# Factor VIII

Chromogenix Monograph Series

CHROMOGENIX

# Factor VIII

## Chromogenic determination of factor VIII activity in plasma and factor VIII concentrates

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# Chromogenic determination of factor VIII activity in plasma and factor VIII concentrates

**Factor VIII is a non-enzymatic plasma protein that is essential for normal blood coagulation. The deficiency of factor VIII activity in humans is associated with a congenital bleeding disorder, called hemophilia A, which affects about 1 in 5000 males. Hemophilia A patients are treated with factor VIII concentrate for maintenance of normal hemostasis but regrettably prophylactic treatment is not in general use worldwide. During later years recombinant factor VIII has been approved for therapeutic use, which minimizes the risk of viral transmission. There is now also growing evidence that elevated factor VIII activity is a risk factor for thrombosis. Hence, factor VIII levels are of importance to measure not only for diagnosing and monitoring hemophilia A but also for thrombophilia investigations. With the advent of chromogenic substrate technology accurate and sensitive methods are available for quality control and for the clinical coagulation laboratory. It is the purpose of this monograph to present an overview of biochemical and clinical data on factor VIII and to provide comprehensive information on methodological aspects and on the use of the Coamatic and Coatest Factor VIII kits.**

## Introduction

Hemostasis is the collective name of all the processes which allows uninterrupted blood flow and which, on demand, has an immediate and considerable capacity to stop leakage of blood from the blood organ and to dissolve small thrombi, should these events occur. Maintenance of normal hemostasis is therefore not surprisingly a complex and delicate balance between procoagulant and anticoagulant events and their regulation.

The bleeding disorder hemophilia A, with its easily recognized and dramatic symptoms already in childhood, has been known among mankind for a very long time.

Most people may be familiar with this disease through its occurrence in the Royal family of Queen Victoria and its links to the Tsar empire and to the Spanish Royal family. However, documentation of this disease stems as long back as the 3rd century, described by the rabbins in the Talmud.[1] Hemophilia A is inherited as an X-linked recessive disease expressed in males and with females being asymptomatic carriers.

This disease was shown to be connected with deficiency of a specific plasma protein in 1937, a protein which we denote as factor VIII but which was earlier called antihemophilic factor or antihemophilic globulin.[2]

Hemophilia B is another bleeding disorder, caused by deficiency of factor IX, which was earlier denoted Christmas Factor.

A simplified overview of blood coagulation and its regulation by antithrombin, tissue factor pathway inhibitor (TFPI) and the protein C anticoagulant pathway is shown in figure 1. Coagulation is triggered by factor VII complexed with tissue factor from exposed subendothelium in the damaged vessel wall.

Factor VIII serves as a cofactor to the enzyme factor IXa in its activation of the zymogen factor X, other accessory components being calcium ions and procoagulant phospholipids expressed on the surface of activated platelets. Factor VIII has to be activated before it can support factor IXa as an effective cofactor. In vitro such activation can be accomplished by thrombin and factor Xa but in vivo it may well be that thrombin is the only activator of physiological relevance to generate factor VIIIa. Under in vivo conditions, the rate increase of factor IXa activity due to factor VIIIa is several thousand-fold.[3]

A similar rate increase of factor Xa activity is found for factor Va.[3] It is no wonder, then, that the down-regulation of blood coagulation through specific inactivation of factor VIIIa and factor Va by activated protein C is a most efficient way of preventing further thrombin generation.

During the last decades much new information has been gained on risk factors for venous and arterial thrombosis and the prevalence of genetic disorders among specific plasma components resulting in an increased risk. Examples on this are deficiencies of antithrombin, protein C and protein S and point mutations in factor V and prothrombin (see ref. 4 for a review).
Indeed, although classically being regarded as a component with abnormalities strictly related to bleeding symptoms, it now appears that if the abnormality is expressed as an elevated activity, factor VIII turns into a risk factor for thrombosis. This was published already 1980 regarding arterial thrombosis[5] and appears now from more recent publications to have been reconfirmed[6] and also to be linked with an increased risk for venous thrombosis.[7,8]

Factor VIII is a labile plasma component and determination of factor VIII activity has to be made in a strictly controlled way in order to ensure accurate results. Blood sampling should be connected with a clean venepuncture in order to avoid activation of blood coagulation and thereby the risk of inadvertent activation of factor VIII. Traditionally, clotting assays are used for assigning factor VIII activities, but chromogenic assays are gaining an increased use, especially in assigning potencies of factor VIII concentrates.
The prime reasons for this is that chromogenic assays can be designed to provide robust assay conditions, offering a high resolution and without any negative interference from preactivated factor VIII molecules, features which have resulted in the selection of chromogenic methodology as the European Pharmacopoeia Reference method for factor VIII concentrates.[9]

# Biochemistry

## The factor VIII gene and synthesis of the factor VIII protein

The factor VIII gene is located on the X chromosome and comprises no less than 26 exons separated by 25 non-coding introns and the complete gene constitutes about 0.1% of the chromosome.

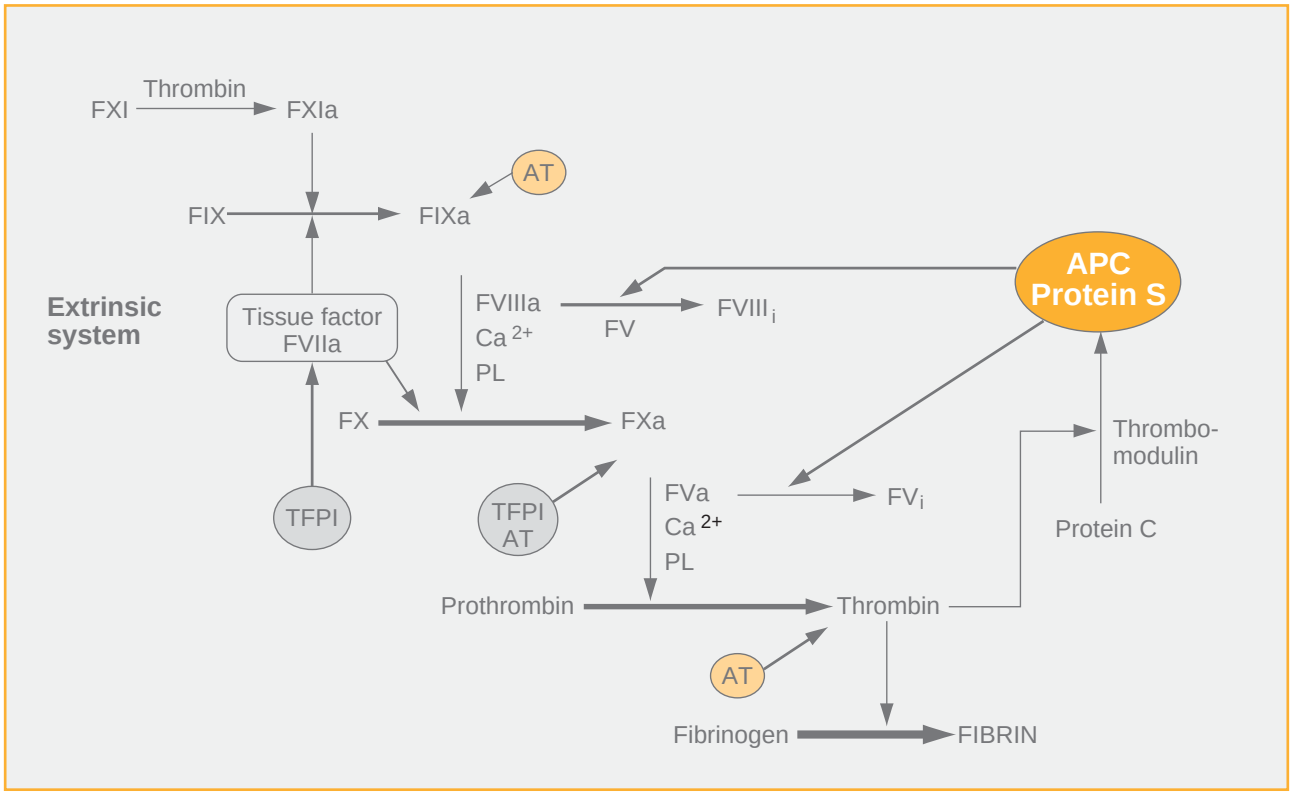



***Figure 1. The Coagulation Cascade***
*Blood coagulation can somewhat simplified be described as a cascade system of proteolytic reactions initiated in response to tissue damage. In each reaction, an inactive zymogen is converted to its active enzyme counterpart, which then participates in the subsequent step of the coagulation cascade. Thrombin activates factors V, VIII and XI, thereby promoting its own generation.*
*The protein C anticoagulant pathway down-regulates blood coagulation by inactivating factors VIIIa and Va.*
*Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) inhibits the generated enzymes factor VIIa, IXa, Xa and thrombin.*

The uncertainty about the site of synthesis and the low abundance of factor VIII, about 0.2 mg/L, made the cloning of the factor VIII gene a most challenging task. At the same time it could be easily understood that the interest was considerable due to the low amount of protein needed to manage a proper hemostasis in hemophilia A patients. Indeed, and quite fascinating, two groups simultaneously announced their successful cloning and expression of the factor VIII gene at the XVIth World Federation of Hemophilia Congress in Rio de Janeiro 1984, accompanied by publications in the same issue of Nature that year.[10,11]

The site of synthesis of factor VIII has remained unclear for a long time but the most likely location is in the hepatocytes although factor VIII mRNA has also been detected in many other cells and tissues.[12,13] Independent support for the involvement of the hepatocytes is the beneficial effect of liver transplantation in severe hemophilia A patients.[14]

## Protein structure

The mature protein contains 2332 amino acids preceded by a 19-residue hydrophobic signal peptide of importance for the secretion.
Factor VIII is synthesized as a single polypeptide chain with a molecular weight of about 330 kDa[15] and it is thus an unusually large protein.
Factor VIII contains three different domains:
an A-domain which is repeated three times, a central B-domain and a twice repeated C-domain (see fig. 2).[11,16]
There are also small acidic peptide regions, one of which joins the A1 and A2 domains and another joining the B-domain with the amino terminal of the A3 domain.
The overall structure has a high homology with factor V, which also is of a similar size. The amino acid sequence homology is, however, limited for the B-domains of the two proteins. In common, though, is that the B-domains are heavily glycosylated through asparagine-linked carbohydrates.
Each of the A-domains share about 30% sequence homology with an A-domain structure also found in ceruloplasmin[17], a copper binding protein, and the C-domains are homologous to proteins which bind negatively charged phospholipids, the latter suggesting that these domains are important for the interaction with procoagulant phospholipids.[18]

The amino-terminal signal peptide is removed upon translocation of factor VIII into the endoplasmatic reticulum and the native factor VIII molecule is then cleaved in the B-domain in connection with its secretion. This results in the release of a heterodimer comprised of a 200 kDa heavy chain and an 80 kDa light chain, the association of which is metal-ion dependent.[19]

During the secretion factor VIII associates with von Willebrand factor (see below).

