

# Antithrombin

Product Monograph 1995



***CHROMOGENIX***

# Antithrombin

## Antithrombin, Product Monograph 1995

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***CHROMOGENIX***

# Antithrombin

<b>Contents</b>	<b>Page</b>
<b>Introduction</b>	<b>3</b>
Antithrombin deficiency – a risk factor for venous thrombosis	3
<b>Biochemistry</b>	<b>5</b>
Biochemistry of antithrombin	5
- Structure	
- Function	
- Interactions with heparin	
<b>Clinical Aspects</b>	<b>7</b>
Antithrombin deficiency	7
- Hereditary	7
- Acquired	9
Management of antithrombin deficiency	10
- Antithrombin levels	
- Prophylaxis and treatment options	
<b>Assay Methods</b>	<b>11</b>
Diagnosis of antithrombin deficiency	11
Antithrombin assays	11
- Activity assays	11
- Immunological assays	12
<b>Products</b>	<b>13</b>
Diagnostic kits from Chromogenix	13
General assay procedures	14
COAMATIC® Antithrombin	15
COAMATIC® Antithrombin 400	16
COAMATIC® LR Antithrombin	17
COACUTE® Antithrombin R	18
COATEST® Antithrombin	19
<b>References</b>	<b>20</b>
<b>Glossary</b>	<b>22</b>



## Antithrombin deficiency— A Risk Factor for Venous Thrombosis

**Antithrombin is a circulating plasma protein that functions as an important regulator of blood coagulation. It inactivates several enzymes of the coagulation cascade, in particular thrombin and factor Xa. Since a link between hereditary antithrombin deficiency and thrombosis was established in 1965, there has been increasing clinical interest in antithrombin and a need for simple and accurate determination. Assays based on chromogenic peptide substrates are now available, allowing photometric detection of antithrombin activity in plasma.**

Thrombin is the key enzyme in blood coagulation (Figure 1).<sup>1,2</sup> It clots blood by converting fibrinogen into clot-forming fibrin monomers and activates factor XIII leading to the strengthening of the blood clot by cross-linking. Thrombin also activates platelets and the cofactors, factor V and factor VIII, thereby accelerating its own generation and providing a quick response to injury. However, the autocatalytic nature of thrombin also entails certain hazards. The complete thrombin generation of only one millilitre of plasma would, unless regulated, clot the blood content of an adult within minutes. It is thus apparent that thrombin activity must be closely controlled in order to prevent abnormal fibrin deposition within the vasculature.

Inhibitory regulation of thrombin is primarily achieved by two principally different mechanisms. When thrombin binds to the membrane protein thrombomodulin present at the surface of intact vascular endothelium, it loses all of its procoagulant properties. Instead, thrombomodulin dramatically accelerates the rate at which thrombin activates protein C. Activated protein C then degrades factors Va and VIIIa, effectively impeding further thrombin generation.

The other regulatory mechanism of thrombin activity generated during blood clotting is provided by a group of circulating enzyme inhibitors (Table 1). Antithrombin (also called antithrombin III) is the major inhibitor, accounting for approximately 80% of the thrombin inhibitory activity in plasma.<sup>3</sup>

Inhibitors	Target protease(s)
Antithrombin	Thrombin, IXa, Xa, others
$\alpha_2$ -macroglobulin	Non-specific
Trypsin inhibitor	Neutrophil elastase, Thrombin, others
Heparin cofactor II	Thrombin

*Table 1. Thrombin inhibitors in plasma*

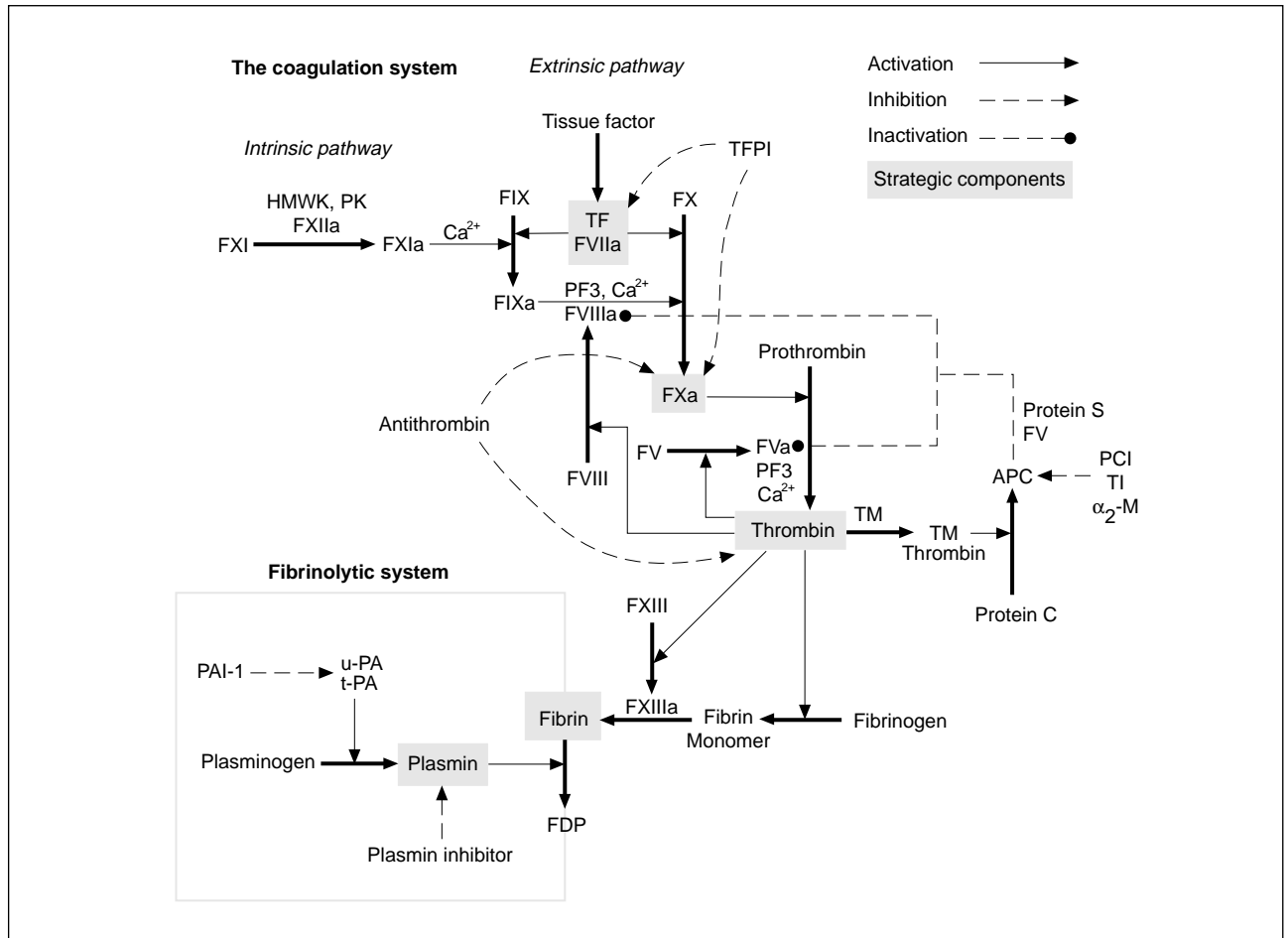
Given the important role of antithrombin it is not surprising that individuals with low antithrombin levels have been reported to have an increase in thrombosis risk. The risk is especially apparent in patients with inherited antithrombin deficiencies, which have been found to be susceptible to developing venous thrombosis and pulmonary embolism.

Antithrombin deficiency is usually transmitted as an autosomal dominant trait and may, in some countries, affect as many as 0.3% of the general population.<sup>4,5</sup> In patients with a history of venous thrombosis presented before the age of 40-45, the incidence is estimated to be 3-5%.<sup>3,6,7</sup> A family history of venous thrombosis with events occurring at a relatively early age are thus strong criteria for selecting patients for antithrombin determination.<sup>7</sup>

This monograph describes five chromogenic substrate assays designed for the specific diagnosis of antithrombin deficiency and provides an up-to-date mini-review of antithrombin. For more detailed information, please contact your local distributor.



# Introduction



**Figure 1. Schematic representation of the coagulation cascade.**

Blood coagulation is initiated by vascular injury and results in the explosive generation of thrombin which clots blood. Coagulation factors are represented by their Roman numerals (a = activated). Abbreviations: HMWK= high molecular weight kininogen, PK= prekallikrein, K= kallikrein, TF= tissue factor, TFPI= tissue factor pathway inhibitor, PF3= phospholipid, TM= thrombomodulin, PC= protein C, APC= activated protein C, PCI= protein C inhibitor, TI= trypsin inhibitor,  $\alpha_2$ -M=  $\alpha_2$ -macroglobulin, FDP= fibrin degradation products. Notes: Factor V has recently been identified as a second APC cofactor.<sup>8</sup>

Factor	Name	Size [kDa]	Concentration [ $\mu$ g/ml]	Factor	Name	Size [kDa]	Concentration [ $\mu$ g/ml]
I	Fibrinogen	340	3000	X	Stuart-Prower factor	59	8
II	Prothrombin	69	100	XI	Thromboplastin antecedent	160	5
III	Tissue factor	47	-	XII	Hageman factor	80	30
IV	Calcium	-	-	XIII	Fibrin-stabilizing	320	10
V	Proaccelerin	330	10	-	Tissue factor	37	-
VI	-	-	-	-	Protein C	57	4
VII	Proconvertin	48	0.5	-	Protein S	75	25
VIII	Antihemophilic factor	330	0.1	-	Antithrombin	58	150
IX	Christmas factor	55	5	-	Heparin cofactor II	66	91

**Table 2. Plasma coagulation factors and regulatory proteins.**



## Biochemistry of antithrombin

### Structure

Antithrombin is a 58 kDa single-chain plasma glycoprotein and a member of the serpin family of serine protease inhibitors. It is synthesized in the liver<sup>9</sup> and occurs in normal plasma at a concentration of about 150 mg/l,<sup>10</sup> with a half-life of 3 days.<sup>11</sup> The primary structure of human antithrombin in plasma comprises 432 amino acid residues, six of which are cysteine that form three intermolecular disulfide bonds. The molecule also contains four carbohydrate side chains which make up around 15% of the molecular mass.

