

ADAMTS-13 Activity Assay (ATS-13®)

INTENDED USE

For the quantitative measurement of ADAMTS-13 protease activity.

For Research use Only.

SUMMARY AND EXPLANATION

It was recently discovered that ADAMTS-13 is the protease responsible for cleaving von Willebrand Factor; deficiency of ADAMTS-13 activity has been demonstrated in the plasma of thrombotic thrombocytopenic purpura (TTP) patients. The lack of ADAMTS-13 activity results in the accumulation of multimers of von Willebrand Factor in the plasma and ultimately intravascular platelet aggregation resulting in the clinical symptoms associated with TTP.^{4,5} Mild or moderately decreased levels of ADAMTS-13 activity have also been associated with other disease states and conditions.²⁻⁵

PRINCIPLE OF THE PROCEDURE

The ATS-13® assay is based on fluorescence resonance energy transfer (FRET) technology. A synthetic fragment of the von Willebrand Factor protein is used as the Substrate. Cleavage of this peptide between two modified residues releases the fluorescence quenching capabilities.

This assay is based on quantifying the cleavage of a small fragment of von Willebrand Factor by the ADAMTS-13 protease. The cleavage of this synthetic substrate is detected by reading the fluorescence that results when the substrate is cleaved.

REAGENTS

Maximum number of tests per kit: 40

All reagents should be stored as directed by the label.

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|-----------------|---|
| ATS-MS | 1. Black Microwell Strips: Once removed from the foil pouch, take care not to expose the strips to dust or particulates. Take care to protect from moisture. Strips should be stored at room temperature. Ready to use. |
| ATS-SUB | 2. Substrate: lyophilized . Keep substrate protected from light. Store lyophilized material at -15 to -30°C. Hydrated substrate should be stored upright in the parafilm original stoppered vial at -15 to -30°C (non-cycling freezer) in the dark. |
| ATS-SD | 3. Specimen Diluent: Ready for use. Store at 2 to 8°C. |
| ATS-SB | 4. Substrate Buffer: Ready for use. Store at 2 to 8°C. |
| ATS-PCH | 5. Positive Control; HIGH: Store at -15 to -30 °C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use. |
| ATS-PCL | 6. Positive Control; LOW: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use. |
| ATS-CALA | 7. Calibrator A: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use. |
| ATS-CALB | 8. Calibrator B: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use. |
| ATS-CALC | 9. Calibrator C: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13® Calibrator and Control Recording Sheet. Discard after single use. |

**ATS-
CALD**

10. Calibrator D: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13[®] Calibrator and Control Recording Sheet. Discard after single use.

**ATS-
CALE**

11. Calibrator E: Store at -15 to -30°C. Contains human source material. Thaw and mix thoroughly before use. Ready for use. Values can be found on ATS-13[®] Calibrator and Control Recording Sheet. Discard after single use.

PRECAUTIONS

- Do not use reagents that are turbid or contaminated.
- Care **MUST** be taken to avoid contamination of Calibrators and Substrate. Inadvertent contamination of these reagents with human plasma will invalidate the assigned values of the calibrators.
- Unopened and lyophilized reagents are stable until the expiration date printed on the box when stored as directed.
- Do not use reagents beyond their expiration date.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- Discard any unused portions of Calibrators, Controls, and used Black Microwell Strips after each run.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.
- When making dilutions, follow pipette manufacturer's instructions for appropriate dispensing and rinsing techniques.
- The enzyme substrate reaction is temperature sensitive and should be performed in a controlled area at 22 to 25°C.
- Only plasma should be used in the assay. Serum will give inaccurate results.

CAUTIONS

- All human plasma used in the Calibrators and Positive Controls for this product has been tested and found negative for antibody to HIV, HCV and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

Sample Collection and Preparation

NOTE: Only platelet poor plasma collected in 3.2% sodium citrate may be used for this assay. Do not use plasma that has been collected in or treated with EDTA. See Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays. Approved Guideline H21-A4 NCCLS, Volume 23, Number 35, December 2003 for details.

Plasma collection should be performed as follows:

1. Collect blood in buffered sodium citrate (light blue top, 3.2%) plastic tubes (available in 4.5 mL, 2.7 mL or 1.8 mL full draw tubes).

NOTE: Partial draw tubes should NOT be processed. Since the tubes are pre-calibrated to draw the specified amount of blood, the resulting sample, will not have the proper 9:1 ratio of blood to anticoagulant if a full sample is not collected.

2. After collection, store tube upright at room temperature until centrifugation.

NOTE: Blood samples should be centrifuged between fifteen minutes and two hours after blood collection for best results.

3. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.
4. Centrifuge blood sample at room temperature in a horizontal rotor (swing-out rotor) for 15 - 20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force) with the brake off.

WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury.

5. Following centrifugation, transfer the top 2/3 of the plasma layer into a new plastic tube.
6. Re-centrifuge the collected plasma at 1500 to 1800 RCF with the brake off for an additional 15 - 20 minutes to remove any red cells or platelets.

7. Transfer the top 2/3 of the plasma into a new plastic tube, taking care not to disturb any cells at the bottom of the tube.

Sample Storage

1. Plasma should be stored at 2 to 8°C and assayed within 4 hours OR aliquoted and frozen at -70°C or colder for up to 6 months.
2. Frozen plasma should be thawed rapidly at 37°C. Thawed plasma should be stored at 2 to 8°C and assayed within 4 hours.