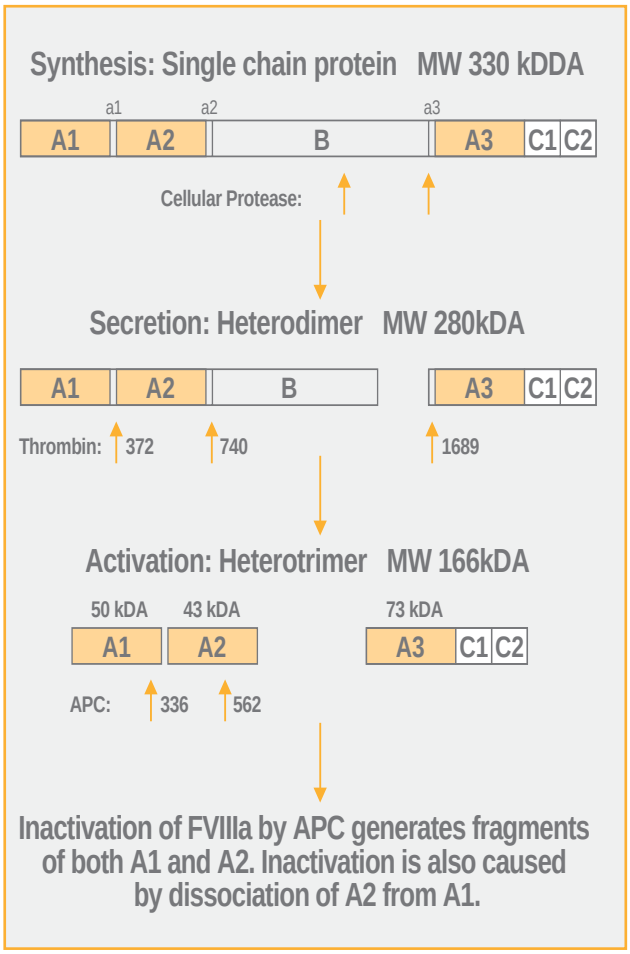



***Figure 2. Schematic presentation of various forms present in the life cycle of FVIII.***
*Factor VIII is synthesized in e.g. the hepatocytes and is then processed by a so far unknown enzyme and secreted into the blood as a single chain protein. The structure shows a high homology with factor V. The short a1, a2 and a3 sequences are acidic regions of importance for stabilization of factor VIII and for interaction with factor IXa and von Willebrand factor. Both factor V and factor VIII contains copper ions. The A-domains are also present in the copper transporting protein ceruloplasmin.*

The homology of the A-domains with ceruloplasmin raised the question whether factor VIII may also be a copper-containing protein. This has been shown to be true for factor V and indeed it was more recently found to be true also for factor VIII and both contain 1 mole copper per mole of protein.[20,21]
Quite fascinating, the nature of copper binding, so called Type-1, seems to be closely related to the ancient, blue copper-containing oxidases found in bacteria, plants and in mitochondria in mammals.[22,23]

| Data on Factor VIII | |
|---|---|
| Name: | Factor VIII |
| Synonyms: | Antihemophilic globulin |
| Gene location: | X-chromosome |
| Plasma concentration: | 0.2 µg/ml |
| Molecular weight: | Synthesized as 330 kDa single chain, secreted as two-chain 280 kDa molecule |
| Primary structure: | 2332 amino acids |
| Carbohydrate: | 5 % |
| Metal ion content: | One mole $Cu^{1+}$ per mole protein |
| Half-life: | 12 hours, reduced several-fold in the absence of vWF |
| Function: | Cofactor to factor IXa in the activation of factor X |
| Importance: | Severe deficiency causes the bleeding disease hemophilia A<br>Elevated activity is a risk factor for thrombosis |

What is then the role of copper?
Reconstitution experiments suggest that Cu(I), but not Cu(II) is important for the proper reassembly of separated heavy and light chains and with restoration of factor VIII activity.[24]
It has also been proposed that the metal ion bridge between the heavy and the light chain in the activated molecule is between the A1 and A3 domains whereas the A2 domain is associated with the heterodimer through electrostatic interactions.[25,26]
The copper ion appears thus to bind to the A1 domain, but the exact coordination and valency state of the copper ion is still surrounded with some uncertainty.[27,28]
Interestingly, data have also been published which suggest that copper increases the cofactor activity of factor VIIIa.[29]
Factor VIII also contains stabilizing disulphide bonds in the A and C domains[30] as well as a number of sulfated tyrosines, claimed to be essential for maximal expression of factor VIII activity.[31]

## Interaction with von Willebrand factor

During the secretion factor VIII associates with von Willebrand factor (vWF), a protein which stabilizes factor VIII and which is of great importance for platelet adhesion to the subendothelium and for platelet aggregation. vWF binds to the B-domain and the N-terminal part of the A3-domain.
vWF serves an important role in targeting and concentrating factor VIII to the injured vascular wall with its exposed subendothelium (for comprehensive reviews, see ref. [32-34]).

Since severe deficiency of vWF activity is associated with bleeding symptoms and low factor VIII activity, it was believed during many decades that factor VIII and vWF activities resided in one and the same protein. Supporting this was also the fact that early preparations of low purity factor VIII concentrates corrected the bleeding time.
However, the situation was confusing since it was known that von Willebrand´s disease is inherited as an autosomal disorder whereas hemophilia A is an X-linked disease.[35]

The association of vWF with factor VIII and the difficulties in establishing these proteins as distinct entities resulted in the immunological terminology for vWF antigen 1971 as “factor VIII related antigen”[36] but a few years later convincing data were published on the non covalent association of these proteins[37,38] and a consensus on their identities and roles was soon reached.[39]
An important reason for the difficulties in arriving at this conclusion was that when purifying factor VIII from plasma, vWF was copurified and constituted easily more than 95% of the total protein content.
Indeed, factor VIII is a trace protein in plasma with a concentration of only about 0.2 mg/L.[39,40]
When vWF is bound to factor VIII it prevents binding of factor VIII to phospholipid surfaces including activated platelets and thereby factor VIII cannot be a part of the tenase complex with factor IXa, phospholipids and calcium ions (see fig. 1).[41-43] vWF also protects factor VIII from inactivation by activated protein C (APC) and from activation by factor Xa but not by thrombin, the reason probably being that both factor Xa and APC requires a phospholipid surface in their action on factor VIII whereas thrombin does not.[44-47]

## Activation of factor VIII

Factor VIII is activated by thrombin through specific proteolytic cleavages in both the heavy and the light chains.[48,49] In the heavy chain one cleavage occurs at the amino acid Arg372 to generate separate A1 and A2 domains and another at Arg740 at the junction between the A2 domain and the B-domain resulting in the release of the B-domain.

In the light chain there is a cleavage at Arg1689 in the acidic region at the amino terminal of the A3-domain (see fig. 2) whereby a new NH2-terminus is created. Through this cleavage, vWF dissociates from factor VIII (fig. 3) and the phospholipid binding region in the C-domains is exposed, which is mandatory for the cofactor activity of activated factor VIII (factor VIIIa).
Interestingly, factor VIII contains two sulfated tyrosines at the positions 1664 and 1680 in the light chain, which are of importance for the binding of vWF.

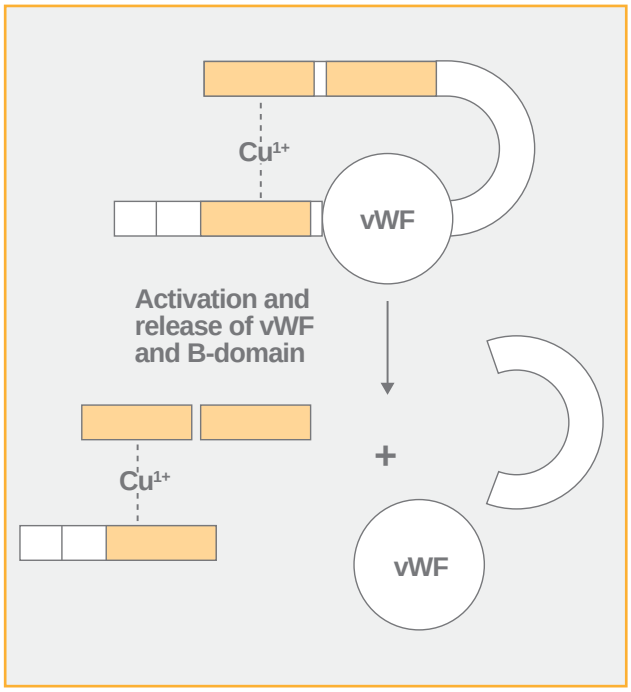



***Figure 3. Activation of factor VIII and dissociation of the B-domain and von Willebrand factor.***
*The figure shows a schematic structure of factor VIII with a tentative binding of one copper(I) ion to the A1 and A3 domains. As a result of cleavage and activation of factor VIII by thrombin, the B-domain and von Willebrand factor are released and binding sites to pro-coagulant platelet phospholipids are exposed in the A3 domain, thereby facilitating formation of the tenase complex on the surface of activated platelets. The figure is not drawn to scale.*

Mutagenesis to obtain a phenylalanine at 1680 resulted in a loss of a high affinity vWF binding site[31] and indeed the corresponding mutation in a patient resulted in moderate hemophilia.[50]

The cleavages at 372 and 1689 are necessary for full activation of factor VIII. The 200 + 80 heterodimer is thus converted to a heterotrimer consisting of A1, A2 and A3-C1-C2. As mentioned above, the metal ion bridge is probably between the A1 and the A3-C1-C2 domains. Factor VIIIa has a molecular weight of about 165 kDa, derived from the A1 and A2 domains with molecular weights of 50 kDa and 43 kDa, respectively, and the 72 kDa light chain.[25,49,51]
Factor VIII is also activated by factor Xa with cleavages occurring at the thrombin cleavage sites at 372 and 1689.[49,52,53]

This suggests, but does not necessarily mean, that factor Xa is important for the physiological activation of factor VIII; it may be that the main role of the initial traces of factor Xa generated through the extrinsic pathway is to generate minute amounts of thrombin which then activates factor VIII.

