Human AT (Antithrombin) Sandwich ELISA Kit

STJE0007355

This ELISA kit used for quantitative determination of AT in Human serum, plasma, tissue homogenates, cell lysates, cell culture supernatant or other biological fluids.

We highly recommended reading this manual thoroughly before using this kit.

St John's Laboratory, Knowledge Dock Business Centre, University Way, London, E16 2RD, UK
T: +44 (0)208 223 3081 E: info@stjohnslabs.com W: stjohnslabs.com

Introduction

This kit is a sandwich enzyme immunoassay (ELISA) for in-vitro quantitative measurement of AT in Human serum, plasma, tissue homogenates, cell lysates, cell culture supernatant or other biological fluids.

Principle of The Assay

This ELISA kit uses the Sandwich-ELISA principle. The micro plate provided in this kit has been pre-coated with an antibody specific to Human AT. Standards or samples are added to the micro plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human AT and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human AT, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human AT. You can calculate the concentration of Human AT in the samples by comparing the OD of the samples to the standard curve.

Sensitivity: 12.6 ng/mL
Detection Limit: 31.2~2000 ng/mL

Materials Provided

Component	Size (48T/96T)	Storage recommendation
Micro-ELISA Coated Plate	4 x 12 strips / 8 x 12 strips	Return unused wells to the foil pouch containing the desiccant pack and store at ≤ -20° C for up to 12 months. Reseal along entire edge of zip-seal.
Standard (Lyophilized)	1 / 2	Aliquot and store at ≤ -20 ° C for up to 12 months. * Avoid repeated freeze-thaw cycles.
Concentrated Biotinylated Detection Antibody (100×)	1 × 60 µL / 1 x 120 µL	May be stored for up to 12 months at -20° C. Protect from light.
Streptavidin-HRP Concentrated (100×)	1 x 60 µL / 1 x 120 µL	
Diluent Buffer	1 × 22.5 mL / 1 x 45 mL	May be stored for up to 6 months at 2-8° C. (Protect TMB Substrate from light)
Wash Buffer(30x)	1 × 10 mL / 1 × 20 mL	
TMB Substrate	1 x 4.5 mL / 1 x 9 mL	
Stop Solution	1 × 3 mL / 1 × 6 mL	
Plate Sealers	5 Strips	

Sample Collection and Storage

1. Cell Culture Supernatant

Centrifuge 1000xg for 10 min and detect; or aliquot and store samples at -20° C to -70° C (Stored at 2-8° C if tested within 24 hours). Avoid freeze/thaw cycles. If cell culture supernatant samples require larger dilutions, perform an intermediate dilution with culture media and the final dilution with the Standard/Sample Diluent.

2. 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 1000xg. Assay freshly prepared serum immediately or store samples in aliquot at -20° C or -80° C for later use. Avoid repeated freeze/thaw cycles.

3. 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 aliquot at -20° C or -80° C for later use. Avoid repeated freeze/thaw cycles.

4. Cell Lysates

Cells need to be lysed before assaying according to the following directions. Adherent cells should be washed by cold PBS gently, and then detached with trypsin, and collected by centrifugation at 1000×g for 5 minutes (suspension cells can be collected by centrifugation directly). Wash cells 3 times in cold PBS. Resuspend cells in fresh lysis buffer with concentration of 10^7 cells/mL. If it is necessary, the cells could be subjected to ultrasonication until the solution is clarified. Centrifuge at 1500×g for 10 minutes at 2-8° C to remove cellular debris. Assay immediately or aliquot and store at ≤-20° C.

5. Tissue homogenates

The preparation of tissue homogenates will vary depending upon tissue type. Tissues should be rinsed thoroughly in ice-cold PBS to remove excess blood and weighed before homogenization. Mince the tissues to small pieces and homogenise them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular

location of the target protein) (E.g., 1mL lysis buffer in 200mg tissue sample) with a glass homogenizer on ice. The resulting suspension should be sonicated with an ultrasonic cell disrupter until the solution is clarified. Centrifuge the homogenates for 5 minutes at 10000 × g and collect the supernatant. Assay immediately or aliquot and store at ≤-20° C.