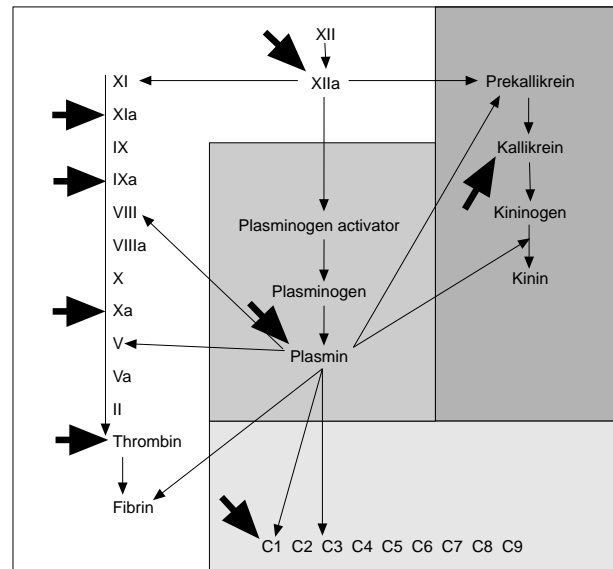
### Function

Antithrombin is the major inhibitor of thrombin, factor IXa, and factor Xa in plasma, but it also inactivates the other serine proteases of the intrinsic coagulation pathway, factors XIa and XIIa, as well as some noncoagulation serine proteases, such as plasmin, kallikrein and the complement enzyme C1 (Figure 2). Most proteases are inactivated much more slowly than thrombin.

Inhibition by antithrombin involves the formation of a stable 1:1 complex between the active domain of the serine protease and the reactive site of antithrombin, which proteases initially recognize as a substrate. During the cleavage of the reactive site bond in antithrombin (located at Arg<sup>393</sup>-Ser<sup>394</sup>), a conformational change occurs in the inhibitor that traps the protease.

### Interactions with heparin

Protease-antithrombin interactions are slow, although enhanced dramatically in the presence of certain sulfated polysaccharides known as glycosaminoglycans (GAGs).<sup>12</sup> It is believed that vascular GAGs, primarily heparan sulfate, bind antithrombin and thrombin and thereby catalyze the antithrombin-thrombin reaction. This would permit the selective enhancement of antithrombin actions at blood-cell interfaces, where the coagulation enzymes are generated.<sup>13</sup>

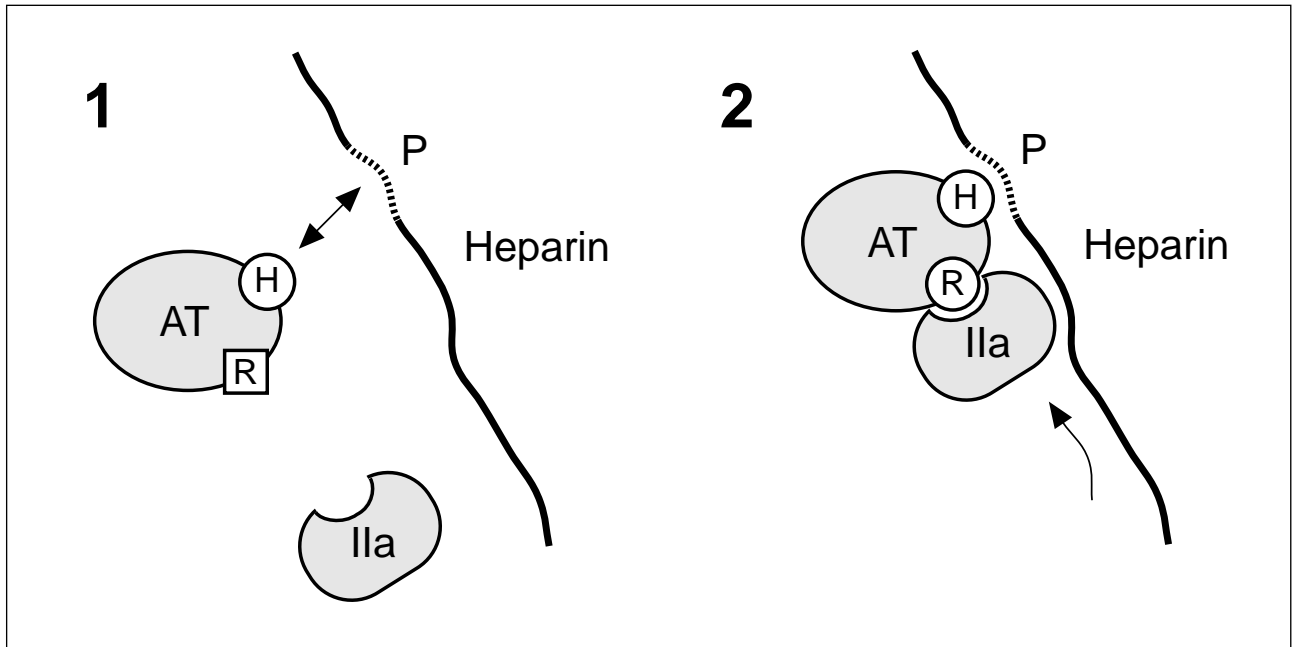


**Figure 2.** Overview of antithrombin targets in plasma enzyme systems. Coagulation (left), fibrinolysis (middle), complement system (bottom) and the Kallikrein-kinin system (right).

Commercial heparin is a mixture of GAGs extracted mainly from bovine or porcine intestinal mucosa and used as an important antithrombotic drug. Both heparin and heparan sulfate catalyze the actions of antithrombin by inducing a conformational change in the antithrombin molecule at the reactive site.<sup>14</sup>

**Figure 3** presents a simplified model describing how heparin catalyzes the formation of an irreversible complex between thrombin (IIa) and antithrombin (AT). [**H**] symbolizes the heparin binding site and [**R**] is the reactive site in antithrombin, normally in an unfavourable conformation for protease inhibition. [**P**] is the unique antithrombin binding segment of heparin. Binding to [**P**] induces a conformational change in antithrombin that facilitates its reaction with thrombin. Thrombin binds to heparin in a non-specific manner and 'slides' along the chain until it encounters the bound antithrombin. The affinity of heparin to the thrombin-antithrombin (TAT) complex is much lower than to free antithrombin. Heparin will therefore dissociate from the TAT complex, which is rapidly removed from the circulation by receptors in the liver ( $t_{1/2} < 5$  min).<sup>15</sup> Factor Xa inactivation is also enhanced by heparin, although the sliding mechanism is not relevant for this enzyme as only the conformation change is required.

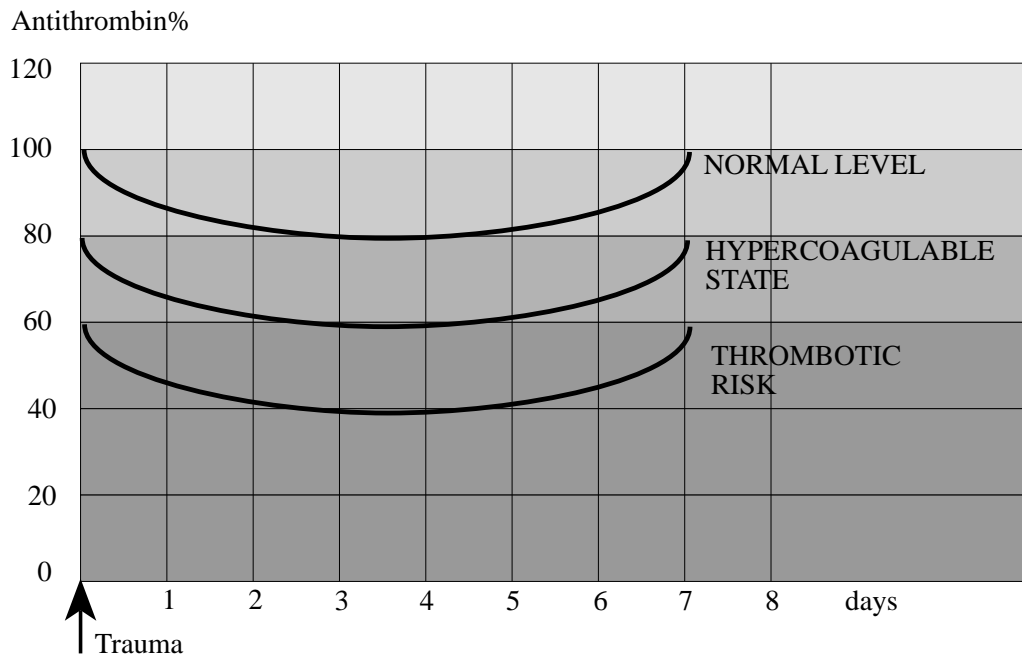




**Figure 3.** Simplified model describing how heparin catalyzes the antithrombin-thrombin reaction. Symbols: AT; antithrombin, IIa; thrombin, [H]= heparin binding site, [R]= reactive site, [P]= antithrombin binding segment