The crucial importance of cleavages at 372 and 1689 for activation of factor VIII is also illustrated by the finding of severe hemophilia A patients with missense mutations at either of these residues. As might be expected, these patients had no measurable factor VIII activity but normal levels of factor VIII antigen.[54,55]

Factor VIIIa is inherently unstable. Initially it was thought that the loss of activity was exclusively related to additional proteolysis by thrombin or factor Xa but it has later been shown to be much due to subunit dissociation. Thus, the loss of factor VIIIa procoagulant activity coincides with the dissociation of the A2 subunit from the factor VIIIa heterotrimer and the activity can also be restored by adding the A2 subunit to A2-deleted factor VIII.[25,56,57] Addition of protease inhibitors does not prevent the loss of cofactor activity but addition of factor IXa and/or phospholipids improves the stability of human factor VIIIa.[58-62] The latter finding may well be relevant in vivo with factor VIIIa bound to the membrane of activated platelets which have a large number, about 400 per platelet, of high affinity binding sites for factor VIII.[63]

## Cofactor activity of factor VIIIa in the tenase complex

Factor VIIIa serves as a most potent procoagulant cofactor in the tenase complex where factor X is activated by factor IXa in the presence of calcium ions and phospholipids. This is in analogy with the role of factor Va in the prothrombinase complex, comprising also factor Xa, calcium ions and phospholipids.
In vivo these processes takes place primarily on the surface of activated and aggregated platelets which accumulate at the site of a vascular damage.
In contrast to resting platelets, activated platelets expose procoagulant phospholipids, such as negatively charged phosphatidylserine.[64,65] Upon platelet activation, binding sites for both factors IXa, VIIIa, X /Xa, Va and prothrombin are exposed, which brings about a high local concentration of the constituents and an efficient generation of thrombin (see fig. 4 for a schematic view).[63,66]
Furthermore, the cleavage in the factor VIII light chain and the release of vWF upon activation results in a considerably higher affinity for factor VIIIa to activated platelets as compared to unactivated factor VIII.[67] In addition, factor X has been shown to increase the affinity of factor IXa for factor VIIIa approximately 10-fold.[68]
Both these features contribute to an optimal assembly of the components. Detailed studies have been performed on the interactions between factor VIII and factor IXa and it has been shown that the A3 domain in factor VIII interacts with the light chain of factor IXa and the A2 domain with the factor IXa protease domain.[69-71]

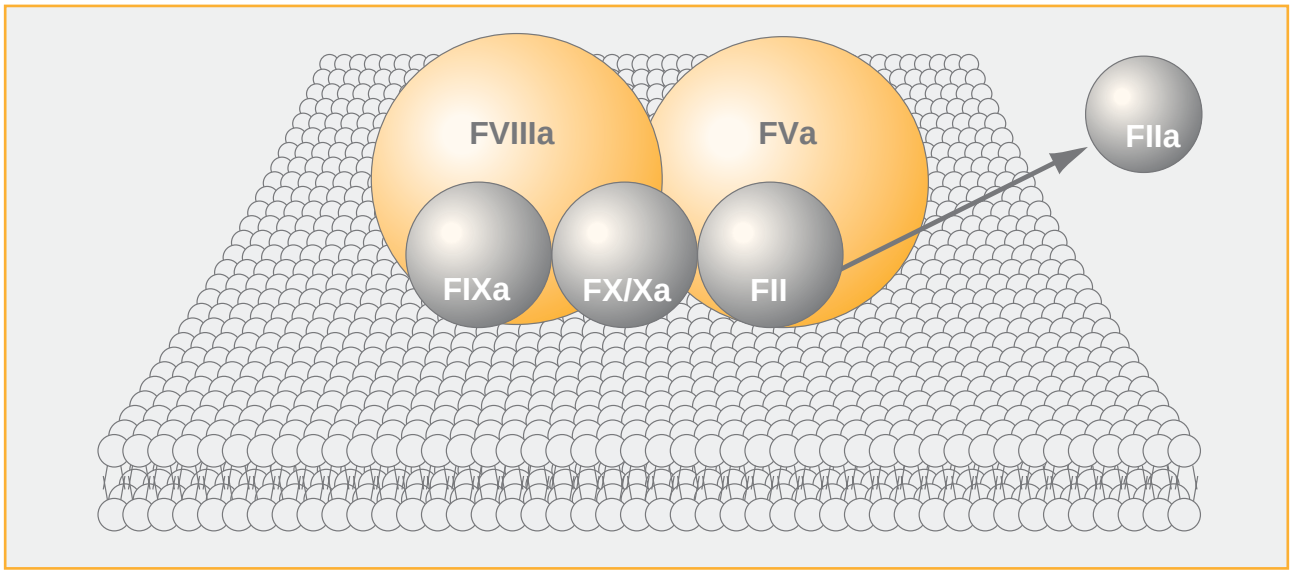



***Figure 4. Schematic view of the tenase and prothrombinase complexes.***
*The tenase complex (factor IXa, VIIIa, X) and the prothrombinase complex (factor Xa, Va, prothrombin) assemble in close conjunction on the membrane surface of activated platelets in the presence of calcium ions. Factor Xa and thrombin are generated by the tenase and prothrombinase complex, respectively. The binding of factors IXa, X/Xa and prothrombin to the platelets is partly directed through Gla-domains in these proteins. Thrombin contains no Gla-domains and is released from the membrane whereafter it will cleave fibrinogen and other substrates.*

The overall increase in the catalytic efficiency of factor IXa in the tenase complex as compared to factor IXa by its own, at physiological concentrations of reactants, is > $10^5$ with factor VIIIa being responsible for a several thousand-fold increase of the reaction rate and where the highly increased affinity of factor X for factor IXa in the presence of procoagulant phospholipids is another major reason (Table 1).[3,72]
The effect of factor VIIIa is very similar to the effect of factor Va in the prothrombinase complex.
As mentioned above, data have also been published which suggest that copper increases the cofactor activity of factor VIIIa.[29]

| *Factor X activator* | *Km factor X, µmol/L* | *Vmax, FXa x min-1 x IXa-1* |
|---|---|---|
| *FIXa, $Ca^{2+}$* | *181* | *0.01* |
| *FIXa, $Ca^{2+}$, PL* | *0.36* | *0.025* |
| *FIXa, $Ca^{2+}$, PL, FVIIIa* | *0.29* | *500* |

***Table 1. Effect of phospholipids and factor VIIIa on Km and Vmax in the tenase complex.***
*Procoagulant phospholipids, such as on activated platelets, dramatically increases the affinity of factor X for factor IXa as expressed by a 500-fold decrease of Km. Factor VIIIa stimulates the FXa generation more than 1000-fold. Altogether this results in efficient catalysis also at the low concentrations of coagulation factors prevailing in blood plasma. Data from ref. 3.*

The physiological triggering of coagulation is most probably caused by the extrinsic pathway whereby factor VII is efficiently activated after complexing with exposed tissue factor (TF) whereupon factor IX and factor X are activated by the factor VIIa/TF complex.[73,74]
It is a fact that hemophiliacs bleed severely and thus the clinical evidence clearly show that activation of factor X by the tenase complex is of great importance in vivo. There are actually considerable research data to support this. Thus, the factor VIIa/TF complex is down-regulated by TFPI after binding factor Xa and hence continued direct activation of factor X is inhibited.[75]
Furthermore the activation of factor X by the factor IXa-factor VIIIa pathway is about 50-fold more efficient than the direct FX activation by factor VIIa/TF.[76]
The contribution by the factor IXa pathway is also efficiently promoted by factor XIa, which activates factor IX (see fig. 1).
The findings that thrombin as well as meizothrombin activate factor XI[77-81] and that such activation also can occur on the platelet surface[82] indicate that this event probably is of importance in vivo.
The activation of factor XI by thrombin may thus result in an increased generation of factor IXa, supporting further the important role of factor VIII in obtaining a sufficient generation of thrombin. Indeed, this fairly recently discovered additional positive feed-back role of thrombin serves as a further beautiful illustration of how thrombin regulates its own activity and fate.

## Proteolytic inactivation of factor VIIIa by APC

A most efficient down-regulation of coagulation is provided by APC, which inactivates factor Va and factor VIIIa through specific proteolytic cleavages. In factor VIIIa, these cleavages occur at Arg336 in the A1 subunit and Arg562 in the A2 subunit[49,83] and it seems that in the human system, the cleavage at 336 is the preferred initial cleavage site with concomitant loss of cofactor activity.[84]
There are fascinating interactions between the components of the tenase system and of the protein C anticoagulant system which affect the rate of inactivation of factor VIIIa. Thus, factor IXa protects the cleavage by APC at Arg562 and factor X protects the 336 cleavage site.[85,86] However, protein S abrogates the protecting effect of factor X and, in the absence of factor IXa, also stimulates the rate of cleavage at 562 considerably.[86] Corresponding regulatory roles for factor Xa and protein S have also been demonstrated for the APC-dependent inactivation of factor V.[87]
The relative importance in vivo of degradation of activated factors V and VIII by APC on the down-regulation of coagulation is not clear. Some data indicate that inactivation of factor Va is the crucial effect of APC[84] and it has also been shown in mutagenesis studies that both APC cleavage sites had to be blocked before a significant impairment of the rate of thrombin generation was registered in an APTT-based method and that indeed the major inactivation of factor VIIIa may be caused by dissociation of the A2 subunit.[88-90]

# Clinical aspects

## Hemophilia A Background and classification

Hemophilia A is a serious bleeding disorder which is caused by a deficiency or complete absence of factor VIII activity which affects about 1 in 5000 males and its prevalence show no ethnic differences[91,92] (for a comprehensive review on various aspects on hemophilia A and its treatment, see ref. 93).
It is inherited as an X-linked disorder but in many cases there is no family history simply due to the fact that about 30% of patients have a recent, spontaneous mutation. The obvious bleeding symptoms mean that this disorder, initially named haemorraphilia (love of bleeding) can be traced back to very early observations and in 1835 the disease was described as a protein disorder[1,94,95]. Within only a few years later the first blood transfusion was administered to a patient for treatment of his bleeding.[96]

Since then tremendous progress has been made in the treatment of hemophilia patients who nowadays in many cases in the Western countries receive highly purified plasma factor VIII or recombinant factor VIII concentrates with a minimal risk of transmittance of viral infections.

| *Factor VIII activity* | *Classification* |
|---|---|
| *< 0.01 IU/mL (< 1% of normal)* | *severe* |
| *0.01 - 0.05 IU/mL* | *moderate* |
| *> 0.05 - < 0.40 IU/mL* | *mild* |

***Table 2. Classification of hemophilia A***
*The bleeding disorder hemophilia A is classified into three categories depending on the level of factor VIII activity. Data from ref. 97.*

Depending on the factor VIII activity, which is related to the bleeding severity, hemophilia A is classified according to Table 2.[97] However, it is not a straightforward relationship, since indeed there are patients with a factor VIII level < 1% who have very little or no spontaneous bleedings and, on the other hand, spontaneous and clinically severe bleeding occur in some patients with 1-5% factor VIII activity.
Since patients with von Willebrand´s disease also have a decreased factor VIII activity, a correct diagnosis of hemophilia A requires determination of vWF antigen or ristocetin cofactor activity.

