase plasminogen activator receptor (suPAR) in acute care: a strong marker of disease presence and severity, re-admission, and mortality. A retrospective cohort study. Emerg Med J. 2016 Nov; 33:769-75.