Human antithrombin data	
<b>Name:</b>	Antithrombin <sup>16</sup>
<b>Synonyms:</b>	AT, antithrombin III, ATIII, heparin cofactor I, thrombin inhibitor I
<b>History:</b>	Discovered by Morawitz in 1905
<b>Plasma concentration:</b>	150 mg/l
<b>Molecular weight:</b>	58,200 daltons
<b>Primary structure:</b>	432 amino acid residues
<b>Carbohydrate content:</b>	15%
<b>Biological half-life:</b>	55-70 h
<b>Gene:</b>	13.5 kb, 7 exons, 6 introns location chromosome 1 at 1q 23-25
<b>Type:</b>	Belongs to a superfamily of about 40 serine protease inhibitors (serpins) which have diverged from a common ancestral molecule.
<b>Function:</b>	Inhibits thrombin, factor Xa and other proteases. Cofactor for heparin.
<b>Importance:</b>	Antithrombin deficiency is associated with an increased risk of developing a thromboembolic disorder





**Figure 4. Illustration of the increased risk of thrombosis in individuals with antithrombin deficiency.** Antithrombin deficiency may be inherited or arise as an acquired condition and represents a hypercoagulable state, a syndrome caused by the disturbance in the delicate balance between procoagulants and anticoagulants within the body. With a major trauma, such as an operation or infection, a significant amount of antithrombin is usually consumed. The combination of an initial antithrombin deficiency and trauma may therefore be detrimental.

## Antithrombin deficiency

### Thrombophilia

Venous thrombosis is a serious medical problem that annually affects 1 in 1000 individuals, causing considerable morbidity and death due to pulmonary embolism. The pathogenesis of the disease is multifactorial and several circumstantial as well as genetic risk factors have been identified.

*Thrombophilia* is the term used for familial or acquired defects of the hemostatic mechanism that increase the tendency to clot. The term encompasses a susceptibility to both venous and arterial thrombosis although, in the majority of patients, venous thrombosis of the lower limbs is the predominant clinical problem. Deficiencies of antithrombin, protein C, protein S, and the recently discovered inherited resistance to activated protein C (APC-resistance), are the most common known genetic causes for thrombosis. Together, they constitute

about 60-70% of cases diagnosed as inherited thrombophilia and may present as predisposing factors in up to one third of all affected patients with thrombosis.<sup>53</sup>

The laboratory diagnosis of these patients is of utmost importance, since it may help determine the type and the duration of treatment.

### Hereditary antithrombin deficiency

Hereditary antithrombin deficiency was the first recognized inherited thrombophilia. It was reported in 1965 by Egeberg, who described a Norwegian family with recurrent episodes of venous thromboembolism.<sup>17</sup> Since then, families with hereditary antithrombin deficiency have been found in many countries and the link between antithrombin deficiency and thrombosis has now been clearly established.<sup>3,18-20</sup> The pattern of inheritance is usually autosomal dominant (non-sex linked), with levels of functionally active protein around 40-70% of normal.



## Prevalence

The prevalence of symptomatic hereditary antithrombin deficiency is estimated at between 1 in 2,000 and 1 in 5,000 in the general population, although the total prevalence, i.e. symptomatic and asymptomatic individuals, may be much higher. Recent studies of healthy blood donors estimate the incidence of hereditary antithrombin deficiency to be between 1 in 250 and 1 in 500.<sup>4,5</sup>

Up to 5% of patients with a history of venous thrombosis, presenting with thrombosis at an age lower than 40-45 years, have a hereditary antithrombin deficiency.<sup>6</sup> In an unselected group of patients with acute venous thrombosis the prevalence is about 1%.<sup>54,55</sup>

## Classification

Various mutations causing hereditary antithrombin deficiency have been found and classified into a number of different types. The most common scheme divides antithrombin deficiency into two types based on the results from functional and immunological assays.<sup>21</sup> Type I antithrombin deficiency is the “classic” form of disorder and is characterized by a 50% reduction in both antigen and functional activity levels. Type II deficiency covers those cases in which approximately half the plasma antithrombin is a variant protein with reduced activity. Type II deficiency is subdivided into: mutations affecting the reactive site (RS), mutations affecting the heparin binding site (HBS), and pleiotropic (multiple) effects (PE). A more precise classification

- Thrombotic episode occurring at an early age
- A family history of venous thrombosis
- Recurrent venous thrombosis
- A thromboembolic event at an unusual site
- Thrombosis during pregnancy
- Heparin resistance

**Table 3.** The clinical features of hereditary antithrombin deficiency<sup>20</sup>

scheme based on the molecular defects has recently been proposed.<sup>22</sup>

## Risks and clinical features

The risk of a thromboembolic event in symptomatic antithrombin deficient individuals is on average approximately 1% per year of life. However, the risk appears to be age-related with the peak incidence occurring between the ages of 15 and 40.<sup>20</sup>

About one-third of thrombotic episodes occur spontaneously, while in the remaining two-thirds identifiable risk factors include pregnancy or childbirth, prolonged immobilization, oral contraceptives containing oestrogen and major trauma.<sup>23</sup>

The most common presentation of antithrombin deficiency is venous thrombosis of the lower limbs. A history of recurrent thrombosis occurs in about 60% of patients and is the clinical feature that usually prompts a search for antithrombin deficiency.

Classification	Activity	Antigen	Prevalence*	Thrombosis risk**
Type I	Low	Low	1:4200	High
Type II	Low	Normal	1:280	High (RS), Low (HBS)

**Table 4.** Classification, prevalence and thrombosis risk of inherited antithrombin deficiency.

\* The prevalence in 4,200 blood donors in the West of Scotland.<sup>5</sup> \*\* Type I deficiency is associated with a high risk of recurrent thromboembolic disease, whereas the risk associated with type II deficiency depends on the location of the mutation. Individuals with mutations at or close to the reactive site (RS) have a high risk of thrombosis. In contrast, heterozygous individuals with mutations affecting the heparin-binding site (HBS) run a relatively low risk.<sup>3</sup>



## Acquired antithrombin deficiency

Acquired antithrombin deficiency<sup>19,24</sup> is due to either *decreased synthesis* (e.g. liver disease,<sup>25</sup> premature neonates), *increased loss* (e.g. nephrotic syndrome), *increased consumption* (e.g. DIC), or is *drug-induced* (e.g. heparin, L-asparaginase<sup>26</sup>) (Table 5). Because acquired antithrombin deficiency is usually accompanied by decreases in other coagulation proteins, it is difficult to estimate the thrombotic risk based on antithrombin alone. However, in some cases the acquired antithrombin deficient condition entails a similar risk of thrombosis as the hereditary state and early recognition is therefore important.

### DIC syndrome

A number of primary events such as trauma, shock, burns, infection, cancer, complicated pregnancy (pre-eclampsia), may cause a loosely-defined syndrome called disseminated intravascular coagulation (DIC). The syndrome is characterized by poorly-controlled protease activity, resulting in accelerated fibrin formation and lysis which ultimately causes multiple organ damage. This happens when large amounts of traumatized or necrotizing tissue in the body releases tissue factor into the blood.

Laboratory indications include a prolonged APTT, thrombocytopenia, hypofibrinogenemia and increased fibrin and fibrinogen degradation products. Finding a low level of antithrombin also suggests DIC, particularly if this value declines over time. In acute DIC, the consumption of antithrombin greatly exceeds the rate of synthesis and the body is unable to replace antithrombin quickly enough. In these cases the antithrombin level is useful as a diagnostic aid and for the evaluation of therapy.<sup>27</sup> A severe initial decrease in antithrombin levels is a strong predictor of death and multiple organ failure in patients with septic DIC.<sup>28</sup>

### Neonate antithrombin levels

Antithrombin activity in healthy term neonates is approximately 50% of adult levels, with an increase to the normal range about 6 months after birth.<sup>29</sup> The inhibitor  $\alpha_2$ -macroglobulin plays a more important role during this time, which partially explains why

#### Physiological deficiency

Pre-term infants and neonates

Old age

#### Consumption coagulopathies

DIC

Pre-eclampsia

Surgical procedures and post-operative period

Acute myeloid leukemia

Massive venous thrombosis

Sick, pre-term infants and neonates

#### Renal disease

Nephrotic syndrome

Hemolytic uremic syndrome

#### Hepatic disease

Acute liver failure

Chronic liver disease

#### Gastrointestinal disease

Inflammatory bowel

Protein-losing enteropathy

#### Drugs

Heparin

L-Asparaginase

Oestrogens, tamoxifen

#### Dilutional effects

Hemodialysis

Plasmapheresis

Cardiopulmonary bypass

#### Other conditions

Diabetes

Behçet's disease

Malnutrition

**Table 5.** Conditions that can lead to an acquired reduction in antithrombin levels.<sup>19</sup>

thrombosis is uncommon in healthy neonates.<sup>30</sup> Plasma levels of antithrombin are about 35% in healthy preterm infants but may be decreased further in complications such as respiratory distress, sepsis or DIC. Sick infants with very low antithrombin levels have been reported to have a higher mortality rate, with a higher incidence of thrombotic and hemorrhagic complications.<sup>31</sup>

### Drug-induced effects

Heparin therapy decreases plasma antithrombin levels by increasing the hepatic clearance of antithrombin.<sup>32,33</sup> In some cases a phenomenon called heparin resistance may develop when antithrombin levels are critically low.



