Innovative RESEARCH Results Driven Research

Human alpha1-Microglobulin ELISA kit

Introduction

Alpha1-microglobulin (1M), also called protein HC, is a tubular plasma and tissue protein that belongs to the lipocalin transport protein superfamily for small hydrophobic molecules. It contains 184 amino acids and weighs 26-kDa (1-2). Mature 1M and bikunin (urinary trypsin inhibitor) result from a common precursor (3). 1M is found in blood both in free form and complex-bound to immunoglobulin A (IgA). It is involved in inflammatory and detoxification processes caused by immune system activation, and extracellular heme catabolism (4-5). While increased excretion was detected in urine or serum shortly after tubular injury, 1M may predict acute kidney injury and the need for renal replacement therapy (6). Urinary 1M is useful for the early detection of nephropathy in type 2 diabetic subjects (7).

Principal of the Assay

The Human Alpha1-microglobulin ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human Alpha1-microglobulin in plasma, serum, urine, saliva, milk, and cell culture supernatant. This assay employs a quantitative sandwich enzyme immunoassay technique that measures human Alpha1-microglobulin in less than 4 hours. A polyclonal antibody specific for human Alpha1-microglobulin has been pre-coated onto a 96-well microplate with removable strips. Alpha1-microglobulin in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for human Alpha1-microglobulin, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **Human 1M Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonall antibody against human 1M.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human 1M Standard:** Human 1M in a buffered protein base (160 ng, lyophilized).
- **Biotinylated 1M Antibody (100x):** A 100-fold concentrated biotinylated polyclonal antibody against 1M (80 µl).
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and use supernatants. Dilute samples 1:10000 with EIA Diluent and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:10000 into EIA Diluent. Store serum at -20^{0}C or below. Avoid repeated freeze-thaw cycles
- **Urine:** Collect urine using sample pot. Centrifuge samples at 600 x g for 10 minutes. Urine dilution is suggested at 1:500 in EIA Diluent; however, the user should determine the optimal dilution factor within the range 200-2000x. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:4 with EIA Diluent and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:100 with EIA Diluent and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 160 ng of 1M Standard with 4 ml of EIA Diluent to generate a solution of 40 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard solution (40 ng/ml) 1:2 with EIA Diluent to produce 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[1M] (ng/ml)
P1	Standard (40 ng/ml)	40.00
P2	1 part P1 + 1 part EIA Diluent	20.00
P3	1 part P2 + 1 part EIA Diluent	10.00
P4	1 part P3 + 1 part EIA Diluent	5.000
P5	1 part P4 + 1 part EIA Diluent	2.500
P6	1 part P5 + 1 part EIA Diluent	1.250
P7	1 part P6 + 1 part EIA Diluent	0.625
P8	EIA Diluent	0.000

- **Biotin 1M Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of 1M standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.

If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.

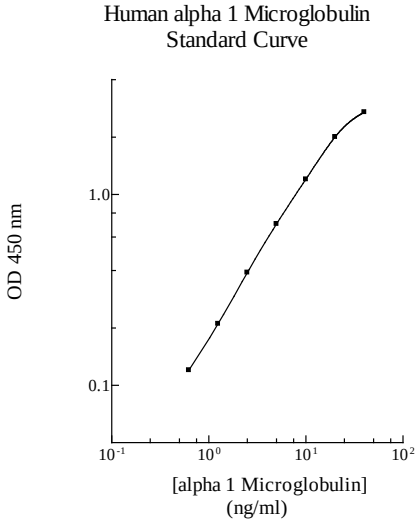
- Add 50 µl of Biotinylated 1M Antibody to each well and incubate for one hour.
- Wash a microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash a microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for about 7 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of 1M is typically less than 0.60 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 5.0% and 7.2% respectively.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:5000	96%	94%
1:10000	102%	100%
1:20000	111%	116%

	Average Percentage of Expected Value
Sample Dilution	**Milk**
1:50	106%
1:100	99%
1:200	93%

Recovery

Standard Added Value	1 – 10 ng/ml
Recovery %	85-105 %
Average Recovery %	96 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	0.1%
Bovine	None
Monkey	> 70% (suggest 1:4000 dilution for plasma)
Mouse	None
Rat	None
Swine	None
Rabbit	None

References

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Version 1.2