These levels are normal in hemophilia patients. Furthermore, the family history also provides important information since von Willebrand´s disease is inherited as an autosomal trait. However, one rare variant, Type 2N, of von Willebrand´s disease in which vWF does not bind to factor VIII, may be misdiagnosed for (autosomal!) hemophilia A and here vWF-factor VIII binding studies must be performed.[98,99]

Severe hemophilia A patients typically meet with bleedings in joints and muscles and sustained and dangerous bleedings after trauma and surgery and these patients may, unless treated, develop permanent disability. In moderate or mild hemophilia
A bleeding episodes are more rare and occur usually in connection with trauma or surgery.[91]

## Clinical management through replacement therapy

The only available treatments for hemophilia A patients for many decades were whole blood or plasma but this was often not sufficient to rapidly achieve proper hemostasis. This situation changed dramatically after it was discovered in 1956 that factor VIII coprecipitated with fibrinogen in the so called Cohn fractionation system for purification of fibrinogen and already the same year a crude factor VIII-vWF preparation, at that time called antihemophilic globulin, was administered to a woman with severe von Willebrand´s disease.[100,101] This preparation was also rapidly introduced for treatment of hemophilia A patients.[102]
After this pioneering work a further important step forward was the discovery that factor VIII is recovered in plasma cryoprecipitate, which allowed an effective replacement therapy.[103] Still, though, the specific activity was low, about 0.5 IU/mg, the major proteins being fibrinogen and fibronectin.

More pure factor VIII preparations, so called intermediate purity concentrates, were developed over the years by the use of different types of chromatography, which resulted in specific activites of up to 10 IU/mg.
The next step forward in purification was the introduction in the late 70s of immunoaffinity purification of factor VIII by using matrix-bound antibodies against vWF and elution of factor VIII with calcium ions.[104]
Within a decade immunoaffinity techniques were used for large-scale purification of factor VIII whereby factor VIII-specific antibodies also were utilized[105,106] and now specific activities close to the theoretical maximum of about 5000 IU/mg could be obtained.[39] However, due to the labile nature of factor VIII, albumin is usually added as a stabilizer, which results in specific activities of about 15 IU/mg.

The dosages of factor VIII concentrate used depend upon the specific bleeding event, but normally the factor VIII level must exceed 0.3 IU/mL in plasma in order to achieve normal hemostasis; in acute serious trauma about 1 IU/mL may be needed.
This requires the infusion of about 15-40 IU/kg bodyweight.[107] Repeated infusions are necessary to maintain sufficient factor VIII levels since the half-life of factor VIII in vivo is only about 12 h.[108]
Much lower doses are used for prophylaxis, whereby the target factor VIII levels are above 0.03 IU/mL.[109]

The availability and introduction of factor VIII concentrates for treatment of acute bleedings and for prophylaxis has had a dramatic improvement of the life expectancy of severe hemophilia A patients. Thus, until the end of the 50s, the median life expectancy of such patients in Sweden was still only about 20-25 years, whereas it had increased to about 57 years twenty years later and is now about 70 years at the entry of the 21st century.[109,110] In parallel, the development and progression of joint disease has decreased significantly.

The use of blood products is connected with a risk of viral transmission such as hepatitis B and C, and many hemophiliacs developed chronic liver disease but the great benefits of the therapy was considered to justify the treatment.[111]
The picture changed dramatically from 1982 when the first hemophilia A patient was identified with AIDS as a result of infusion with factor VIII concentrate.
A gruel decade followed with thousands of hemophilia patients killed by AIDS due to HIV virus contamination in factor VIII concentrates. This was a tragedy not only to the patients and their families but certainly also to all clinicians and other medical staff who showed a great engagement and commitment to good hemophilia care.

Development of concentrates with maximal safety against viral transmission was made by two routes, which luckily has resulted in no new HIV infections of hemophilia patients.
One route was through introduction of extensive heat treatment or use of solvent-detergent treatment of plasma derived factor VIII concentrates[112], the other through the development of recombinant factor VIII concentrates. From the cloning of the factor VIII gene in 1984[10,11], ten years passed until recombinant factor VIII concentrates were registered for clinical use, preceded by publications on their safe and successful applications.[113,114]
More recently, a recombinant truncated form of factor VIII (ReFacto®, Pharmacia), lacking most of the B-domain, has been developed and characterized and shown to be equally clinically effective.[115-117] This preparation has no addition of human albumin as a stabilizer, thereby showing a possibly even lower risk of viral transmission.
In mild and moderate hemophilia A patients, sufficiently high factor VIII activity levels can be reached in most patients by administering desmopressin, an analogue to the diuretic hormone vasopressin.[118,119]
This agent has not only the benefit of having no risk of viral transmission but it can also be provided to the patient intranasally.[120]

## Factor VIII inhibitors

Unfortunately a fairly large proportion of hemophilia A patients develop antibodies against factor VIII, denoted factor VIII inhibitors. This occurs in 5-15% of patients with mild or moderate severity and in about 25% of patients with severe hemophilia A after being treated with factor VIII concentrates.[110,121-126]

The factor VIII inhibitor titer vary greatly between patients. The titer is expressed in so called Bethesda units, defined by the use of a specified test system (see below) and may vary between 0.5 -15.000 units (!).[127]
Patients are classified as high or low responders[128] and it has recently been decided to use the term high responder for a patient who at any time presents with an antibody titer above 5 Bethesda units whereas patients who persistently have below 5 Bethesda units in spite of repeated treatments with factor VIII concentrates are denoted low responders.[97]

Does the type of mutation affect the risk of developing factor VIII inhibitors? Yes, apparently patients with large deletions and nonsense mutations or gene inversions develop inhibitors to a larger extent than those with frameshift or missense mutations.[129] It also seems that the greatest risk of raising inhibitors is during the initial treatment.

Does the type of factor VIII concentrate affect the development of inhibitors? Generally no, although it has been reported that modification of a concentrate during manufacturing resulted in antibody development in a patient.[130]
On the other hand the type of concentrate to choose for treatment may play a role. Thus, if a patient has developed antibodies against the light chain of factor VIII, it may be preferable to administer a concentrate which is rich in vWF, since factor VIII then appears to be more slowly neutralized and hence more efficient.[131]
Since, understandably, high responding patients may meet with life-threatening conditions, other treatment regimes have been developed. Thus, a high dosage of porcine factor VIII concentrate may be successfully used, although usually a patient can not receive many infusions until new antibodies appear.[132]
Another avenue is to use immunosuppression therapy in combination with extracorporeal adsorption of IgG and administration of factor VIII.[133,134] This also seems to be one way of inducing immune tolerance in high-responding patients, a desired but difficult goal and which is more often obtained in low-responding patients, sometimes by “merely” infusing high doses of factor VIII. A third treatment regime with quite successful results with rapid achievement of normal hemostasis in many instances is infusion of recombinant factor VIIa.[135] This will probably gain increasing use in the future.

## The future - gene therapy?

Cost is a prime issue in the treatment of hemophilia and it is a main obstacle in providing proper treatment worldwide. Indeed, the yearly cost is roughly in the order of US$ 100.000 for a severe hemophilia A patient with no inhibitor development.[136]
For patients with inhibitors the cost is approximately four-fold higher. Thus the prospects of bringing efficient, modern treatment into global use are very meager and, sadly, in many countries transfusions of blood or plasma is the only option available, if at all.
Since only minute amounts of factor VIII have to be present in plasma to warrant a proper hemostasis, great efforts are made in gene therapy research.
The real challenge, apart from important safety issues, is to achieve a sustained production of factor VIII at a low level. The first attempts were made in the early 90s[137,138] and now a number of different approaches are being explored, including retroviral, adenoviral and non-viral gene deliveries and utilizing different target cells.[139,140] Progress is being made and it seems possible that gene therapy may be available within a decade.

## Elevated factor VIII activity as a risk factor for thrombosis

It has been known since long that arterial thrombosis is a multifactorial disease and this has later been shown to be true also for venous thrombosis (see 4 for a review). Thus, combined abnormalities of factor V:Q506 (factor V Leiden) and inherited deficiency of either of antithrombin, protein C or protein S results in a significantly higher incidence of venous thrombosis.[141-144]
Abnormalities of other plasma components are also being investigated as possible risk factors for thrombosis, such as hyperhomocysteinemia, dysfibrinogemia, factor XII deficiency, thrombomodulin mutants and elevated factor VIII activity.[4]
From a prevalence point of view, hyperhomocysteinemia and elevated factor VIII seem most important and much data have now accumulated on elevated factor VIII levels as an important risk factor.
In 1980 a prospective study indicated factor VIII to be a risk factor for arterial disease[5] and other studies also suggested association of elevated factor VIII with both cardiac and cerebral vascular disease and increased morbidity or earlier fatal outcome.[145,146]
This has later been supported also in other studies[6,147-149] as well as by an experimental study in mice with controlled mild carotid artery injury and who received infusion of factor VIII, which study suggested a direct thrombogenic role for factor VIII.[150]

In 1995 elevated factor VIII activity was found to be quite frequent also in patients with venous thrombosis[7] and this was later confirmed in other studies.
Thus, elevated factor VIII activity has been shown to be an independent, higly prevalent risk factor with an odds ratio of up to 6 and it is recommended to be included in the laboratory screening test panel on analysis of plasma from thrombotic patients.[8,151,152]

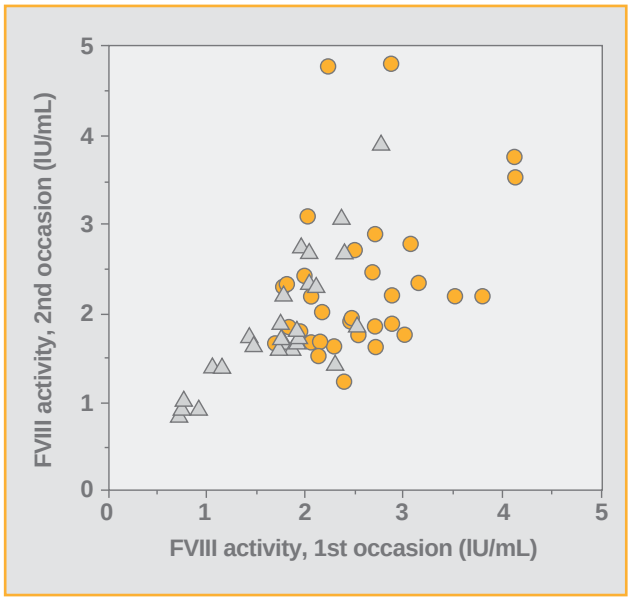



***Figure 5. Persistence of high factor VIII activity upon repeated determinations.***
*The figure shows a comparison of results from a second determination of factor VIII activity made between 3 months and 4 years after the first determination in patients with venous thromboembolism. Data are from ref. 151 (triangles) and ref. 154 (circles).*

In more than 20% of the patients, factor VIII activities were >1.5 IU/mL and occasionally levels as high as 4-5 IU/mL were found. There was also a close correlation to factor VIII antigen, demonstrating that there was an increased synthesis of the factor VIII protein and thus the rise in activity is not due to generation and circulation of activated factor VIII.
However, factor VIII is an acute phase reactant and there were, quite understandable, early doubts as to whether elevated factor VIII levels has any causal role but perhaps rather were an effect of the disease.