# Management of antithrombin deficiency

### Antithrombin levels

A normal antithrombin range is assumed to be 80-120%,<sup>23</sup> although there is a difficulty in knowing precisely what constitutes the lower limit of the normal range and what is the significance of levels just above or around this limit. Because of this, investigation of antithrombin must always include laboratory results as well as the patient's clinical history. Higher concentrations than normal appear to have no clinical significance.

### Prophylaxis and treatment options

The role of prophylaxis for individuals with hereditary antithrombin deficiency is at present unclear and controversial. However, it is agreed that individuals with symptomatic hereditary antithrombin deficiency should be offered prophylactic anticoagulants in situations that present a recognized risk of thrombosis (e.g. pregnancy, immobilisation and surgery). Attempts should be made to classify the type of deficiency in both symptomatic and asymptomatic individuals, in order to properly assess the risk of thrombosis and the need for prophylaxis. In general, patients with antithrombin deficiency type I and type II (RS) run a high risk of thrombosis.

### Heparin

Heparin is the first line of treatment for acute thrombosis in antithrombin deficient patients. Heparin resistance and extension of thrombosis have been reported<sup>35</sup> although in general this is not a major

problem. It is probably not cost-effective to give antithrombin substitution to patients requiring heparin, in order to overcome presumed heparin resistance.<sup>34</sup>

### Warfarin

In the absence of contra-indications (e.g. hemorrhagic diathesis, severe hypertension, pregnancy) the choice of a long-term prophylactic is the oral anticoagulant warfarin. Warfarin is a vitamin K antagonist and interferes in the vitamin K-dependent biosynthesis of several clotting factors by reducing their procoagulant activity.

### Antithrombin concentrates

Although plasma may be used as a source of antithrombin, the large volume required for therapeutic replacement represents a major limitation. Unlike plasma, a pure antithrombin concentrate can restore balance to an unbalanced system. Antithrombin concentrates are usually prepared by affinity chromatography and are carefully treated to destroy infectious agents.

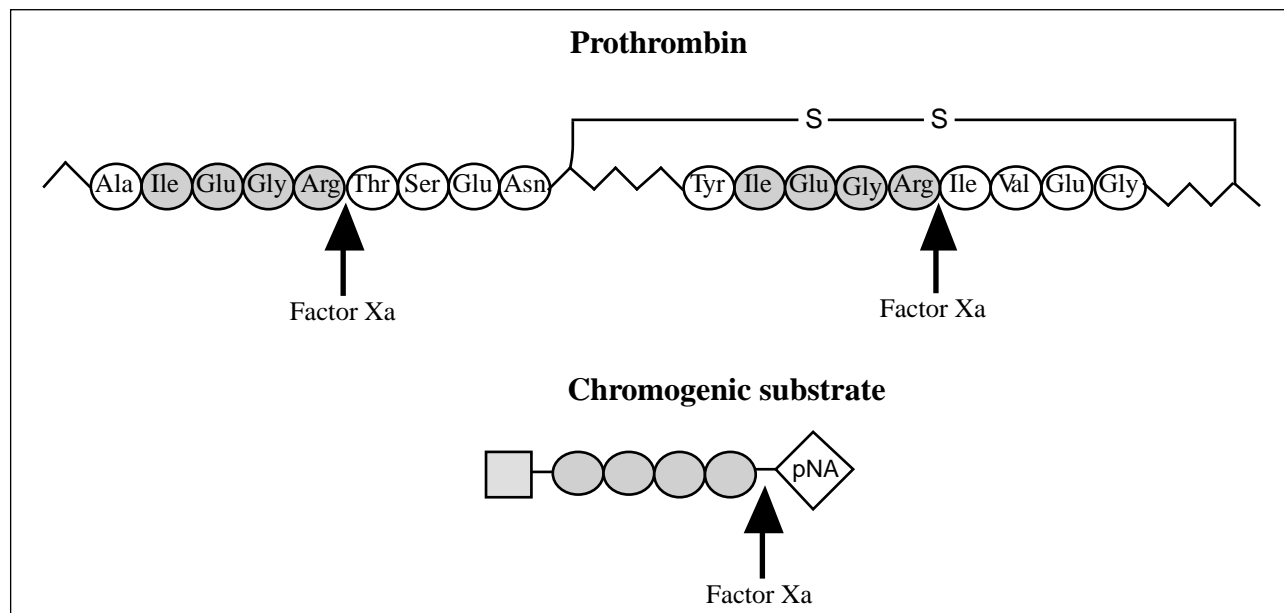
The successful use of antithrombin concentrates as prophylactics during surgical procedures or in the management of various consumptive coagulopathies have been reported by several groups.<sup>35-37</sup> However, the definitive advantage of using antithrombin concentrates has still not been adequately proven.<sup>38</sup>

Because of the moderate survival time of antithrombin in the blood circulation, alternate day infusions of antithrombin concentrates are usually necessary. Plasma levels should be monitored regularly to achieve effective treatment at a reasonable cost.

Condition	Range	Ref.	Condition	Range	Ref.
Normal	84-116%	23	Liver disease	25- 135%	25
Healthy neonates	39-87%	28	DIC	31-137%	26
Hereditary def.	40-70%	19	Heparin*	40-78%	32

Table 6. Antithrombin levels in various clinical states. \*Continuous infusion of therapeutical dose





**Figure 5.** Chromogenic synthetic peptide substrates mimic the cleavage sites of a natural substrate. Above: the natural substrate prothrombin, which can be cleaved by factor Xa at two locations. Bottom: a chromogenic factor Xa substrate.

## Antithrombin assays

### Diagnosis of antithrombin deficiency

The diagnosis of antithrombin deficiency is primarily based on the determination of antithrombin levels in plasma by using a functional activity assay or an immunological assay. For screening purposes the functional antithrombin assays are most relevant since they can detect both types of hereditary antithrombin deficiencies.<sup>39</sup>

Activity levels are usually expressed relative to a normal plasma pool with the designated value of 100%, which is equal to one unit of antithrombin in one millilitre of reference plasma.

### Activity assays

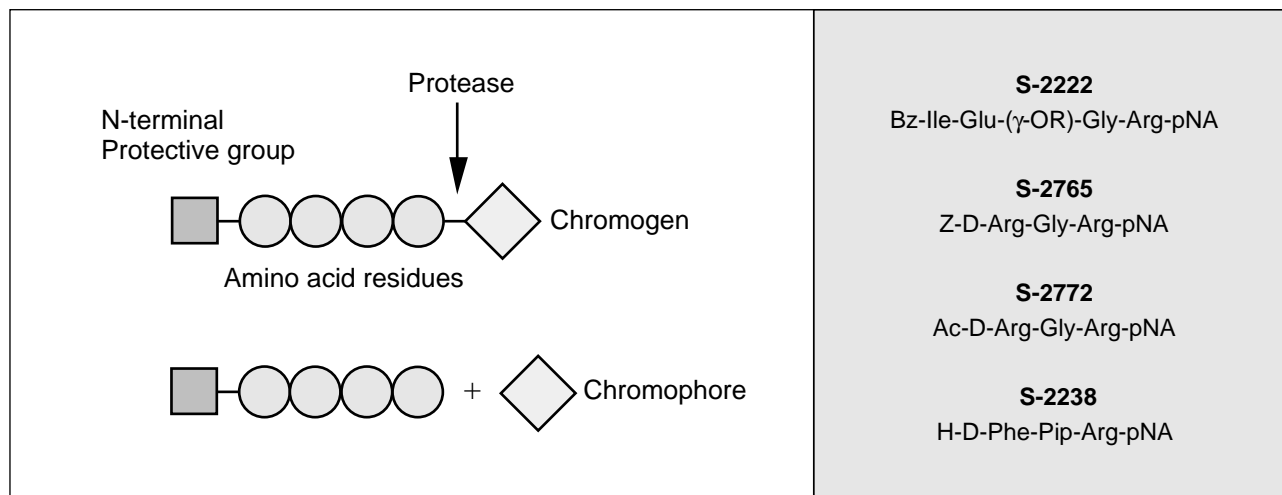
Functional assays are based on the capacity of a plasma sample to inhibit a known amount of either thrombin or factor Xa added in excess, in the presence of heparin (heparin cofactor assay) or in the absence of heparin (progressive activity assay). The residual enzyme is measured by a clotting-based assay or by using chromogenic peptide substrates.

### Heparin cofactor assays

In the absence of heparin, the inhibition rates are slow and progressive antithrombin assays are influenced by other plasma inhibitors such as trypsin inhibitor and  $\alpha_2$ -macroglobulin.<sup>40</sup> These assays are therefore used mainly for corroborative purposes.

