#EKL55707, 96 Tests

Human Antithrombin (AT) ELISA Kit

FOR IN VITRO AND RESEARCH USE ONLY. NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

INSTRUCTION MANUAL

Last Updated 2024-12-20

The manual may be updated as a result of continuous improvements.

Please always refer to the hard copy manual included in the kit for your experiment.

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Human Antithrombin (AT) ELISA Kit

Catalog No: EKL55707

96 Tests

INTENDED USE

This kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of AT in human serum, plasma, tissue homogenates, cell lysates, cell culture supernatants and other biological fluids.

REAGENTS AND MATERIALS PROVIDED

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	2
Standard	2	Standard Diluent	1 × 20 mL
Detection Solution A	1 × 12 mL	TMB Substrate	1 × 9 mL
Detection Solution B	1 × 12 mL	Stop Solution	1 × 6 mL
Wash Buffer (30× concentrate)	1 × 20 mL	Instruction manual	1

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution.

STORAGE OF THE KITS

1. ***For unopened kits:*** All the reagents should be stored at **-20°C** upon receipt.
2. ***For opened kits:*** Once the kit is opened, the remaining reagents still need to be stored according to the above storage conditions. In addition, return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

Note:

For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this date.

It is highly recommended to use the remaining reagents within 1 month of opening.

SAMPLE COLLECTION AND STORAGE

- **Serum** - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000 × g. Assay freshly prepared serum immediately or store samples in aliquots at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.
- **Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2-8°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquots at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.
- **Tissue homogenates** - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues should be rinsed in ice-cold PBS (0.01 mol/L, pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Mince the tissues to small pieces and homogenize them in 5-10 mL of PBS with a glass homogenizer on ice (Micro Tissue Grinders also work). The resulting suspension should be sonicated with an ultrasonic cell disrupter or

subjected to 2 freeze/thaw cycles to further break the cell membranes. Then, centrifuge the homogenates for 5 minutes at 5000 × g. Remove the supernatant and assay immediately or aliquot and store at ≤-20°C.

- **Cell Lysates** - Cells must be lysed before assaying according to the following directions.

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells 3 times in cold PBS.
3. Resuspend cells in PBS (1×) and subject the cells to ultrasonication 4 times (or Freeze cells at ≤-20°C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle 3 times.).
4. Centrifuge at 1500 × g for 10 minutes at 2-8°C to remove cellular debris.

- **Cell culture supernatants and other biological fluids** - Centrifuge samples for 20 minutes at 1000 × g. Remove particulates and assay immediately or store samples in aliquots at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

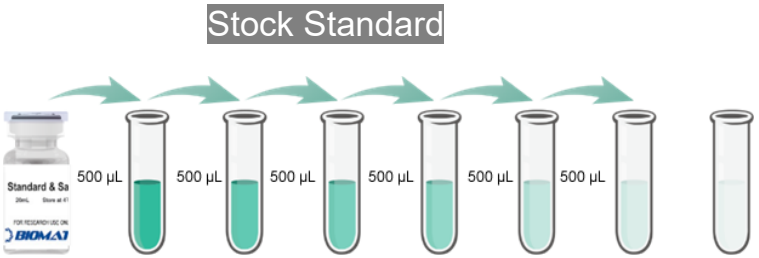
Note:

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/or contamination.
2. Noticeable hemolysis will affect antibody-antigen reactions. Samples with any sign of hemolysis are not acceptable for this assay.
3. When performing the assay, bring samples to room temperature.

REAGENT PREPARATION

1. **Bring all kit components and samples to room temperature (18-25°C) before use.**
2. **Standard** - Reconstitute the Standard with 1.0 mL of Standard Diluent, keep for 10 minutes at room temperature, shake gently (avoid bubbles).

The concentration of the standard in the stock solution is 2000 ng/mL. Prepare 7 tubes containing 0.5 mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Prepare a dilution series with 7 points, for example: 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, and the last EP tube with Standard Diluent is the blank at 0 ng/mL.