Thus, elevated levels are associated with conditions such as trauma, infection and exercise and, in common to many other coagulation factors, factor VIII activity is also increased during pregnancy.
It was shown, however, that elevated factor VIII activities were not linked to any acute response[8,153,154] and that they were indeed persistent with similarly high levels demonstrated upon repeated analysis after 3 months to 4 years (fig. 5).[151,154] Furthermore, heritability for elevated factor VIII activity has been demonstrated which remained after adjustment for blood group and vWF but so far no polymorphism of the factor VIII gene promoter has been found.[155-158]
The search for a genetic contribution is actively pursued, though, and it should be expected that our knowledge in this field is expanded within the next few years.
In addition to an inheritance for elevated factor VIII activity, there may possibly also be an association with cytomegalovirus infection, which seem to be linked with high factor VIII levels and with thrombosis.[159,160]

In conclusion, there is now a substantial amount of data which points to a causal role for elevated factor VIII activity and at least venous thrombosis (table 3) and it should be expected that analysis of factor VIII activity will be increasingly introduced in routine laboratory investigations of thrombotic patients.

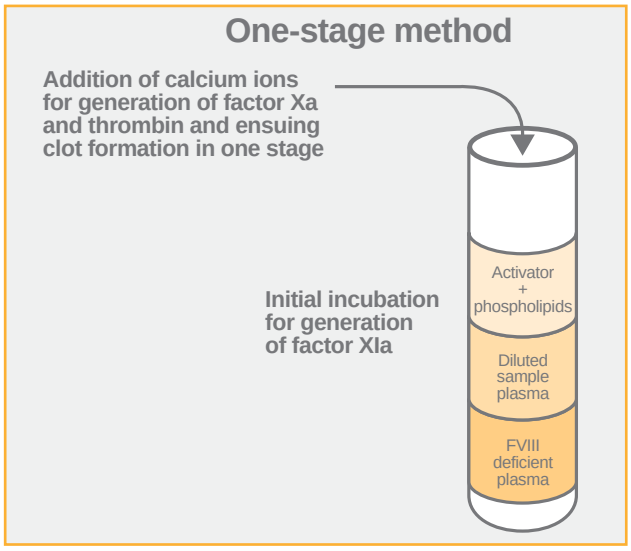
## Determination of factor VIII activity

### *The one-stage clotting method*

The so far most widely used method for factor VIII activity determination in plasma is the one-stage clotting method which is based upon the activated partial thromboplastin time (APTT) and using congenital severe hemophilia A plasma or artificially prepared factor VIII deficiency plasma as a substrate.

| | Odds ratio | | Note |
|---|---|---|---|
| *Koster T et al. (ref. 7)* | *5* | *>1.5 IU/mL:* | *In 25% of patients vs 11% of controls* |
| *O'Donnel J et al. (ref. 8)* | *-* | *>1.5 IU/mL:* | *In 25% of patients (no control group)* |
| *Kraaijenhagen RA et al. (ref. 151)* | *5* | *>1.75 IU/mL:* | *In 19% (single event) and 33% (recurrency) of patients vs 10% of controls* |
| *Kamphuizen PW et al. (ref. 153)* | *6* | *>1.5 IU/mL:* | *In 24% of patients vs 10% in controls* |
| *O'Donnel J et al. (ref. 154)* | *-* | *>1.5 IU/mL:* | *In 14% of patients after exclusion of any other abnormality* |
| *Schambeck CM et al. (ref. 157)* | *-* | *>2.0 IU/mL:* | *In 17% of patients vs 2% of controls* |

***Table 3. Elevated factor VIII activity as a risk factor for venous thrombosis.***
*A survey of results from different studies on elevated factor VIII as a risk factor for venous thrombosis. Note that raised levels of factor VIII are not due to acute phase reactions (see text for further details).*




This method for factor VIII activity was developed already in the early 50s and is based on the ability of a plasma sample to shorten the prolonged APTT of factor VIII deficient plasma, the effect being related to the amount of factor VIII activity in the sample.[161,162]
In the assay system, phospholipids are added to citrated plasma along with a negatively charged surface activator, e.g. kaolin, which thereby activates the contact factors and leads to the generation of factor XIa.

Upon addition of calcium ions, factor IX is activated by factor XIa and factor Xa is then generated by the tenase complex (intrinsic system), in which factor VIII serves as a cofactor, and the time for fibrin clot formation due to cleavage of fibrinogen by generated thrombin is recorded (see fig. 1 for the flow of events).
A standard curve is constructed from assaying different dilutions of a normal plasma with a known factor VIII activity and it is most common to express the results in a double logarithmic plot with log factor VIII activity vs log clotting time. The factor VIII activities of assayed plasma samples are then derived from the standard curve.

The importance of optimizing the contact activation step was realized in the early 60s[163] and the one-stage method has remained essentially unchanged since then, but for the use of mixtures of purified well-defined phospholipids as an alternative to crude phospholipid extracts from brain or soybean.

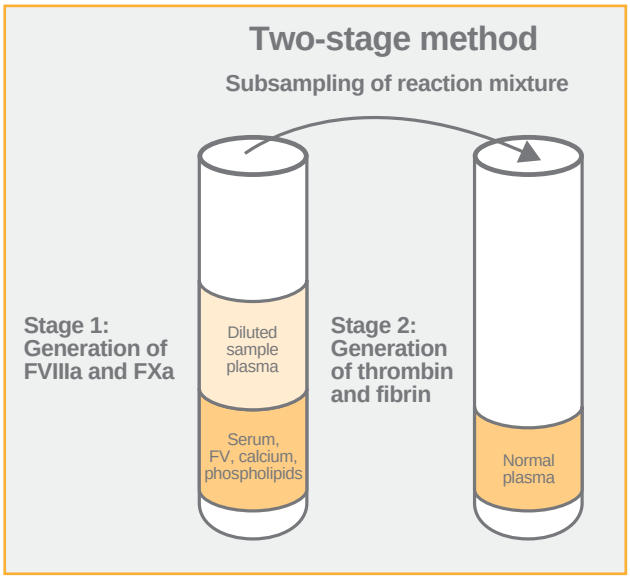
The one-stage method has the advantage of being rapid and easy to perform. At the same time it must be realized that the assay is deceptively simple since it indeed comprises a complex biochemical system with a short total reaction time and there are built-in short-comings such as a great sensitivity to preactivated clotting factors which will result in overestimation of factor VIII activity and furthermore Lupus anticoagulants will interfere. It is also clear from several surveys that there is an appreciable variability in results, with interlaboratory coefficients of variation sometimes reaching 45%.[164-166] Some of this variability could be explained by improper standardization and less satisfactory instrument performance.

With the introduction of international standards of plasma and factor VIII concentrate and with the increasing use of automated coagulation instruments, the situation has improved but there is still a considerable variability which probably can be explained by the use of different sources of phospholipid, contact activator and factor VIII deficiency plasma.
It is also possible that the increasing use of prophylaxis for severe hemophilia A patients will limit the availability of congenital factor VIII deficiency plasma and therefore the quality of artifically prepared factor VIII deficiency plasma may be of increasing importance.

### *The two-stage clotting method*

This method was also developed in the 50s and it is based upon a first step whereby activated factors V and X are generated in an amount which is related to the sample factor VIII activity. In a second step, prothrombin and fibrinogen are added, usually in the form of normal plasma, and the clotting time is recorded.[167,168]

This method makes no use of factor VIII deficiency plasma. It is claimed to show a better precision in general and also to be more sensitive than the one-stage method.[169] It is also less prone to interference than the one-stage method and its design makes it e.g. insensitive to preactivation of factor VIII.



These properties resulted in the two-stage method being selected as the reference method for determination of the factor VIII potency of factor VIII concentrates for several decades. However, it is more cumbersome to perform and it appears that expert laboratories are best suited to explore the full potential of the method; hence it is not commonly used today.

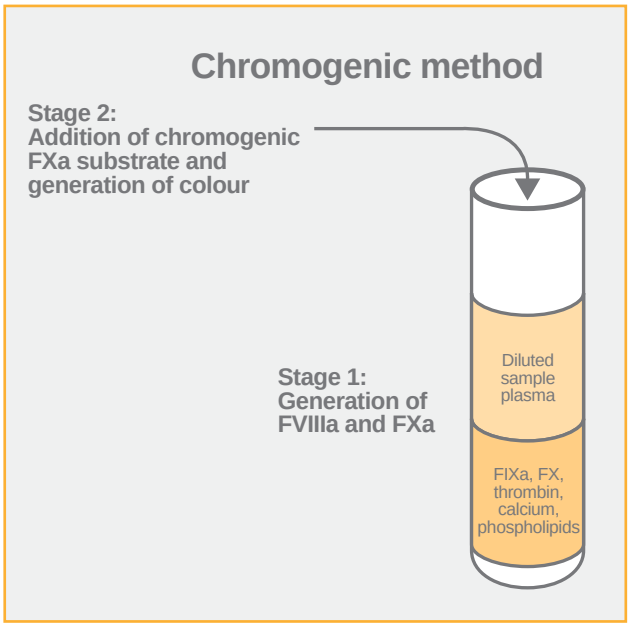
### *Additional factor VIII clotting methods*

Other approaches than the one-and two stage clotting methods have also been utilized, such as different thrombin generation tests. These vary from straight-forward recalcification of plasma[170] to more elaborate methods in which partially purified factors were utilized.[171,172] With the use of lyophilized mixtures of well-defined reagents the thrombin generation test was, however, made much more convenient and seemingly suitable for routine use.[173] None of these methods, however, gained widespread use.

### *Chromogenic methods*

With the invention of chromogenic substrates a new avenue was opened for designing methods for hemostatic factors and their general suitability for determination of enzymes, proenzymes, inhibitors and cofactors was shown as well as their applicability to automated instruments.[174-177]
The availability of the highly selective chromogenic substrate S-2222 (Benzoyl-Ile-Glu-Gly-Arg-pNA) for factor Xa[178] triggered the development of chromogenic methods for factor VIII activity[179-181] and a specific method with a high resolution and with stabilized reagents was published in 1983.[182]
The assay principle is similar to the two-stage assay in being based upon an incubation step for generation of factor Xa and with no need of factor VIII deficiency plasma but rather utilizing purified bovine factors IXa and X. However, instead of using a fibrin clotting time as the end-point, the amount of factor Xa is determined from the hydrolysis of S-2222.
Furthermore, the amount of generated factor Xa was directly proportional to the factor VIII activity warranting a high resolution. This feature makes the method suitable for both measurement of very low levels, such as in the classification of hemophilia A patients, as well as of very high levels by proper adjustment of sample dilution and incubation time.