The introduction of heparin in the heparin cofactor assays speeds up the reaction rates and makes them more specific and practical. Antithrombin is often determined by measuring its activity in the presence of an excess of thrombin.<sup>41</sup> Although, heparin cofactor assays that use human thrombin as the target protease may overestimate the antithrombin concentration due to heparin cofactor II; a thrombin-specific serpin with heparin cofactor abilities. It is present in plasma at a concentration about half that of antithrombin and, in the presence of high heparin levels, may represent up to 20% of the thrombin inhibitory activity in plasma.<sup>42</sup> Interference from heparin cofactor II is therefore particularly apparent when analyzing plasma from heparin-treated patients.<sup>43</sup> The use of bovine thrombin, low heparin concentrations or factor Xa as a substrate instead of thrombin, minimizes the influence.





**Figure 6.** Left: Principle structure of synthetic peptide substrates. Right: Chromogenic substrates for factor Xa (S-2222, S-2765, S-2772) and thrombin (S-2238). Abbreviations: Bz= benzoyl, Z= benzyloxycarbonyl, Ac= acetyl, Pip= pipercolinic acid, Arg= Arginine, Glu= Glutamic acid, Gly= Glycine, Ile= Isoleucine, Phe= Phenylalanine, pNA= para-nitroaniline. S-2222; R= H (50%) and CH<sub>3</sub> (50%).

### Chromogenic heparin cofactor assays

The most frequently applied routine method for measuring antithrombin activity is a heparin cofactor assay based on chromogenic peptide substrates. These mimic either the cleavage site of fibrinogen cleaved by thrombin, or prothrombin cleaved by factor Xa.<sup>41</sup> Considering the influence by heparin cofactor II on assays based on thrombin inhibition, one can expect the chromogenic factor Xa-based assays to completely dominate in the near future.

Chromogenic peptides are composed of 3-4 amino acids, with the chromogenic group 4-nitroaniline (pNA) attached to the end (Figures 5 and 6). When the peptide is incubated with the active protease it is cleaved and pNA (yellow colour) is liberated. The release is measured at 405 nm, either during the reaction in a photometer cuvette, or discontinuously by stopping the reaction with acetic or citric acid. The photometric signal, i.e. the change in absorbance, is inversely proportional to the antithrombin activity.

### Coagulometric heparin cofactor assays

As the name implies, antithrombin clotting assays are based on the ability of antithrombin to prolong the clotting time of blood and plasma.

Clotting assays are dependent on manipulation of the plasma sample (defibrination), using pro-

longed heat treatment or ancred.<sup>45</sup> Defibrinated plasma is incubated with a fixed amount of thrombin in excess and heparin is added to accelerate the inactivation reaction. This mixture is then added to a fibrinogen solution, and the clotting time, which is directly proportional to the antithrombin activity, is measured. In some assays, factor Xa is used instead of thrombin.<sup>46</sup>

Although clotting assays can be used to measure antithrombin activity accurately, chromogenic assays are preferable as they are more rapid and do not require a defibrination step.<sup>47</sup>

### Immunological assays

Immunological antithrombin assays such as the radial immunodiffusion method of Mancini and the electroimmunodiffusion method of Laurell, quantify the amount of antithrombin antigen present in plasma.<sup>48,49</sup> The molar concentration of antithrombin protein material in normal plasma, determined using these methods, is approximately 4  $\mu$ mol/l.

An immunological method in combination with a chromogenic method can be used to diagnose and classify the deficiency states. If both antigen and activity levels are decreased, a quantitative (type I) deficiency can be assumed. They can also provide important information about the consumption of antithrombin in connection with a thrombotic event.



## Diagnostic kits from Chromogenix

### We invented the technology

The development of the first chromogenic peptide substrate S-2160 in the early Seventies, initiated the introduction of photometry in hematology.<sup>50</sup> Today, there are many tests based on chromogenic substrates for coagulation factors, fibrinolytic factors as well as for inhibitors of both enzyme systems and endotoxin (Table 8).<sup>51,52</sup> The tests can be performed manually or on automated analytical systems with high specificity, sensitivity and accuracy.

Important, non-chromogenic-based products include kits for APC resistance (APTT-test) and several ELISA kits (Table 9).

### Antithrombin kits

Chromogenix has a well-documented range of chromogenic test kits for the determination of antithrombin activity in human plasma. The first commercial kit developed was COATEST® Antithrombin, which over the years has become an established assay worldwide. The COACUTE® and COAMATIC® Antithrombin kits are the latest developments in a line of chromogenic products. A major advantage is that they are based on factor Xa instead of thrombin, thereby eliminating the risk of overestimating the antithrombin level due to the influence of heparin cofactor II. All products offer a broad measuring range and good reagent stability.

#### HEMOSTASIS

Prekallikrein  
Factor VII  
Factor VIII  
Factor X  
Soluble fibrin  
Antithrombin  
Heparin/LMW heparin  
Protein C  
 $\alpha_2$ -macroglobulin  
 $\alpha_1$ -antitrypsin  
Plasminogen  
t-PA  
PAI-1  
Plasmin inhibitor

#### ENDOTOXIN

Endotoxin

#### HEMOSTASIS

APC resistance  
Anti-Cardiolipin IgG, IgM  
D-dimer  
Lipoprotein(a)  
t-PA  
PAI-1

#### INFLAMMATION/ SEPSIS

EndoCAB  
IL-6  
TFNa  
Endotoxin

**Table 8.** Substances that can be determined with diagnostic kits based on synthetic peptide substrates (Examples from Chromogenix product range 1995).

**Table 9.** Substances that can be determined with kits from Chromogenix, based on either ELISA or clotting techniques.

### Diagnostic relevance of antithrombin

- **Hereditary antithrombin deficiency**  
- diagnosing thrombophilia

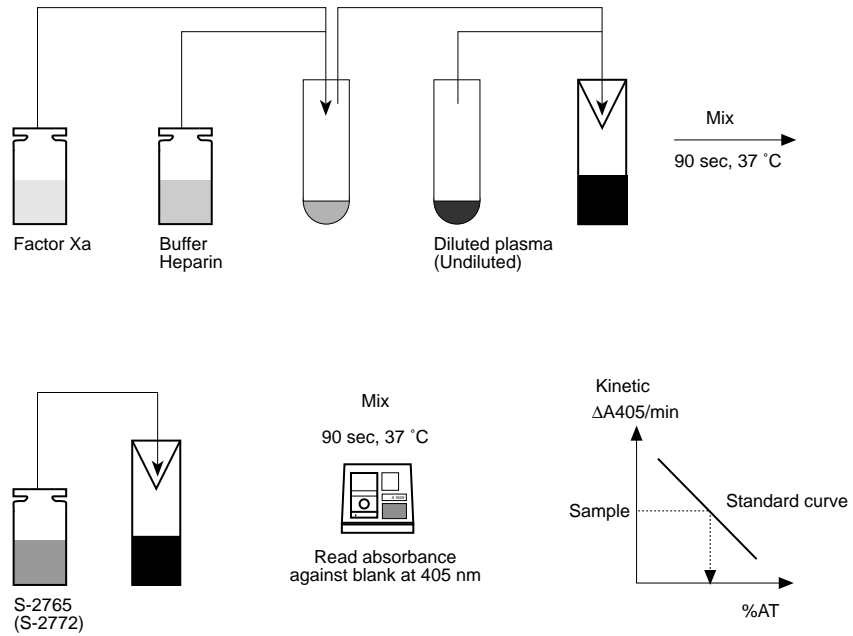
- **Acquired antithrombin deficiency**  
- prognostic marker in patients with DIC, septic shock, nephrotic syndrome, liver disease  
- diagnosis and evaluation of therapy for DIC patients
- **Antithrombin substitution therapy**  
- criterion for initiation of therapy  
- monitoring substitution



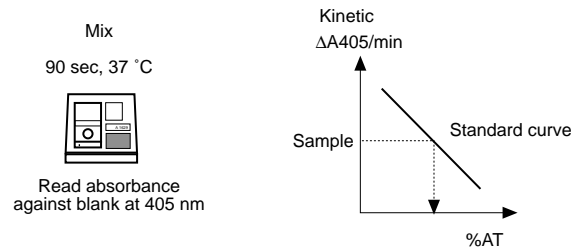
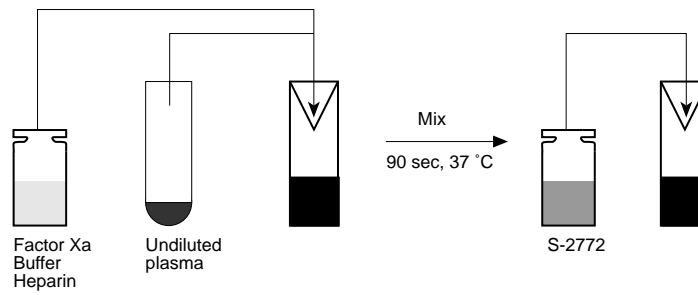
Assay procedures

