Status: Currently Official on 13-Feb-2025
Official Date: Official as of 01-Dec-2024
Document Type: USP Monographs
DocId: GUID-3078520B-75E1-457A-9F8A-13E09F818D6B_5_en-US
DOI: https://doi.org/10.31003/USPNF_M5390_05_01
DOI Ref: v2a4e



# Antithrombin III Human

CAS RN®: 9000-94-6; UNII: T0LTO7L82X.

## DEFINITION

Antithrombin III Human is a glycoprotein, which is the major inhibitor of thrombin and other activated clotting factors, including factors IX, X, XI, and XII. It is obtained from human plasma of healthy donors who have been tested and shown to be free from detectable agents of infection transmissible by transfusion of blood or blood derivatives. The manufacturing steps are shown to remove or inactivate known agents of infection. If substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to an acceptable level and that any residues are such as not to compromise the safety of the preparation for patients. When reconstituted in the recommended volume of diluent, the potency is NLT 25 International Units (IU)/mL. It contains 80%–120% of the potency stated on the label.

## IDENTIFICATION

• **A.** Meets the requirements of the *Assay*

## ASSAY

• **Procedure**

**Solution A:** Dissolve tris(hydroxymethyl)aminomethane, edetic acid, and sodium chloride in water to obtain a solution having concentrations of 0.050, 0.0075, and 0.175 M, respectively. Adjust with hydrochloric acid or sodium hydroxide solution to a pH of 8.4.

**Solution B:** 0.05% (w/v) of albumin human in *Solution A*

**Solution C:** 10 mg/mL of polybrene in *Solution B*

**Solution D:** Reconstitute thrombin bovine (factor IIa), and dilute with *Solution B* to obtain a solution having a concentration of 4 Thrombin IU/mL.

**Solution E:** Prepare a solution of chromogenic substrate for amidolytic test for thrombin bovine (factor IIa) in *Solution C* to obtain a solution having a concentration of about 40.0 mM.

**Solution F:** Resuspend USP Heparin Sodium for Assays RS according to the USP Certificate and dilute to 3 USP Heparin Units/mL in *Solution A*.

**Standard solutions:** Prepare seven dilutions from USP Antithrombin III Human RS within the linear range of the assay in *Solution F* (for example, 1.7, 1.5, 1.2, 1.0, 0.8, 0.6, and 0.4 IU/mL).

**Sample solutions:** Prepare three or more dilutions in *Solution F* within the linear range of the assay.

**Blank:** *Solution A*

**Analysis:** [Note—The procedure also can be performed using alternative platforms.]

Each dilution of the *Standard solution* and *Sample solution* should be tested at least in duplicate. Label a suitable number of tubes depending on the number of replicates that will be tested. For example, if five blanks will be used: B1, B2, B3, B4, and B5 for the blanks; T1, T2, and T3 each at least in duplicate for the dilutions of the *Sample solutions*; and S1, S2, S3, S4, S5, S6, and S7 each at least in duplicate for the dilutions of the *Standard solutions*. Distribute the blanks over the series in such a way that they accurately represent the behavior of the reagents during the experiments. [Note—Treat the tubes in the order B1, S1, S2, S3, S4, S5, S6, S7, B2, T1, T2, T3, B3, T1, T2, T3, B4, S1, S2, S3, S4, S5, S6, S7, B5.]

Prewarm *Solution D* and *Solution E* at 37°. Pipet 50 µL each of the *Standard solutions*, *Sample solutions*, and *Blank* into suitable tubes placed in a water bath set at 37°. Add 350 µL of prewarmed *Solution D* to each tube, mix, and incubate for 1 min. Add 100 µL of prewarmed *Solution E* to each tube in the same order and mix. Follow the change in absorbance for each solution over 1 min at 405 nm using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩). Calculate the change in absorbance/min. Plot standard concentrations against resulting absorbance values and determine potency by interpolating from the standard curve using mean sample absorbances.