Tube	1	2	3	4	5	6	7	8
ng/mL	2000	1000	500	250	125	62.5	31.25	0

3. **Detection Solution A and Detection Solution B** - Detection Solutions A and B are already at the correct concentrations and do not need to be diluted further.
4. **Wash Solution** - Dilute 20 mL of Wash Solution concentrate (30×) with 580 mL of deionized or distilled water to prepare 600 mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips. Do not dump the residual solution back into the vial.

Note:

1. Do not perform a serial dilution directly in the wells.
2. Prepare standard within 15 minutes of performing the assay.
3. Carefully reconstitute Standards according to the instruction, avoid foaming and mix gently until the crystals are completely dissolved.

4. The reconstituted Standards can be used only once.
5. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Any contaminated water or container used during reagent preparation will influence the detection result.

SAMPLE PREPARATION

1. Biomatik INC. is only responsible for the kit itself, not for the samples consumed during the assay. The user should calculate the possible amount of samples used in the whole assay. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their specific experiments. Samples should be diluted with the Standard Diluent provided in the kit. Samples can also be diluted with 0.01 mol/L PBS (pH 7.0-7.2) in case of insufficient Standard Diluent.
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared using a chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
5. Due to the possibility of mismatching between antigens from other origin and antibodies 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.
6. Samples from cell culture supernatant may not be detected by the kit due to influence from factors such as cell viability, cell number and/or sampling time.

7. Fresh samples are recommended for the assay. Protein degradation and denaturation may occur in samples stored over extensive periods of time and may lead to inaccurate or incorrect results.

ASSAY PROCEDURE

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for the standards, 1 well for blank. Add 100 µL each of dilutions of standard (read Reagent Preparation), blank, and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 90 minutes at 37°C.
2. Remove the liquid from each well.
3. Add 100 µL of Detection Solution A to each well. Incubate for 45 minutes at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 300 µL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto-washer, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by tapping the plate onto absorbent paper. Wash thoroughly 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 Solution B to each well. Incubate for 45 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for a total of 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 15-25 minutes at 37°C (Do not exceed 30 minutes). Protect from light. The liquid will turn blue with the addition of the Substrate Solution.
8. Add 50 µL of Stop Solution to each well. The liquid will turn yellow with the addition of the Stop solution. Mix the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drops of water and fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Run the microplate reader and take measurements at 450 nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Remaining wells should be resealed and stored at -20°C.
2. **Samples or reagents addition: Please use the freshly prepared Standard.** Carefully add samples to wells and mix gently to avoid foaming. Do not touch the well walls. 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 standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. In addition, 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 washing procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting, and remove any drops of water or fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and false 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 an inaccurate absorbance reading.

6. **TMB Substrate** is light sensitive and easily contaminated by oxidizing agents. Please protect it from light exposure and potential sources of contamination.
7. The environment humidity may influence the results obtained from the kit. If the humidity in your facility is less than 60%, using a humidifier is recommended.

TEST PRINCIPLE

The microtiter plate provided in this kit has been pre-coated with an antibody specific to AT. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific to AT. 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 AT, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 10 nm. The concentration of AT in the samples is then determined by comparing the O.D. of the samples to the standard curve.

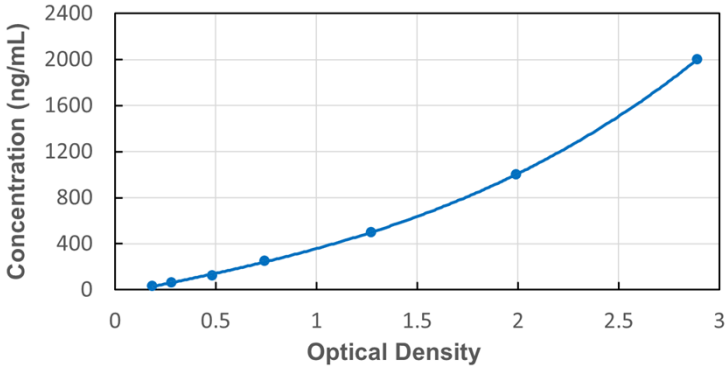
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control and sample, then subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with AT concentration on the *y*-axis and absorbance on the *x*-axis. Using plotting software, (for instance, curve expert 1.30), is also recommended. If samples have been diluted, the

concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

To make the calculation easier, we plot the O.D. value of the standard (*x*-axis) against the known concentration of the standard (*y*-axis), although concentration is the independent variable and O.D. value is the dependent variable. However, the O.D. values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique or temperature effects), plotting the log of the data to establish a standard curve for each test is recommended. The typical standard curve below is provided for reference only.



Typical Standard Curve for Human AT ELISA

DETECTION RANGE

31.25-2000 ng/mL. The concentrations used for creating the standard curve were 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL.

SENSITIVITY

The minimum detectable dose of AT is typically less than 12.6 ng/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 standard replicates and calculating the corresponding concentration.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of AT. No significant cross-reactivity or interference between AT and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible to perform all possible cross-reactivity detection tests between AT and all analogues, therefore, cross reactivity may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level AT were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level AT were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean × 100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

STABILITY

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage conditions.

Note:

To minimize unnecessary influences on the performance, operation procedures and lab conditions, especially room temperature, air humidity, and incubator temperatures should be strictly regulated. It is also strongly suggested that the whole assay is performed by the same experimenter from the beginning to the end.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards.
2. Add 100 µL standard or sample to each well. Incubate 90 minutes at 37°C.
3. Aspirate and add 100 µL Detection Solution A. Incubate 45 minutes at 37°C.
4. Aspirate and wash 3 times.
5. Add 100 µL Detection Solution B. Incubate 45 minutes at 37°C.
6. Aspirate and wash 5 times.
7. Add 90 µL Substrate Solution. Incubate 15-25 minutes at 37°C.
8. Add 50 µL Stop Solution. Read at 450 nm immediately.

IMPORTANT NOTE

1. Limited by the current conditions and scientific technology, it is impossible to conduct comprehensive identification and analysis tests on the raw materials provided by suppliers. As a result, it is possible there are some qualitative and/or technical risks.
2. The final experimental results will be closely related to the validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available to obtain accurate results.
3. 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 manual included in your kit. The electronic ones on our website are for reference only.

4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by the manufacturer.
5. Protect all reagents from strong light during storage and incubation. All bottle caps of reagents should be closed tightly to prevent evaporation of liquids and contamination by microorganisms.
6. There may be a 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 microtiter plate from the storage bag until needed.
7. Incorrect procedures during reagent preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10 nm wavelength is acceptable for use in absorbance measurement. Please read the instructions carefully and adjust the instrument prior to the experiment.
8. Even the same experimenter may get different results from two separate experiments. To get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before the general assay for each batch is recommended.
9. Each kit has undergone several rigorous quality control tests. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipment. Intra-assay variance among kits from different batches could arise from the above factors as well.
10. Kits from different manufacturers with the same item might produce different results since we have not compared our products with other manufacturers.
11. The standard in this kit, as well as the antigens used in antibody preparation are typically recombinant proteins. Differently expressed

sequences, expression systems, and/or purification methods can be used in the preparation of recombinant proteins. There is also the possibility of differences in the screening technique of antibodies and antibody pairs in our kits. As a result, we cannot guarantee that our kit will be able to detect recombinant proteins produced by other companies. We do NOT recommend using Biomatik ELISA kits for the detection of other recombinant proteins.

12. Validity period: 16 months.
13. The instruction manual also works with the 48 T kit, but all reagents in the 48 T kit are reduced by half.

PRECAUTION

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this reagent.