Product name	Enzyme	Substrate	Plasma	Method
COAMATIC® Antithrombin	FXa	S-2765™	Diluted	End-point/Kinetic
COAMATIC® Antithrombin 400	FXa	S-2772™	Undiluted	Kinetic
COAMATIC® LR Antithrombin	FXa	S-2772™	Undiluted	Kinetic
COACUTE® Antithrombin	FXa	S-2772™	Diluted	End-point
COATEST® Antithrombin	Thrombin	S-2238™	Diluted	End-point/kinetic

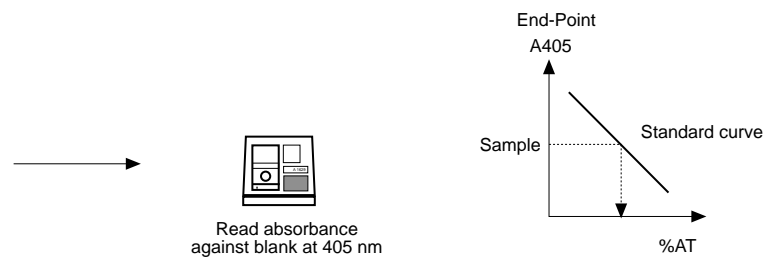
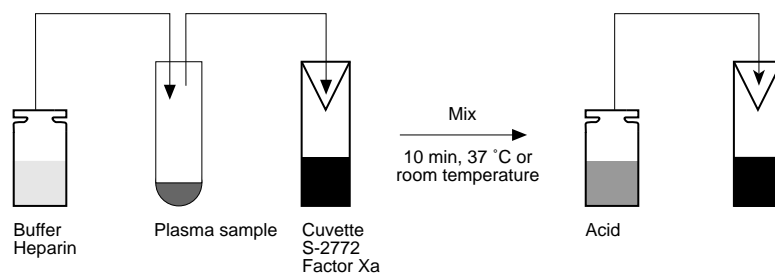
COAMATIC® Antithrombin  
(COAMATIC® Antithrombin 400)



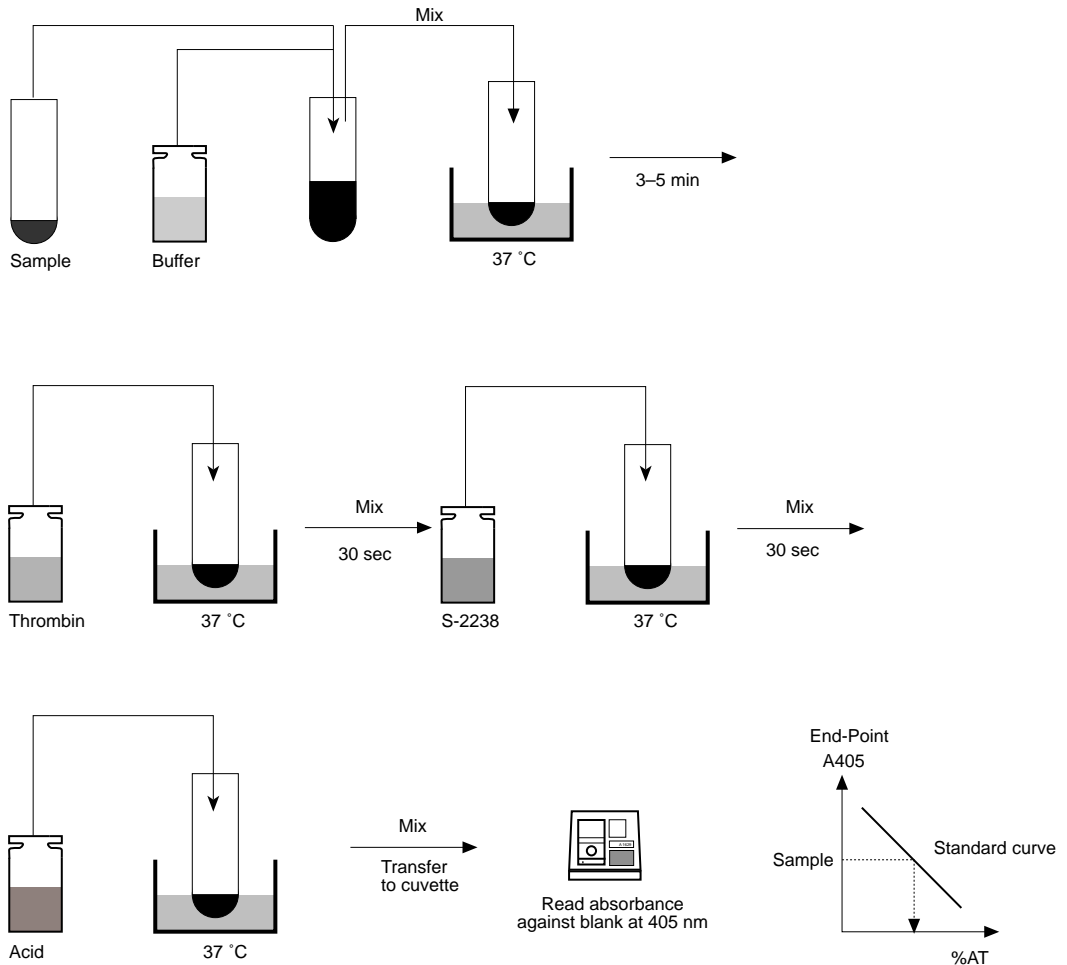
## COAMATIC® LR Antithrombin



## COACUTE® Antithrombin



# COATEST® Antithrombin



## COAMATIC® Antithrombin

COAMATIC® Antithrombin is a chromogenic heparin cofactor assay for the specific determination of antithrombin activity in plasma. The use of factor Xa eliminates interference from heparin cofactor II and allows accurate antithrombin determination in patients receiving heparin therapy. The assay also discriminates better between antithrombin-deficient and non-antithrombin-deficient individuals compared to a conventional thrombin-based assay.

The kit is adapted for use with a wide range of automatic analyzers, achieving comparable reaction conditions on the different analyzers.

### Measurement principle

Diluted plasma is incubated with an excess of factor Xa in the presence of heparin. The residual quantity of factor Xa is determined by the rate of hydrolysis of the chromogenic substrate S-2765. The pNA release measured at 405 nm is inversely proportional to the antithrombin level in the range 0-125% of normal plasma.

Article number: 82 19 91

#### The kit contains

FXa (bovine)	1 vial
Buffer with heparin	1 vial
S-2765	1 vial

#### Storage and stability in solution

FXa	2–8 °C, 3 months
Buffer with heparin	2–8 °C, 3 months
S-2765	2–8 °C, 6 months

#### Measuring range

0-125%

#### Detection limit

5%

#### Repeatability

Microplate method:

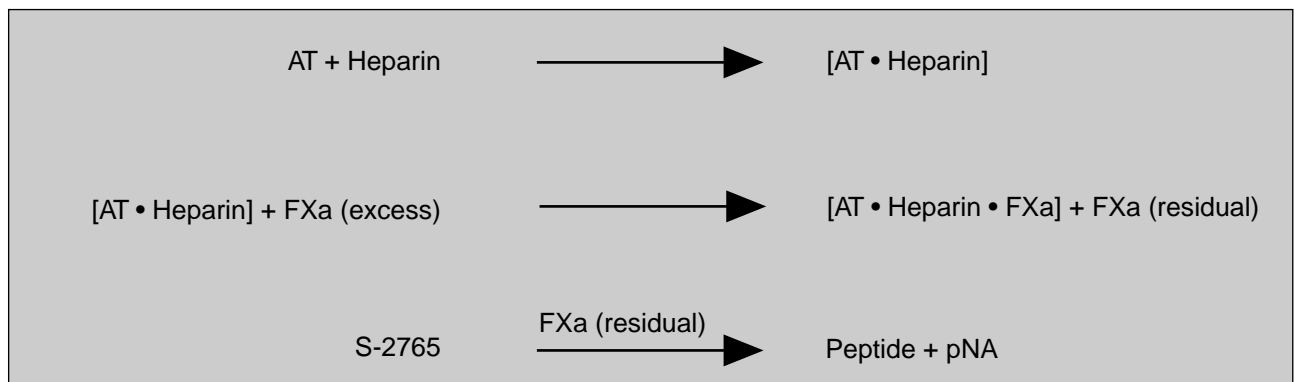
AT	CV within series	CV between series
50%	3.1%	2.6%
100%	4.8%	4.3%

#### Specificity and interfering factors

No influence from heparin cofactor II,  $\alpha_2$ -macroglobulin or trypsin inhibitor. No drug interference reported. Contact activation of plasma may result in the underestimation of antithrombin. Bilirubin, haemoglobin and plasma from hyperlipaemic patients interfere in absorbance readings in the stopped method. In these cases individual plasma blanks are necessary.

#### Determinations per kit

Manual method 50, microplate method 200, automated methods up to 130



The measurement principle of COAMATIC® Antithrombin



# COAMATIC® Antithrombin 400

COAMATIC® Antithrombin 400 is a chromogenic heparin cofactor assay for the determination of antithrombin activity in undiluted plasma. The kit is specifically designed for applications on Hitachi and Cobas instruments.

The use of factor Xa eliminates interference from heparin cofactor II and allows accurate antithrombin determination in patients receiving heparin therapy.

### Measurement principle

Plasma is incubated with an excess of factor Xa in the presence of heparin. The residual activity of factor Xa is determined by the rate of hydrolysis of the chromogenic substrate S-2772. The pNA release measured at 405 nm is inversely proportional to the antithrombin level in the range 0-125% of normal plasma.