**System suitability**

**Samples:** *Standard solutions* and *Sample solutions*

The $R^2$ value of the standard curve is NLT 0.99. The initial and final blanks differ by NMT 10%. The absorbances of the three dilutions of the *Sample solution* must fall within the range of absorbances of the standard curve. The three dilutions of the *Sample solution* give potency estimates that differ by NMT 10%.

**Acceptance criteria:** 80%–120% of the potency stated on the label

**IMPURITIES**

***Change to read:***

• **Heparin Content**

**Solution A:** 9 g/L of sodium chloride

**Sheep plasma substrate:** Use sheep plasma suitable for the test procedure. If frozen, thaw at 37°.

**APTT reagent:** Use a suitable activated partial thromboplastin time (APTT) reagent containing phospholipid and a contact activator at a dilution giving a suitable blank recalcification time not exceeding 60 s.

**Calcium chloride solution:** 3.7 g/L of calcium chloride

**System suitability solution:** ▲Reconstitute USP Antithrombin III Human RS in 1.0 mL of water, then add 10 µL of 500 USP Heparin Units/mL of USP Heparin Sodium for Assays RS.▲ (USP 1-Dec-2024)

**Standard solutions:** Make three or more dilutions of USP Heparin Sodium for Assays RS to known concentrations in USP Heparin Units/mL that are in the expected range of the sample (for example, 0.5–1.5 USP Heparin Units/mL).

**Sample solutions:** Make three or more dilutions of Antithrombin III Human in the range of the *Standard solution* dilutions.

**Blank:** *Solution A*

**Analysis:** [Note—The procedure also can be performed using alternative platforms.]

Each *System suitability solution*, *Standard solution*, and *Sample solution* should be tested at least in duplicate. Label a suitable number of tubes depending on the number of replicates that will be tested. For example, if five blanks will be used: B1, B2, B3, B4, and B5 for the blanks; T1, T2, and T3 each at least in duplicate for the dilutions of the *Sample solutions*; and S1, S2, and S3 each at least in duplicate for the dilutions of the *Standard solutions* and SS for the *System suitability solution*. Distribute the blanks over the series in such a way that they accurately represent the behavior of the reagents during the experiments. [Note—Treat the tubes in the order B1, S1, S2, S3, B2, SS, T1, T2, T3, B3, T1, T2, T3, SS, B4, S1, S2, S3, B5.] In the following order add 1.0 mL of thawed *Sheep plasma substrate* to 1.0 mL of the *Standard solution* dilutions or the *Sample solution* dilutions or the *System suitability solution*. After each addition, mix but do not allow bubbles to form. Transfer each tube to a water bath at 37°, allow to equilibrate at 37° for about 15 min, and add to each tube 1 mL of *APTT reagent* previously heated to 37°. After an appropriate time for the *APTT reagent* used, usually 2–5 min, add 1 mL of *Calcium chloride solution* previously heated to 37° and determine the clotting time. Plot standard concentrations against resulting clotting times and determine heparin content by interpolating from the standard curve using mean sample clotting times. For samples with clotting times longer than the lowest standard dilution, report the result as NMT the lowest *Standard solution* concentration.

**System suitability**

**Samples:** *Standard solutions* and *Sample solutions*

The $R^2$ value of the standard curve is NLT 0.99. The three dilutions of the *Sample solution* give heparin content estimates that differ by NMT 10%. The heparin content in the *System suitability solution* is in the range of 4.0–7.5 IU/mL.

**Acceptance criteria:** NMT 0.1 USP Heparin Unit/Antithrombin III IU

**SPECIFIC TESTS**

• **Sterility Tests** ⟨71⟩, *Test for Sterility of the Product to Be Examined, Direct Inoculation of the Culture Medium***:** Meets the requirements

• **Water Determination** ⟨921⟩, *Method I***:** NMT 3.0%

• **Pyrogen Test** ⟨151⟩**:** Inject 50 Antithrombin III IU/kg of the rabbit's weight, calculated from the activity stated on the label.