6. Other biological fluids

Centrifuge samples for 20 minutes at 1000 × g. Collect the supernatant and assay immediately or store samples in aliquot at -20° C or -80° C for later use. Avoid repeated freeze/thaw cycles. **Avoid haemolytic and hyperlipidaemia samples for serum and plasma.**

Dilution: Dilute samples at the appropriate multiple (recommend carrying out a pre-test to determine the dilution factor).

Note

Samples should be assayed within 7 days when stored at 2-8℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months), avoiding freeze-thaw cycles. We recommend predicting the concentration before assaying. If the sample concentration is not within the range of the standard curve users should determine the optimal sample dilutions for their particular experiments. If the sample type is not included in this manual, a preliminary experiment is advised to verify the validity. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation to the results. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

Precautions

1. This kit is for RESEARCH USE ONLY.
2. Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
3. Variations in sample collection, processing, and storage may cause sample value differences.
4. Reagents may be harmful. If contact made with skin, rinse with an excess amount of tap water.
5. Stop Solution **contains strong acid**. Wear eye, hand, and face protection.
6. For long term storage kit standards should be kept refrigerated, other components should be frozen.
7. Please perform centrifugation to collect liquid before use.
8. Do not mix or substitute reagents with those from other lots or other sources.
9. Adequate mixing is very important for a good result. Use a mini-vortex at the lowest frequency.
10. Mix each sample and all components in the kits adequately and use a clean plastic container to prepare diluent.
11. Samples and standards should be assayed in duplicate, and the sequence of the regents should be added consistently.
12. Reuse of the dissolved standard is not recommended.
13. The kit should not be used beyond the expiration date on the kit label.
14. The kit should be kept away from light when it is stored or incubated.
15. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with appropriate regulations.
16. To avoid cross contamination, please use disposable pipette tips.
17. Please prepare all kit components according to the specification. If the kits will be used several times, keep unused strips sealed and preserve with desiccants. Use within 2 months.

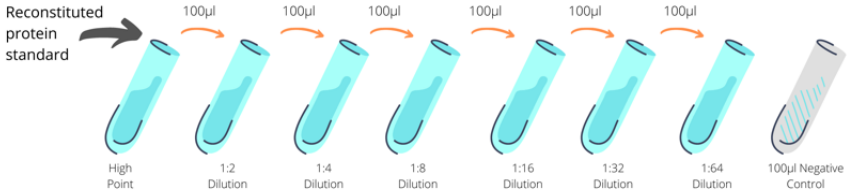
Experiment Materials

The following materials are required to carry out the aforementioned assay but are not included with this kit.

1. Microplate reader (measuring absorbance at 450 nm, with the correction wavelength set at 570 nm or 630 nm).
2. Pipettes and pipette tips: 0.5-10, 2-20, 20-200, 200-1000 µL.
3. Microplate washer, Squirt bottle.
4. Micro-oscillator.
5. Deionized or double distilled water graduated cylinder.
6. Polypropylene Test tubes for dilution.
7. Incubator.

Reagent Preparation

1. Bring all reagents to room temperature before use. If crystals have formed in the concentrate bring the reagent to room temperature and mix gently until the crystals have completely dissolved. It is recommended to test in duplicates.
2. Standard Solution: Add 1 mL Diluent Buffer to freeze-dried standard, sit for a minimum of 15 minutes with gentle agitation prior to making dilutions (2000 ng/mL), Prepare EP tubes containing Diluent Buffer, and carry out a serial dilution according to the picture shown below (recommended concentration for standard curve: 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL). Any remaining Standard Solution can be aliquoted and stored at -20° C to -70° C.