The chromogenic method was shown to correlate closely with the one-stage clotting method including recovery studies after administration of factor VIII concentrate to hemophilia A patients and, similar to the two-stage method, it was demonstrated to be insensitive to preactivation by thrombin.[183-186]




It was also shown to be quite suitable for analysis of factor VIII concentrates and for screening of blood donors by performing the analyses in microplates, thereby allowing a high throughput as well as providing a high precision and also being cost-competitive as compared to the one-stage method.[187-189]
The high sensitivity of the chromogenic method was demonstrated in the first expressions of functional recombinant factor VIII in cell cultures[10,11] as well as in later studies on secretion of recombinant factor VIII[19] and the method has also proven valuable in research studies on factor VIII activity in purified factor VIII and of separate or combined subunits.[190-192]
It has furthermore been used for studies on the effect of factor VIII activity after intranasal administration of DDAVP.[193]

Other variants of the chromogenic method have also been developed and commercialized utilizing human factors IXa and X or adding thrombin to obtain an immediate activation of factor VIII and shorter assay incubation times. This also allowed the chromogenic method to be conveniently applied on automated instruments.[194-196]
Furthermore, highly elevated levels of factor VIII (< 4 IU/ml) can be determined with maintenance of a high accuracy by making an initial sample predilution (see Product Section).

The high dilution used in the chromogenic method as compared to clotting methods also means that interferences can be minimized. One illustration of this is the lack of influence of Lupus anticoagulants, whereby the added procoagulant phospholipid override the inhibitory effect of anti-protein-phospholipid antibodies.[197]

## Diagnosis and classification of hemophilia A

Both one-stage, two-stage and chromogenic methods are today used for diagnosis and classification of hemophilia A patients and presently the majority of tests are made with the one-stage method.
Can a clinician then be certain of obtaining a correct classification irrespective of the method used?
Generally this seems to be the case and that is also one reason why the one-stage method is so frequently used in spite of the fact that the two-stage method has been the reference method for decades.
Also, it is clear that the chromogenic assay correlates well with the one-stage method on analysis of plasmas from hemophilia A patients.[182-184,187,196]
Still, patients with the same factor VIII activity as measured with one method may show quite different clinical symptoms (see above).
There are indeed also reports on some cases with discrepancy between methods. In a comprehensive survey from Australia, where the one-stage and the two-stage clotting methods were used in parallel, there were a number of index patients and also their affected family members who showed significantly higher results with the one-stage method than with the two-stage method and the latter method correlated much better with the clinical severity.[198]
Later, several single point mutations causing alterations of amino acids in the A1, A2 and A3 domains were identified in a number of those families and those mutations were not found in patients who showed similar results in the two methods.[199]
It may not be quite surprising in light of the fact that the one-stage method is based upon contact factor activation of the coagulation system, which does not appear to reflect the physiological situation.
More recently, a discrepant result was also reported between the one-stage method and the chromogenic method, caused by a single point mutation Glu720Lys in the A2 domain, where the one-stage method showed lower result and in this case correlated better with the clinical picture.[200]

Thus, it appears that, similar as for other hemostasis analytes, there seems to exist no method which can safely claim to reflect accurately the clinical picture for all hemophilia A patients. A humble attitude is therefore recommended which should encourage the clinician to use more than one method when the first line results are not in concordance with the clinical severity.

## Determination of factor VIII inhibitor activity in plasma

As mentioned above, about 25% of severe hemophilia A patients develop factor VIII inhibitors.
The proper treatment of these patients requires correct determination of the inhibitor titer.
Initially, incubations were made of patient plasma with factor VIII concentrate (the New Oxford method[201]) but the instability of factor VIII activity in concentrates at that time and the lack of standardization resulted in the development and acceptance of another method (the Bethesda method) in which patient plasma is incubated with normal plasma for a defined time followed by determination of the residual factor VIII activity.[127]
Usually clotting time determination is used as the end-point but the applicability of chromogenic methods have also been shown for both the New Oxford and Bethesda methods.[202-204]

In spite of the introduction of an international standard for plasma factor VIII activity[205], the interlaboratory variability of factor VIII inhibitor determinations was still quite significant but an improved reliability and specificity has been obtained with the use of buffered normal plasma which increased the stability of the factor VIII activity and with control incubation in the presence of factor VIII deficiency plasma.[206]
The benefits of this approach, denoted the Nijmegen modification, were later demonstrated in a large study of close to 900 patient samples.[207]
The Nijmegen-Bethesda method was also recently successfully used in a chromogenic application in connection with clinical studies on hemophilia A patients receiving the truncated, B-domain deleted factor VIII concentrate ReFacto.[208]

## Determination of factor VIII concentrate potency

Discrepancies between methods for determination of factor VIII potency in factor VIII concentrates was a concern already with low or intermediate purity concentrates[209] and persisted also for plasma concentrates with higher purity in spite of the introduction of international concentrate standards.[210]
However, the introduction of such standards in combination with harmonization of assay conditions has gradually resulted in more reliable assays.
Important improvements were predilution of the concentrate to 1 IU/mL in factor VIII deficiency plasma as well as the incorporation of high quality bovine or human albumin in the buffer used for final dilutions[210] and subsequent multicenter studies confirmed the beneficial effect obtained by these modifications.[211]

The two-stage clotting method was the European Pharmacopoeia reference method for factor VIII potency determination of concentrates for several decades. Although the one-stage method is easier to perform, it was not selected as the reference method due to the interferences in this method from lipids or traces of heparin and its sensitivity to preactivation of factor VIII.[211] Indeed, substantially higher potencies (up to 40%) were obtained with the one-stage method.

The chromogenic method has a principal similarity to the two-stage method and a high agreement with this method was also obtained in several labs in potency determinations of concentrates.[187,210,212]
This feature together with its higher precision[212] resulted in the recommendation of the ISTH Subcommittee for factor VIII and factor IX to adopt the chromogenic method as the reference method[213] and it was later also selected as the reference method by the European Pharmacopoeia.[9]

Has the more stringent requirements on how to assay factor VIII concentrates and the selection of a new reference method finally resulted in satisfactory assay performance and agreements between the different methods on analysis of the new generation of very high purity full length recombinant factor VIII concentrates and the B-domain deleted ReFacto?
Unfortunately this does not seem to be the case so far and it even seems that the different types of recombinant factor VIII concentrates has increased the variability and complexity in accurate potency assignments but it is also possible that the assay recommendations are not yet universally adhered to.[214,215]
The greatest discrepancies are obtained between the one-stage and the chromogenic method with up to two-fold lower potencies obtained with the one-stage method on analysis of ReFacto.

One complicating feature with one-stage clotting methods is that depending upon the source of phospholipid used in the APTT reagent, different results will be obtained on analysis of recombinant factor VIII. For ReFacto, this can possibly be due to different phospholipid binding kinetics for this material as compared to other factor VIII concentrates[216] and realizing the short reaction times in one-stage clotting methods as compared to chromogenic methods such differences may well result in invalid potency assignments when using one-stage methods. Indeed, by either decreasing the phospholipid concentration or by using platelet-rich severe hemophilia A plasma or by using a certain phospholipid source the results obtained with the one-stage clotting method on analysis of ReFacto approached those of the chromogenic method.[217,218]

In support for the validity of the chromogenic method is also the finding that the ratio between activity and antigen levels has been found to be in the range 0.8-1.0, which is within the expected range for a factor VIII preparation with only limited amounts of degraded material.[219] Also, and importantly, the chromogenic method showed a high agreement with the two-stage method (the earlier reference method) in thrombin and APC interaction studies on ReFacto.[117]

It is therefore obvious that work still remains until concordant results are obtained between clotting, especially one-stage, and chromogenic methods.
In the meantime it seems clear that dosing hemophilia A patients with factor VIII concentrates with potency assignments according to the chromogenic method offers a safe and cost-effective treatment.[116,117]

## Summary

The last century has showed a most impressive increase in our knowledge of factor VIII including biochemistry, methodology and the treatment of hemophilia A patients. This fascinating molecule has rightly attracted and continues to attract numerous researchers and clinicians. The 20th century ended with the launch of factor VIII concentrates with high safeguarding against transmittal of infectious diseases and the 21th century has entered with promises of gene therapy in the not too distant future.
It remains though as an important task to make modern treatment more accessible in the majority of countries to the benefit of the hemophilia A patients.

# Chromogenic kits for determination of factor VIII activity

## COAMATIC Factor VIII

**Article number: 82 25 85**

COAMATIC Factor VIII is a chromogenic-based kit for the photometric determination of factor VIII activity in human plasma, blood fractions and purified preparations. The test has been adopted to a wide range of automated instruments and fulfills the requirements of the European Pharmacopoeia for factor VIII concentrate testing.

### Assay procedure

Factor X is activated to factor Xa by factor IXa in the presence of calcium ions and phospholipids. This reaction is greatly stimulated by the cofactor protein, factor VIIIa.
By using optimal amounts of $Ca^{2+}$, phospholipids and factor IXa toghether with an excess of factor X, the rate of factor X activation is linearly related to the factor VIII activity in the sample.
Factor X hydrolyses the chromogenic substrate S-2765 thus liberating the chromophoric group, pNA.
The colour intensity, wich is proportional to the factor VIII activity, is then read photometrically at 405 nm.
Hydrolysis of S-2765 by thrombin formed is prevented by the addition of the synthetic thrombin inhibitor I-2581 together with the chromogenic substrate.

### Measuring range

- Elevated 1 - 4 IU/ml
- Normal 0.05 - 1.5 IU/ml
- Low 0.005 - 0.05 IU/ml

### The kit contains

1. ***S-2765 + I-2581*** ***1 vial***
   Chromogenic substrate N-α-Z-Arg-Gly-Arg-pNA, 7.7 mg and synthetic thrombin inhibitor, 0.2 mg with Mannitol (bulking agent).
2. ***Factor reagent*** ***2 vials***
   Bovine factor IXa (0.3 U), bovine factor X (1.8U) and thrombin (1 NIH-U) colyophilized with $CaCl_2$ (40 µmol) and phospholipid (0.2 µmol).
3. ***Buffer stock solution*** ***1 vial***
   24 ml concentrated Tris buffer containing NaCl and bovine serum albumin (BSA).
   Characteristics of diluted buffer (1:10):
   Tris 0.025 mol/L, pH 7.9, I=0.08, 1% BSA.