PROCEDURE

Materials Provided:

Box A

1. 6 x 130 µL Positive Control: High
2. 6 x 130 µL Positive Control: Low
3. 6 sets of Calibrators, 5 levels, 130 µL each:
Calibrator A, Calibrator B, Calibrator C, Calibrator D, Calibrator E.
4. 1 x 0.12 mg Substrate

Box B

1. 2 Microwell frames, each containing 6 – 2 x 8 Black Microwell Strips
2. 1 x 14 mL Specimen Diluent
3. 1 x 14 mL Substrate Buffer

Additional Materials Required:

1. Polypropylene plastic test tubes for patient sample dilutions and substrate dilution
2. Transfer pipets
3. Adjustable micropipets to deliver 10 - 100 µL and 100 – 1000 µL
4. Disposable tips
5. DMSO (Reagent Grade)
6. Fluorescent plate reader capable of measuring fluorescence at
Excitation = 340 - 350 nm and Emission = 440 - 450 nm
7. Timer
8. Centrifuge
9. Aluminum Foil

Test Procedure

1. Allow all reagents to warm to room temperature.

NOTE: Only remove one foam strip set of Calibrators and Controls per assay.

2. Determine the number of patient samples to be tested. Using the Recording Sheet, assign each sample to a location consisting of two (duplicate) wells.
Record the identity of each sample on the Recording Sheet. Place the sample replicates horizontally (e.g. CALA in wells A1 and A2).
3. Remove microwell frame from pouch. Promptly remove unneeded strips from frame and reseal in the protective pouch.
4. In a plastic test tube, dilute each patient plasma sample to be tested by adding 18 µL plasma into 132 µL Specimen Diluent.
5. Add 50 µL of each Calibrator (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.
6. Add 50 µL of Positive Control: Low (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.
7. Add 50 µL of Positive Control: High (in duplicate) to the appropriate microwells of the black microwell strips as designated on the Recording Sheet. Do not dilute.

- Add 50 μL of the prediluted sample plasma solution (prepared in step 4) in duplicate to the appropriate microwells of the black microwell strips as designated on the Recording Sheet.

NOTE: If multiple patient samples are tested at the same time, only one set of calibrators and controls are required.

- Prepare Stock Substrate Solution. Remove stopper carefully as some Substrate may cling to the plastic. Reconstitute the lyophilized Substrate by adding 37 μL of reagent grade DMSO to the Substrate vial. Mix solution and add 113 μL reagent grade H_2O . Replace the stopper and close the cap tightly. Mix well by gently swirling until all contents are dissolved.

- Prepare the assay Substrate Solution (3%) in a plastic tube according to the table below:

Patient Samples to Test	Volume Stock Substrate Solution (μL)	Volume Substrate Buffer (μL)
1	25	795
5	37	1193
10	53	1714
* 40	150	4850

* This can be prepared by adding substrate buffer directly to stock substrate vial if being used for testing within one assay.

NOTE: A repeating pipette should not be used.

- Mix the solution thoroughly. Protect from light. Immediately following preparation, add 50 μL of Substrate Solution into each microwell containing a patient sample, calibrator, or control. Gently tap the sides of the microwell frame to ensure even distribution of the Substrate Solution.

NOTE: Remaining Stock Substrate Solution should be stoppered and stored upright in the original vial with original stopper (sealed with parafilm) at -20°C (non-cycling freezer) in the dark. Re-hydrated stock can be used for up to 6 months following re-hydration.

- Place plate in fluorimeter with Excitation = 340 - 350 nm and Emission = 440 - 450 nm at room temperature. Read and record results as time zero.

NOTE: Reading must be taken within 5 minutes of addition of substrate.

- Set timer for 25 minutes and start.

- Remove plate from fluorimeter. Store plate at room temperature (not in plate reader) and protect from light for 25 - 35 minutes.

NOTE: Do not cover plate with paper or cardboard. Fibers in the plate can cause random fluorescence. Cover with aluminum foil.

- Between 25 - 35 minutes, place plate in fluorimeter with Excitation = 340 - 350 nm and Emission = 440 - 450 nm at room temperature. Read and record results.

RESULTS

Subtract time zero fluorescence values from the 25 - 35 minute fluorescence values for all results. Construct a calibration curve by plotting the mean fluorescence (n=2) value for each calibration standard versus its corresponding concentration of ADAMTS-13 activity. A calibration curve should be generated each time the assay is performed.

CALCULATIONS

Determine the amount of ADAMTS-13 Activity in the plasma sample using the Microsoft[®] Excel spreadsheet provided on the enclosed CD. Instructions for the spreadsheet are on the first worksheet of the file. The spreadsheet assists with background corrections, graphing, and solving the equation to obtain calculated results (% Normal ADAMTS-13 Activity) for the controls and plasma samples.

Plasma samples with calculated ADAMTS-13 activities greater than 100% should be reported as >100% Normal ADAMTS-13 Activity.

REFERENCES

1. Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays. Approved Guideline H21-A4 NCCLS, Volume 23, Number 35, December 2003
2. K. Kokame, M. Matsumoto, Y. Fujimura, and T. Miyata, *Blood*, **103**, 607 (2004).
3. K. Kokame, Y. Nobe, Y. Kokubo, A. Okayama, and T. Miyata, *Br. J. Haematol.*, **129**, 93 (2005).
4. Bernhard Lämmle and James N. George, *Seminars in Hematology*, **41**, 1, 1 (2004).
5. Lämmle B, Kremer Hovinga JA, Alberio L., *J Thromb Haemost*, **3**, 1663 (2005).



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REF ATS-13

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- STORE AT 2 to 8°C for Box B