**Acceptance criteria:** Meets the requirements

***Delete the following:***

▲• **General Safety**▲ (CN 1-Dec-2026)

• **Osmolality and Osmolarity** ⟨785⟩

**Osmolality:** Reconstitute with the diluent according to the manufacturer's instruction.

**Acceptance criteria:** NLT 240 mOsmol/kg for the solution

• **pH** ⟨791⟩**:** Reconstitute with the diluent according to the manufacturer's instruction.

**Acceptance criteria:** 6.0–7.5

• **Molecular Weight Distribution**

**Mobile phase:** 0.05 M sodium phosphate (dibasic), 0.05 M sodium phosphate (monobasic), 0.4 M arginine hydrochloride and 0.05% sodium azide. Adjust with 1 N sodium hydroxide to a pH of 6. Degas and filter.

**Solution A:** 4–5 mg/mL of thyroglobulin in *Mobile phase*

**Sample solution:** 8–10 mg/mL of Antithrombin III Human

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Columns**

**Guard:** 7.5-mm × 7.5-cm; packing L59

**Analytical:** 7.5-mm × 30-cm; packing L59

**Temperatures**

**Autosampler:** 7°

**Column:** Ambient

**Flow rate:** 0.5 mL/min maintained constant to ±1%

**Injection volume:** 20 µL

**System suitability**

**Sample:** *Sample solution*

**Suitability requirements**

**Column efficiency:** NLT 2000 theoretical plates

**Tailing factor:** 0.9–1.3

**Analysis**

**Samples:** *Solution A* and *Sample solution*

**Acceptance criteria:** Note the retention times of the major peak in the *Solution A* chromatogram. The relative peak area of the high molecular weight peak eluting at about the same retention time as the major peak in the *Solution A* chromatogram, or earlier, is NMT 13%.

• **Total Protein Content**

**Solution A:** 1000 mg/mL of trichloroacetic acid in water

**Sample solution:** 7.5 mg/mL of Antithrombin III Human in 0.15 M sodium chloride solution

**Blank:** 0.15 M solution of sodium chloride

**Analysis:** To each of 2.0 mL of the *Sample solution* and the *Blank* in suitable centrifuge tubes, add 1.5 mL of *Solution A*. Mix, allow to stand for at least 10 min, centrifuge for 5 min, and decant the supernatant. Resuspend the precipitates in 1.5 mL of *Solution A*, centrifuge for 5 min, decant the supernatant, and hold the tubes inverted on a filter paper to drain. Quantitatively transfer the residues with a minimum quantity of water to a micro-Kjeldahl flask, and determine the nitrogen content (see *Nitrogen Determination* ⟨461⟩, *Method II*). Multiply the result, corrected for the *Blank*, by 6.25 to calculate the quantity of protein.

## ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Use a Type I glass container, preferably Type I, with an appropriate stopper and seal. Store protected from light between 2° and 8°, excursions permitted up to 25°.

• **Labeling:** The labeling should state the content of antithrombin III in Antithrombin III IU. The diluent and the volume to be used to reconstitute the preparation are indicated.

• **USP Reference Standards** ⟨11⟩

USP Antithrombin III Human RS

USP Heparin Sodium for Assays RS

**Auxiliary Information** - Please check for your question in the FAQs before contacting USP.

| Topic/Question | Contact | Expert Committee |
|---|---|---|
| ANTITHROMBIN III HUMAN | Rebecca C. Potts<br>Associate Scientific Liaison | BIO2 Biologics Monographs 2 - Proteins |

**Chromatographic Database Information:** Chromatographic Database

**Most Recently Appeared In:**
Pharmacopeial Forum: Volume No. 49(2)

**Current DocID: GUID-3078520B-75E1-457A-9F8A-13E09F818D6B_5_en-US**

**DOI: https://doi.org/10.31003/USPNF_M5390_05_01**

**DOI ref: v2a4e**