Article number: 82 23 20

#### The kit contains

FXa (bovine)	6 vials
Buffer with heparin	6 vials
S-2772	2 vials

#### Storage and stability in solution

FXa	2–8 °C, 3 months
Buffer with heparin	2–8 °C, 3 months
S-2772	2–8 °C, 6 months

#### Measuring range

0-125%

#### Detection limit

5%

#### Repeatability

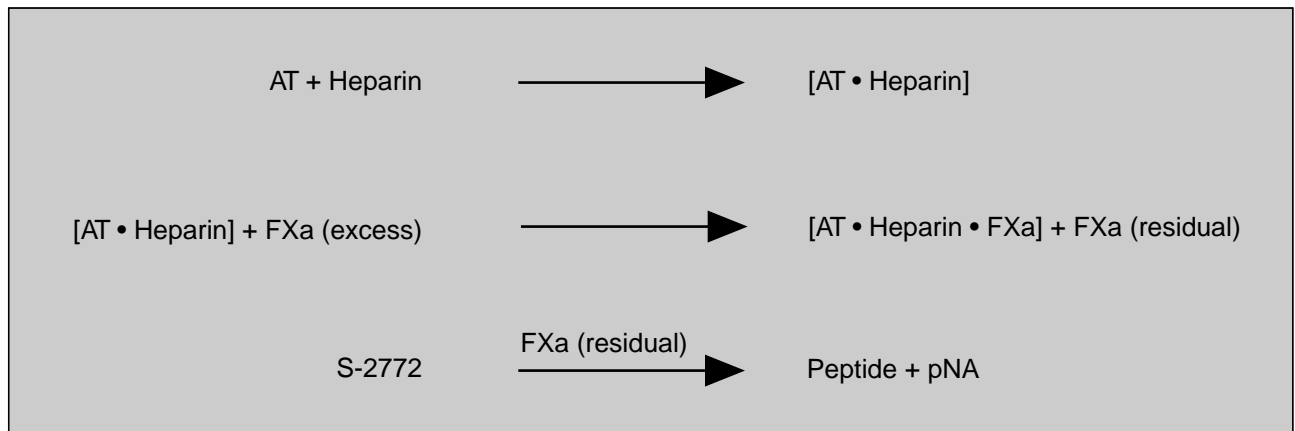
Cobas Mira:		
AT	CV within series	CV between series
50%	3.1%	2.2%
100%	1.6%	2.2%

#### Specificity and interfering factors

No influence from heparin cofactor II.  
No drug interference reported.

#### Determinations per kit

Cobas 430, Hitachi 420



The measurement principle of COAMATIC® Antithrombin 400



# COAMATIC® LR Antithrombin

COAMATIC® LR (liquid reagents) Antithrombin is a chromogenic heparin cofactor assay for the determination of antithrombin activity in undiluted plasma. The reagents of the kit are both in liquid formulation and can be used on the Cobas Fara and Mira instruments, as well as on the Hitachi 704, 705 and 717 instruments.

The use of factor Xa eliminates interference from heparin cofactor II and allows accurate antithrombin determination in patients receiving heparin therapy.

### Measurement principle

Plasma is incubated with an excess of factor Xa in the presence of heparin. The residual activity of factor Xa is determined by the rate of hydrolysis of the chromogenic substrate S-2772. The pNA release measured at 405 nm is inversely proportional to the antithrombin level in the range 0-125% of normal plasma.

Article number: 82 29 57

#### The kit contains

FXa (bovine) with heparin	6 vials
S-2772	2 vials

#### Storage and stability when opened

FXa with heparin	2–8 °C, 1 month
S-2772 solution	2–8 °C, 6 months

#### Measuring range

0-125%

#### Detection limit

10%

#### Repeatability

Hitachi 717:

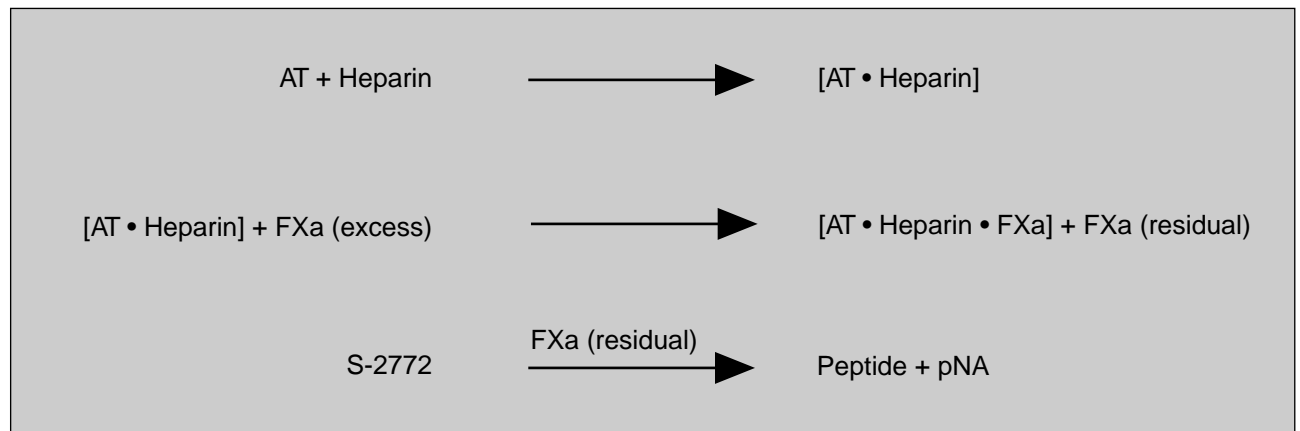
AT	CV within series	CV between series
50%	1.6%	0.7%
100%	1.1%	0.4%

#### Specificity and interfering factors

No influence from heparin cofactor II.  
No drug interference reported.

#### Determinations per kit

Cobas 430, Hitachi 420



The measurement principle of COAMATIC® LR Antithrombin



# COACUTE® Antithrombin R

COACUTE® Antithrombin R (robust) is designed for the convenient, rapid and reliable determination of antithrombin activity in acute situations and in laboratories performing small numbers of antithrombin tests.

## Measurement principle

Plasma is diluted in a buffer containing an excess of heparin. Antithrombin present in the plasma forms an [AT•heparin] complex. An aliquot of this plasma dilution is added to a cuvette containing a lyophilized mixture of factor Xa (FXa) and the FXa specific chromogenic substrate S-2772.

The lyophilized mixture is reconstituted upon addition of the plasma dilution and the solution obtained is allowed to stand for five minutes at room temperature. Two competing reactions thereby occur simultaneously: a) inhibition of FXa by [AT•heparin] complex and b) reaction of FXa with the chromogenic substrate resulting in cleavage of pNA. The amount of cleaved p-NA, determined spectrophotometrically at 405 nm after addition of dilute acetic acid, is inversely proportional to the amount of antithrombin in the sample.

Article number: 82 21 22

### The kit contains

FXa (bovine) + S-2772	2 x 5 cuvettes
Buffer with heparin	10 tubes
Acetic acid 5%	1 vial

### Storage and stability when opened

FXa (bovine) + S-2772	2–8 °C, 1 month
Buffer with heparin	exp. date
Acetic acid	exp. date

### Measuring range

0-110%

### Detection limit

10%

### Repeatability

Manual method:

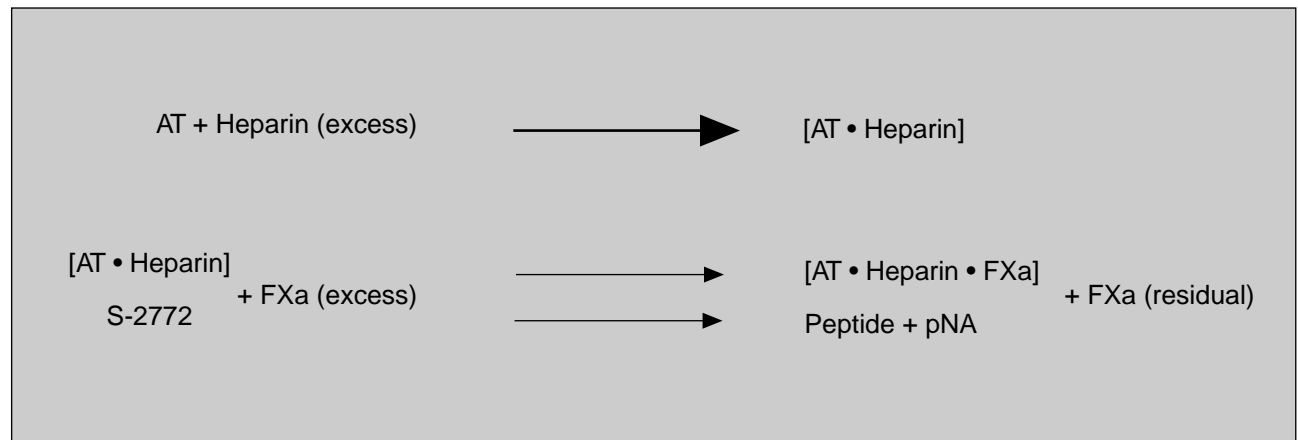
AT	CV within series	CV between series
45%	3.5%	1.7%
69%	2.6%	2.3%
95%	1.4%	1.4%

### Specificity and interfering factors

No influence from heparin cofactor II.  
No drug interference reported

### Determinations per kit

Manual method 10



The measurement principle of COACUTE® Antithrombin R



## COATEST® Antithrombin

COATEST® Antithrombin is a thrombin-based chromogenic heparin cofactor assay for the specific determination of antithrombin activity in plasma. This kit was the first commercial chromogenic antithrombin assay and has become well established over the years.