### Stability after reconstitution (2-8°C)

| | | |
|---|---|---|
| 1. | S-2765 + I-2581 | 1 month |
| 2. | Factor reagent (2 weeks at –30°C, 1 month at –70°C) | 12 hours |
| 3. | Buffer stock solution, opened vial | 1 month |

### Repeatability

| *Factor VIII* | *CV % Within series* | *CV % Between series* |
|---|---|---|
| 1.0 IU/ml | 2.4 (n = 42) | 2.1 (n = 6 N = 7) |
| 0.25 IU/ml | 2.6 (n = 42) | 5.9 (n = 6 N = 7) |
| 0.03 IU/ml | 3.0 (n = 42) | 3.9 (n = 6 N = 7) |

The repeatability has been determined with the microplate method.
n = number of replicates in each series
N = number of series

### Number of determinations per kit

Manual 30, Microplate 120, Automated up to100

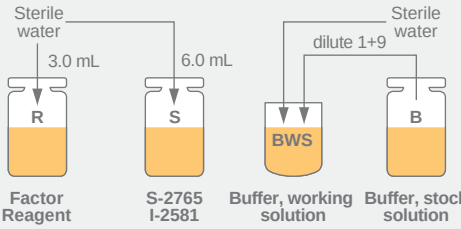
### Available instrument applications*

ACL 200,300,3000,6000,7000
ACL Futura
BCS / BCT
COBAS BIO / FARA / MIRA
ELECTRA 900C, 1000C, 1400C, 1600C, 1800C
HITACHI 704,717,911
PACKS-4
STA/ STA Compact / STA-R
CA 6000, 530, 540
TECHNICON RA
THROMBOLYZER/ HEMOLAB
MICROPLATE (general)

* At the time of print

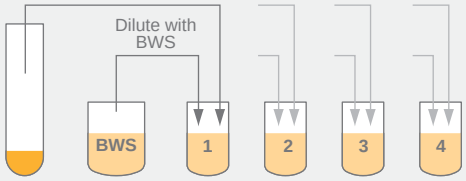
# COAMATIC Factor VIII
# Assay procedure (manual)

## Reconstitution of reagents




Note: The reconstitution volumes of the reagents wary between different automate applications

## Standardization

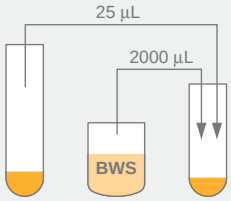



**Reference plasma 1.0 IU/mL (100%)**

BWS = 0 IU/mL

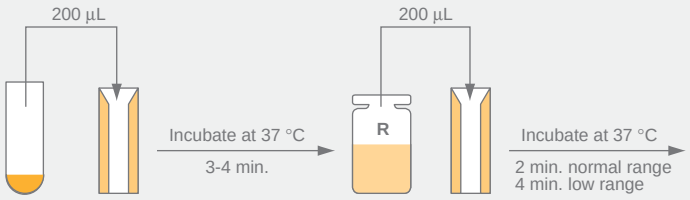
**Low range:** 0.006, 0.012, 0.024, 0.05 IU/mL
**Normal range:** 0.25, 0.5 1.0, 1.42* IU/mL
* A relative standard of 1.42 is obtained by diluting 25 µL with 1400 µL BWS.

## Dilution of test plasma

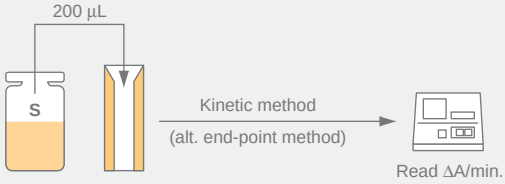



## Assay procedure

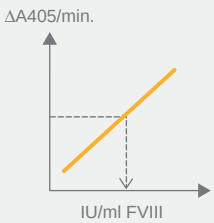
For microplates use 50 µL for all pipetting steps




**Diluted plasma sample (or standard)**




Plot adsorbance readings for standard against their concentration of factor VIII on linear graph paper.
Read sample value from curve.



### Determination of elevated factor VIII activity

A specific adaptation of Coamatic Factor VIII has been developed to allow accurate determination of elevated factor VIII activity.
The advantages in using a chromogenic method as compared to one stage clotting methods are numerous (see p.13). Determination of factor VIII activities up to 4 IU/ml is accomplished by prediluting the plasma samples 1+3 and assaying the diluted samples following the protocol described for the normal assay range but restricting this range to 0-1 IU/ml:

- predilute the samples using the buffer contained in the Coamatic factor VIII kit as follows:
  1 vol plasma sample + 3 vol diluted buffer
- dilute further as detailed in the package insert
- follow the instructions contained in the Coamatic factor VIII package insert (manual microplate procedure) or in the instrument application sheet (automated instruments).

The results should be multiplied by 4 to obtain the final value of factor VIII activity.

### Manual microplate method

*Reagent preparation*
Factor reagent: 3.0 ml of sterile water
Substrate: 6.0 ml of sterile water
Buffer: dilute 1+9 with sterile water

### Standard curve

The standard curve 0-1 IU/ml is prepared by using a human normal plasma calibrated against an International Standard for plasma factor VIII.
In case the normal plasma does not contain exactly 1 IU/ml factor VIII, the values of the standard must be recalculated accordingly.

| | *Predilution* | | *Final dilution* | |
|---|---|---|---|---|
| ***FVIII IU/ml*** | ***Plasma µl*** | ***Buffer µl*** | ***Diluted Plasma µl*** | ***Buffer µl*** |
| 1.00 | - | - | 25 | 2000 |
| 0.70 | 100 | 100 | 25 | 1400 |
| 0.50 | 100 | 100 | 25 | 2000 |
| 0.25 | 50 | 150 | 25 | 2000 |
| 0.00 | - | - | - | 2000 |

### Sample Dilution

1) Predilute the sample by mixing 1 vol plasma with 3 vol of Coamatic Factor VIII Buffer
2) Dilute further as follows:
   Sample 25 µl
   Buffer 2000 µl

### Assay procedure

| | |
|---|---|
| Diluted samples/controls/standards | 50 µl |
| Incubate at 37°C | 3-4 min |
| Factor reagent (37°C) | 50 µl |
| Incubate at 37°C | 2 min |
| Substrate (37°C) | 50 µl |
| Incubate at 37°C | 2 min |
| Acetic acid, 20% | 50 µl |

Read the absorbance at 405 nm, using a reference wavelength of 490 nm.

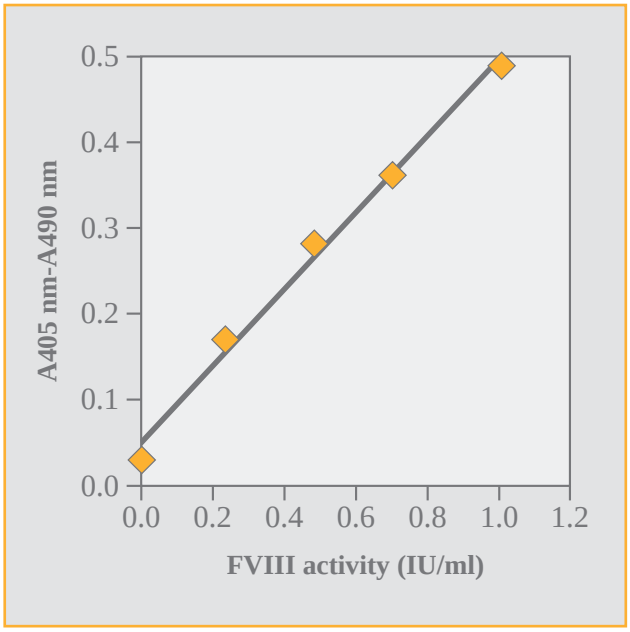



*Fig. 6. Standard curve with the microplate method.*

### ACL method

This method is applicable to the ACL™ 200/300/3000/6000/7000.

*Reagent preparation*
Factor reagent: 3.0 ml of sterile water
Substrate: 5.25 ml of sterile water
Buffer: dilute 1+9 with sterile water

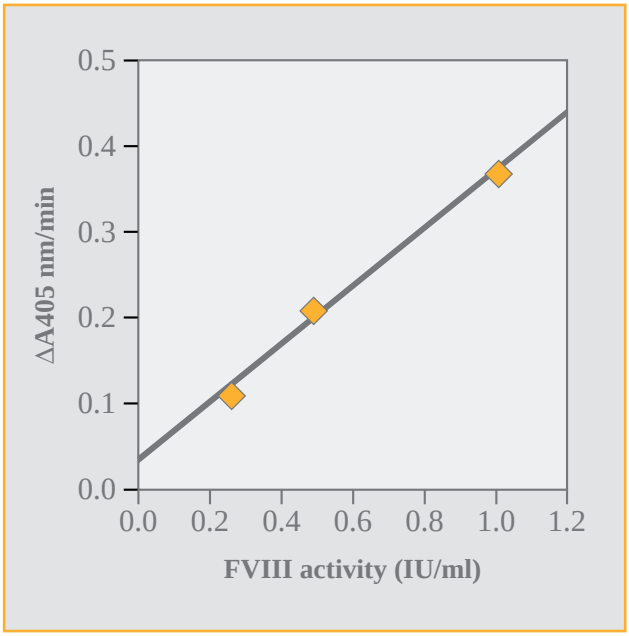



*Fig. 7. Standard curve with the ACL method.*

### Standard curve

The standard curve is prepared by using a human normal plasma calibrated against an International Standard for plasma factor VIII. Dilute the standard as follows: 25 µl plasma + 2000 µl buffer

### Sample Dilution

1. Predilute the sample by mixing 1 vol plasma with 3 vol of Coamatic Factor VIII Buffer
2. Dilute further as follows:
   Sample 25 µl
   Buffer 2000 µl

### Assay procedure

| |
|---|
| Select the test Plasminogen (channel). |
| Place diluted normal plasma in POOL position. |
| Place buffer working solution in DIL position. |
| Place factor reagent in position 2. |
| Place substrate in position 3. |
| Place sample cups with diluted plasmas. |

### Measuring range

With predilution of the sample the measuring range is 1-4 IU/ml with both the microplate and the ACL method.