Two-fold dilution series: Each microvial starts with 100µl diluent. 100µl is carried over from the reconstituted protein standard, thoroughly mixed and then 100µl is transferred to the next. The process is repeated in each microvial in the sequence. No treatment is carried out on the negative control, which should hold 100µl of diluent.

Dilution Method

3. Biotin-Conjugated Antibody Working Solution: Dilute Concentrated Biotin-Conjugated Antibody 1:99 with Diluent Buffer before use. The diluted solution should be used within 30 min.

No. of strips	Concentrated Biotin-Conjugate antibody (100x)	Biotin-Conjugate antibody diluent
2	20 µL	1980 µL
4	40 µL	3960 µL
6	60 µL	5940 µL
8	80 µL	7920 µL
10	100 µL	9900 µL
12	120 µL	11880 µL

4. Streptavidin-HRP Working Solution: Dilute Concentrated Streptavidin-HRP 1:99 with Diluent Buffer before use. The diluted solution should be used within 30 min.

No. of strips	Concentrated Streptavidin-HRP (100x)	Streptavidin-HRP antibody diluent
2	20 µL	1980 µL
4	40 µL	3960 µL
6	60 µL	5940 µL
8	80 µL	7920 µL
10	100 µL	9900 µL
12	120 µL	11880 µL

5. Wash buffer: Dilute 1:29 with double distilled or deionized water before use.

Wash Method

Aspirate each well and wash, repeating the process 2 times for a total of 3 washes. Wash by filling each well with Wash Buffer (300ul) using a squirt bottle, manifold dispenser, or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Assay Procedure

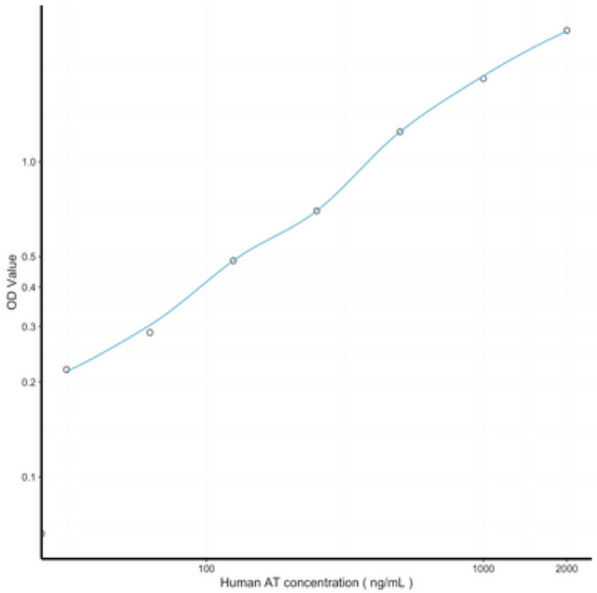
1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack and reseal.
2. Keep a blank well free.
3. Add 100 µL of each different concentration of standard and the samples to each of the other wells to be used. Cover with the adhesive strip provided and incubate for 90 minutes at 37° C. (Note: solution should be added to the bottom of ELISA plate well, avoid touching the inside wall and foaming.)
4. Remove all the liquid from each well. **Do not wash.**
5. Add 100µL Biotin-Conjugated Antibody Working Solution to all the wells. Cover with new adhesive strip provided and incubate for 1 hour at 37° C.
6. Aspirate the solution from all the wells.
7. Add 300 µL wash buffer to each well, and aspirate after 1-2 minutes, repeating the process 2 times for a total of 3 washes.
8. Add 100 µL Streptavidin-HRP Working Solution to each of the wells. Cover with a new adhesive strip and incubate for 30 minutes at 37° C.
9. We recommend that you ensure the Microplate reader is set up during this incubation stage.
10. Add 300 µL wash buffer to each well, and aspirate after 60-120 seconds. Repeat the process 4 more times, to total 5 washes.
11. Add 90 µL TMB Substrate to each well and incubate for 15-20 minutes at 37° C. Protect from light.
12. Add 50 µL Stop Solution to each well. Determine the optical density of each well within 5 minutes, using a Microplate reader set to 450 nm. If wavelength correction is available, set to 570 nm or 630 nm. If wavelength correction is not available, subtract readings at 570 nm or 630 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
13. Upon completion of the experiment ensure you return unused reagents to their appropriate storage locations.