### Measurement principle

Diluted plasma is incubated with an excess of thrombin in the presence of heparin. The residual quantity of thrombin is determined by the rate of hydrolysis of the chromogenic substrate S-2238. The pNA release measured at 405 nm is inversely proportional to the antithrombin level in the range 0-125% of normal plasma.

Article number: 25 55 21

#### The kit contains

Thrombin (bovine)	4 vials
Buffer with heparin	1 vial
S-2238	1 vial
Normal plasma	4 vials

#### Storage and stability in solution

Thrombin	2–8 °C, 4 weeks
Buffer with heparin	2–8 °C, 2 months
S-2238	2–8 °C, 6 months
Normal plasma	-20 °C, 1 month
	2–8 °C, 1 week

#### Measuring range

0-125%

#### Detection limit

5%

#### Repeatability

Manual method

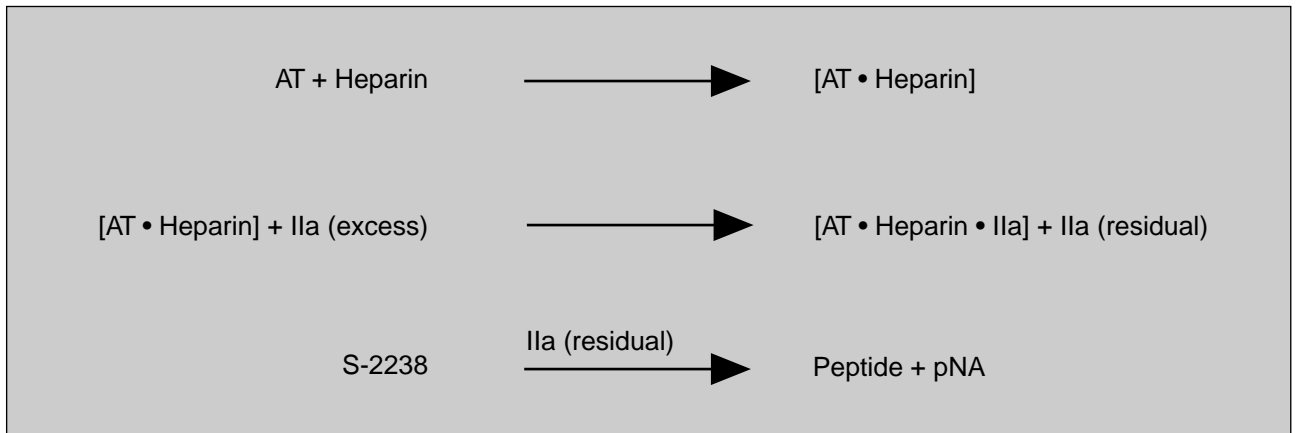
AT	CV within series	CV between series
50%	5.5%	6.7%
100%	4.1%	4.9%

#### Specificity and interfering factors

No drug interference reported. May be influenced by heparin cofactor II in plasma from heparinized patients.

#### Determinations per kit

Manual method 45, microplate method 260, automated methods up to 300



The measurement principle of COATEST® Antithrombin. IIa = Thrombin



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# Glossary

**Allele.** One of an array of possible mutational forms of a gene at a specific locus

**Amino acids.** Basic building blocks of all proteins

**Antibody.** A molecule produced by animals in response to antigen.

**Antigen.** A molecule that induces the formation of an antibody.

**APC resistance.** An hereditary defect caused mainly by a point mutation in the gene coding for factor V and characterized by a poor anticoagulant response to activated protein C.

**Autosome.** A chromosome other than a sex chromosome.

**Chromosome.** The darkly staining bodies within the cells made up of a large number of genes and a centromere region.

**Embolism.** Obstruction or occlusion of a vessel by a transported clot.

**Endothelium.** Cells lining blood vessels and lymphatics which control the passage of materials into and out of the bloodstream.

**Enzymes.** A protein with catalytic power.

**Exon.** Gene segment encoding protein.

**Fibrin.** An elastic filamentous protein derived from fibrinogen by the action of thrombin, which releases fibrinopeptides A and B from fibrinogen.

**Fibrinogen.** Factor I; a globulin of the blood plasma that is converted into the coagulated protein, fibrin, by the action of thrombin in the presence of calcium ions.

**Fibrinolysis.** The hydrolysis of fibrin by plasmin.

**Gene.** The unit of inheritance, located at a specific region on the chromosome.

**Glycoprotein.** One of a group of protein-carbohydrate compounds

**Hemostasis.** Process that arrests the escape of blood from injured vessels.

**Homozygous.** Condition of having identical alleles at one or more loci under consideration

**Heparin cofactor II.** Serpin with heparin cofactor abilities. Specific inhibitor of thrombin.

**Hepatocytes.** Cells in the liver that are arranged in folded sheets. They produce many of the blood proteins.

**Heterozygous.** Having a dissimilar alleles at one or more loci.

**Intron.** Gene segment between exons not encoding protein.

**Locus.** The position on a chromosome at which a particular gene is found.

**Platelet.** A small disk-shaped blood cell, containing granules in the central part and peripherally, clear protoplasm, but no nucleus. Numbering 200,000 to 300,000/ $\mu$ l.

**Platelet factor 4.** A heparin antagonist released from activated platelets. It interacts equally with high and low affinity heparin and like most heparin-binding proteins the interactions decrease with molecular weight.

**Protamine.** Protein derived from fish and used as a clinical antagonist for heparin.

**Proteases, proteinases.** Enzymes hydrolyzing native protein, or polypeptides, making internal cleavages; they include pepsin, chymosin, trypsin, papain etc.

**Proteins.** A class of macromolecules that are built from a repertoire of twenty amino acids.

**Proteoglycan.** A macromolecular glycoconjugate composed of sulfated glycosaminoglycans covalently linked to a protein core.

**Proteolysis.** Enzymatic cleavage of protein.

**Prothrombin.** Factor II, zymogen of thrombin; a glycoprotein formed and stored in the parenchymal cells of the liver. Present in blood at approximately 100  $\mu$ g/ml.

**Receptor.** A cell surface molecule which binds specifically to particular proteins or peptides in the fluid phase.

**Sepsis.** A clinical syndrome of serious bacterial infection.

**Serine protease.** Proteolytic enzyme with a serine residue at its enzymatically active site.

**Serpin.** Serine protease inhibitor.

**Serum.** The watery portion of blood remaining after fibrinogen has been removed from the plasma

**Thrombocyte.** Blood platelet

**Thrombocytopenia.** A condition in which there is an abnormally small number of platelets in the circulating blood (usually less than 150,000/ $\mu$ l).

**Thromboembolism.** Refers to either thrombosis or embolism or a combination of both.

**Thrombolytics.** Biological and synthetic substances capable of activating the fibrinolytic system in plasma.

**Thrombophlebitis.** Thrombus formation with inflammation.

**Thrombin.** Active protease deriving from prothrombin (factor II). Induces conversion of fibrinogen into clot-forming fibrin monomers resulting in the coagulation of blood.

**Thrombophilia.** A disorder in which there is a tendency to develop thrombosis.

**Thrombosis.** The formation of a thrombus (blood clot).

**Thrombotic.** Relating to, caused by, or characterized by thrombosis.

**Zymogens.** The enzymatically inactive precursors of proteolytic enzymes.



# Notes



# Antithrombin

## Chromogenix Product Monographs

### Antithrombin

COAMATIC® Antithrombin  
COAMATIC® AT 400  
COAMATIC® LR Antithrombin  
COATEST® Antithrombin  
COACUTE® Antithrombin R

### APC resistance

COATEST® APC Resistance  
COATEST® APC Resistance – C  
COATEST® APC Resistance – S  
COATEST® APC Resistance – SC  
COATEST® APC Resistance V  
COATEST® APC Resistance V-S  
COASET® FV-506

### D-dimer

SimpliRED® D-dimer  
DIMERTEST® GOLD EIA

### Factor VIII

COAMATIC® Factor VIII  
COATEST® Factor VIII  
COATEST® VIII:C/4

### Heparin

COATEST® Heparin  
COATEST® LMWHeparin/Heparin  
COACUTE® Heparin

### Plasminogen

COAMATIC® Plasminogen  
COATEST® Plasminogen

### Protein C

COAMATIC® Protein C

### t-PA

COASET® t-PA  
COALIZA® t-PA

### COAMATIC®

The latest techniques adapted specifically for the use with automated laboratory equipment.

### COATEST®

Innovative and well-documented products with a range of applications for automated instruments.

### COALIZA®

Complete enzyme-immunoassay (ELISA)-based kits for antigen determinations.

### COACUTE®

For a small number of tests. All the reagents are freeze-dried in a single test cuvette.

### COASET®

A group of products aimed for research applications.

### COAMAB®

Monoclonal antibodies for research purpose.

# **CHROMOGENIX**