### Results

The evaluation of Coamatic Factor VIII with samples from thrombotic patients has been performed both with the microplate and the ACL applications.
The standard curves are shown in figures 6 and 7 respectively. The upper limit of the standard curve is 1 IU/ml in both methods resulting in an upper measurement limit of 4 IU/ml, with plasma samples prediluted 1+3.
The precision of the method has been evaluated by using plasma samples diluted according to the protocol described above.

| FVIII *IU/ml* | Within series *CV%* | *n* | Between Series *CV%* | *n* | *N* |
|---|---|---|---|---|---|
| 1 | 3.0 | 35 | 6.0 | 5 | 7 |
| 4 | 3.0 | 35 | 6.0 | 5 | 7 |

The factor VIII activity of 130 patient samples has been determined with Coamatic Factor VIIII on ACL, by prediluting or not the plasma samples.
The samples have been obtained from patients about three months after the thrombotic episode.
The following results were obtained from linear regression analysis (figure 8):

Slope = 1.52
Intercept = -0.57
R = 0.96
Range (x) = 0.45 – 3.28 IU/ml factor VIII
Range (y) = 0.33 – 4.50 IU/ml factor VIII

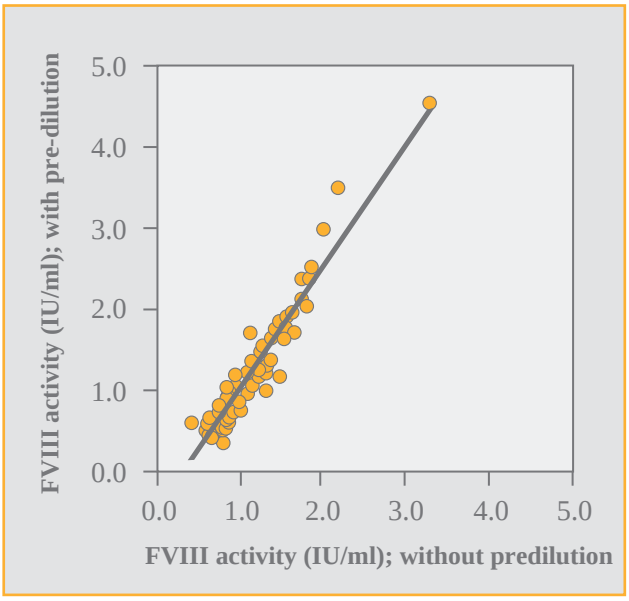



*Fig. 8. Comparison of factor VIII activities obtained with and without sample predilution using Coamatic Factor VIII.*

For factor VIII activities higher than 1 IU/ml, the samples can be underestimated if the predilution is not performed.
Coamatic Factor VIII has been compared with a one-stage clotting method on the ACL analyser. For the Coamatic Factor VIII assay, the samples were pre-diluted 1+3 as recommended in the protocol described above.
For the clotting method the plasma samples were pre-diluted 1+3 (with 0.05 mol/l imidazol, 0.1 mol/l NaCl, pH 7.3; buffer recommended by the clotting reagent manufacturer) followed by the prescribed sample dilution 1+4.
71 plasma samples from thrombotic patients were analysed. The results are shown in figure 9.

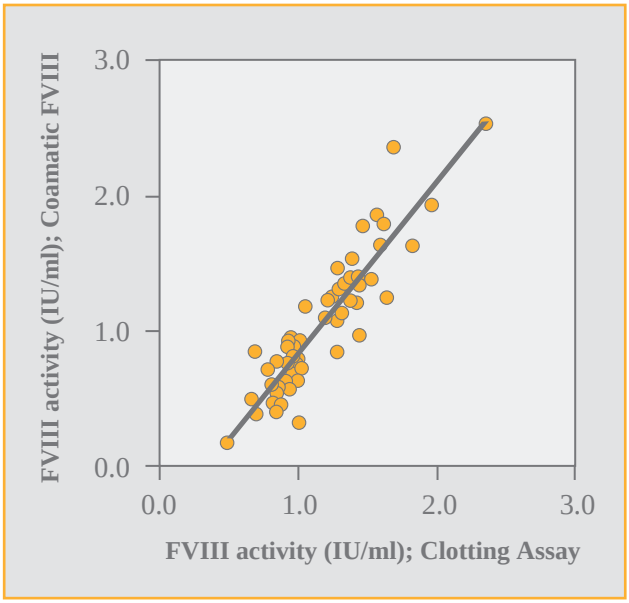



*Fig. 9. Comparison of factor VIII activities obtained by a clotting assay and Coamatic Factor VIII.*

The following results were obtained from linear regression analysis:
Slope = 1.28
Intercept = -0.43
R = 0.92
Range (x) = 0.50 – 2.32 IU/ml factor VIII
Range (y) = 0.18 – 2.51 IU/ml factor VIII

**Conclusions**

The results described here represent a preliminary evaluation of Coamatic Factor VIII applied for the screening of samples from thrombotic patients. From the population of samples tested, about 25% had a factor VIII activity higher than 1.4 IU/ml, thus confirming earlier published data. These results have been obtained by a simple modification of the existing applications and protocols, consisting in the predilution 1+3 of the plasma samples. Coamatic Factor VIII is a kit suitable for use on a number of automated instruments as well as on microplates. The data presented here show its applicability on the ACL instrument for determination of elevated FVIII activity.
In case the predilution is done manually, the current application notes for automated instruments can then be adhered to, with the only exception of restricting the assay range to 0-1 IU/ml.
Indeed, some instruments offer the possibility of also performing the pre-dilution step.

# COATEST SP FVIII
***Article number: 82 4086 63***

# COATEST SP4 FVIII
***Article number: 82 4094 63***

They are the classic chromogenic-based kits for the photometric determination of factor VIII activity in human plasma, blood fractions and purified preparations.
The formulation of the two kits is the same.
They are optimized for different test volumes.

### Assay procedure

Factor X is activated to factor Xa by factor IXa in the presence of calcium ions and phospholipids.
This reaction is greatly stimulated by the cofactor protein, factor VIIIa.
By using optimal amounts of $Ca^{2+}$, phospholipids and factor IXa toghether with an excess of factor IXa and factor X, the rate of factor X activation is linearly related to the factor VIII activity in the sample.
Factor Xa hydrolyses the chromogenic substrate S-2765 thus liberating the chromophoric group, pNA.
The colour intensity, wich is proportional to the factor VIII activity, is then read photometrically at 405 nm.
Hydrolysis of S-2765 by thrombin formed is prevented by the addition of the synthetic thrombin inhibitor I-2581 together with the chromogenic substrate.

### Measuring range

- 20-150% (0.2 -1.5 IU/ml)
- 1-20% (0.01 - 0.2 IU/ml)

### The kits contain

***1. S-2765 + I-2581*** ***1 vial***
Chromogenic substrate Bz- Ile-Glu (γ-OR)-Gly-Arg-pNA 20 mg, and synthetic thrombin inhibitor, 335 µg with mannitol (bulking agent).

***2. Factor IXa + factor X*** ***1 vial / 4 vials ****
Lyophilized bovine factors IXa and factor X with bovine serum albumin added as a stabilizing agent

***3. $CaCl_2$*** ***1 vial***
Calcium chloride solution, 0.025 mol/L.

***4. Buffer stock solution*** ***1 vial***
20 ml concentrated Tris buffer.
Characteristics of diluted buffer (1:10):
Tris 0.05 mol/L, pH 7.3, 0.2% bovine albumin.

***5. Phospholipid*** ***1 vial***
Mixture of highly purified phospholipids.

### Stability after reconstitution (2-8°C)

| | |
|---|---|
| 1. S-2765 + I-2581 | 3 months |
| 2. Factor IXa + factor X (3 months at –20°C) | 12 hours |
| 3. $CaCl_2$ | exp.date |
| 4. Buffer stock solution, opened vial | 3 months |
| 5. Phospholipid, opened vial | 3 months |

### Repeatability

*Range 1-20 %*

| *Factor VIII* | *CV % Within run* | *CV % Total* |
|---|---|---|
| 14 % | 4.3 (n = 2) | 5.6 (n = 80) |

*Range 20-150 %*

| *Factor VIII* | *CV % Within run* | *CV % Total* |
|---|---|---|
| 83 % | 3.4 (n = 2) | 5.3 (n = 80) |

The repeatability has been determined with the microplate method.
n = number of replicates in each series
N = total number of replicates

### Number of determinations per kit

| | |
|---|---|
| Manual | 60 |
| Microplate | 240 |
| Automated | up to 200 |

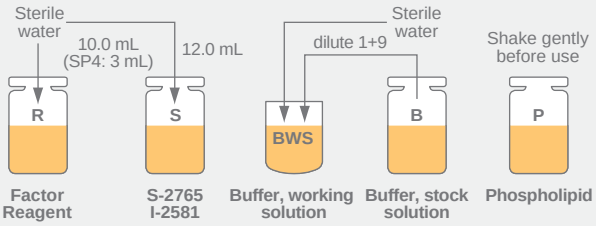
### Available instrument applications*

ACL 200,300,3000,6000,7000
COBAS BIO / MIRA
ELECTRA 900C, 1000C
LABSYSTEMS FP-910
MULTISTAT III/PLUS
MICROPLATE (general)

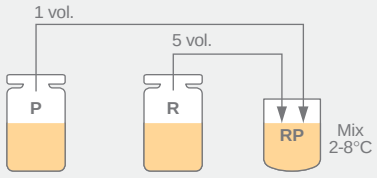
* At the time of print

# COATEST SP FVIII, COATEST SP4 FVIII
# Assay procedure (manual)

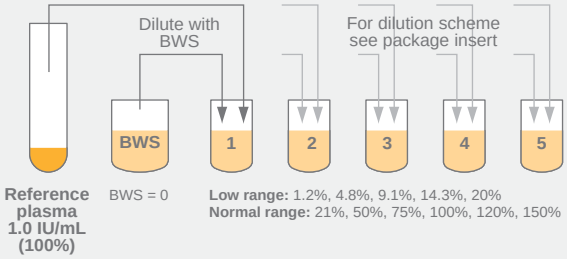
## Reconstitution of reagents



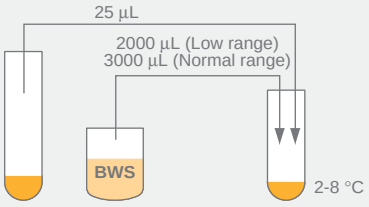

## Preparation of Factor Reagent



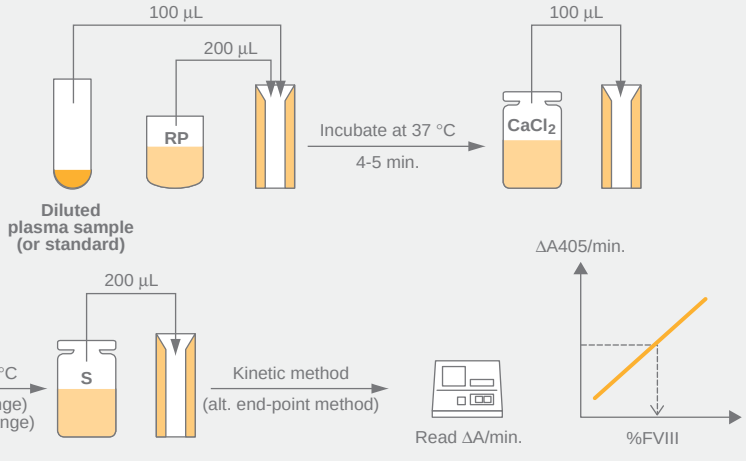

## Standardization




## Dilution of test plasma




## Assay procedure




Plot adsorbance readings for standard against their concentration of factor VIII on linear graph paper. Read sample value from curve.

# Products' Summary

## Coamatic Factor VIII
## Coatest SP FVIII
## Coatest SP4 FVIII

**Unique products features:**

- Highly precise and accurate factor VIII activity measurements in both plasma and factor VIII concentrates
- Highly sensitive, with a detection limit less than 1% factor VIII
- Fulfills all the requirements set by the European Pharmacopoeia for factor VIII concentrate testing
- Applications for a wide range of automated instruments
- Convenient reagent handling
- Good stability of reagents
- Rapid test results

**Clinically recommended for:**

- Potency determination of FVIII concentrates in Quality Control
- Diagnosis, classification and management of hemophilia A
- Thrombophilia investigations (elevated factor VIII activity)

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# Notes

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