Calculation of Results

1. Average the duplicate readings for each standard, control and sample, and subtract the average zero standard optical density (O.D.).
2. Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y- axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the AT concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.
3. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical Data

The standard curves are provided for demonstration only. A standard curve should be generated for each set of AT assayed.



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.

Intra-plate Precision

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

Inter-plate Precision

Inter-assay Precision: 3 samples with low, mid-range and high-level Human AT were tested on 3 different plates, 20 replicates in each plate.

CV	Intra-Assay %	Inter-Assay %
Low concentration	6.10	5.71
Middle concentration	5.94	5.84
High concentration	7.21	7.39

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of AT and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16
Serum (n=5)	84-90 %	89-94 %	85-89 %	102-106 %
EDTA plasma (n=5)	92-109 %	82-95 %	97-108 %	86-109 %
Heparin plasma (n=5)	97-106 %	82-86 %	101-103 %	80-91 %

Recovery

The recovery of Human AT spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Matrix	Recovery range (%)	Average (%)
Serum (n=5)	85-102 %	93 %
EDTA plasma (n=5)	84-103 %	93 %
Heparin plasma (n=5)	98-101 %	99 %

Troubleshooting

Problem	Causes	Solutions
Poorly developed standard curve	Inaccurate pipetting.	Check pipetting volume consistency and accuracy.
	Improper standard dilution.	Gently mix the standard solution and dissolve the powder thoroughly in solution.
	Wells were not fully aspirated.	Completely aspirate wells in between stages.
Low fluorescence readings	Insufficient incubation time.	Ensure sufficient incubation time.
	Incorrect assay temperature.	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes or inconsistent dilution.	Check pipettes and ensure correctly prepared.
Large CV	Inaccurate pipetting.	Check pipettes and technique.
High background	Concentration of target protein is too high.	Use recommended dilution factor.
	Plate is insufficiently washed.	Review the manual's washing process. If using a plate washer, check that the ports are not obstructed.
	Contaminated wash buffer.	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the kit.	All the reagents should be stored according to the instructions.
	Too long incubation time.	Ensure precise incubation time.

Declaration

1. Due to limitations in scientific technology, we are unable to conduct comprehensive identification and analysis on all raw materials provided in this kit, which may result in qualitative and technical risks for users of this kit.
2. This assay has been designed to eliminate interference by factors present in biological samples, but until all factors have been conclusively tested in the ELISA immunoassay the possibility of interference cannot be excluded.
3. Users of this kit can expect the results to be closely related to the validity of both the kit itself, the individual's technical skill, the experimental environments used, and so on.
4. **We will only accept responsibility for the kit itself.**
5. We cannot accept responsibility for the samples consumed during the assay. Users must calculate the required volume of samples to be used before initiating this ELISA experiment.
6. For best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions. Any concerns should be raised with the manufacturer before commencing with the assay.
7. Incorrect results may come about because of incorrect application of the steps during reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
8. The same operator may see different results over two separate experiments. To get reproducible results, the operation of every step in the assay should be controlled.
9. Each kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to variables such as transportation conditions, differing laboratory equipment, and so on. This may also lead to intra-assay variance among kits from different batches.
10. Kits from different manufacturers - or other methods for testing the same analyte - could bring out inconsistent results, since we haven't compared our products with those from other manufacturers.
11. The kit is designed for research use only, we will not accept responsibility for any issues if the kit is applied in clinical diagnosis or any other related procedures.

NOTES