



The Corporate Organization

In 1996 Chromogenix became a part of the Instrumentation Laboratory group.

Instrumentation Laboratory is a worldwide corporation providing laboratory reagents, instruments and services in a number of key diagnostic areas, including Hemostasis, Critical Care and Clinical Chemistry.

R&**D**, **M**anufacturing, **M**arketing

Research, development and production of all reagent products is based in the Instrumentation Laboratory plant located in Orangeburg NY, USA, where all Chromogenix brand products are manufactured.

Located in Milan, Italy, the Chromogenix Marketing Group manages all aspects of the business and is the heart of the technical support network.

The Distribution Network

The Group Chromogenix and its independent distributors work as one team.

A complete detailed list of our distributors is shown on our web site: <http://www.chromogenix.com>

Quality Statement

At Chromogenix, we believe that the quality of our products and services are critical, not only for today's good reputation, but also for tomorrow's success. We want our customers to know that:

- Chromogenix' name represents a high quality of service, which is integral to the company's concept of total quality.
- Chromogenix constantly strives to manufacture quality products that our customers will find useful, effective and reliable.
- Chromogenix' products reflect not only the customer's needs but also local regulatory requirements.
- Chromogenix' approach to development, manufacturing and marketing is governed by ethical business principles.
- Chromogenix emphasises the importance of a high level of quality consciousness among staff at all levels within the organisation.

Chromogenix' quality policy extends not only to our products but also to our organisation, the way we work, and the service we provide to our customers.

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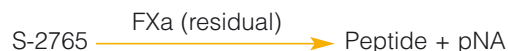
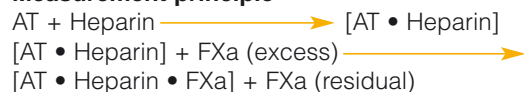
For the USA:
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for the FDA clearance status of our kits.
Please contact your local distributor for
updated information.

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COAMATIC® ANTITHROMBIN

A chromogenic kit for the determination of antithrombin activity in human plasma. The use of factor Xa in preference to thrombin eliminates interference from heparin cofactor II and thrombin inhibitors.

Measurement principle



Reagents and their stability when opened

Substrate S-2765	1 vial	6 months	2-8°C
Buffer with heparin	1 vial	3 months	2-8°C
FXa	1 vial	3 months	2-8°C

Number of determinations

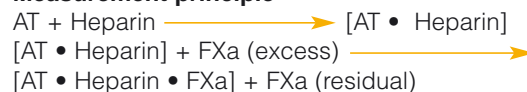
Test tube method	50
Microplate method	200
Automated methods	up to 130

Article number: 82 1991 63

COAMATIC® AT 400

A chromogenic kit for the determination of antithrombin activity in human plasma. The use of factor Xa in preference to thrombin eliminates interference from heparin cofactor II and thrombin inhibitors.

Measurement principle



Reagents and their stability when opened

Substrate S-2772	2 vials	6 months	2-8°C
Buffer with heparin	6 vials	3 months	2-8°C
FXa	6 vials	3 months	2-8°C

Number of determinations

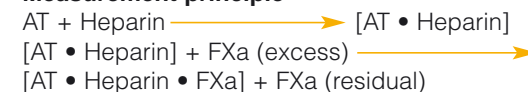
Automated methods	up to 450
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Article number: 82 2320 63

COAMATIC® LR ANTITHROMBIN

A chromogenic kit for the determination of antithrombin activity in human plasma. The use of factor Xa in preference to thrombin eliminates interference from heparin cofactor II and thrombin inhibitors. Reagents in liquid formulation.

Measurement principle



Reagents and their stability when opened

Substrate S-2772	2 vials	6 months	2-8°C
FXa with heparin	6 vials	1 month	2-8°C

Number of determinations

Automated methods	up to 450
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Article number: 82 2957 63

LARGE KIT

**COATEST®
APC™ RESISTANCE**

An APTT-based assay for the detection of the APC resistance phenotype, i.e. the poor anticoagulant response to activated protein C (APC). The test result (APC ratio) gives an estimation of the anticoagulant function in vivo and provides information on the thrombotic risk associated with inherited and acquired APC resistance.

Measurement principle

Plasma is incubated with the APTT reagent for a standard period of time. Coagulation is initiated by the addition of CaCl₂ in the absence and presence of APC and the time for clot formation is recorded.

Reagents and their stability when opened

CaCl ₂	1x8 ml	1 week 1 month	15-25°C 2-8°C
APTT reagent	1x16 ml	1 week 1 month	15-25°C 2-8°C
APC/CaCl ₂	4x2 ml	8 hours 5 days 3 months	15-25°C 2-8°C -20°C

Number of determinations

Automated methods 80-160

Article number: 82 2643 63

SMALL KIT

**COATEST®
APC™ RESISTANCE - S**

An APTT-based assay for the detection of the APC resistance phenotype, i.e. the poor anticoagulant response to activated protein C (APC). The test result (APC ratio) gives an estimation of the anticoagulant function in vivo and provides information on the thrombotic risk associated with inherited and acquired APC resistance.

Measurement principle

Plasma is incubated with the APTT reagent for a standard period of time. Coagulation is initiated by the addition of CaCl₂ in the absence and presence of APC and the time for clot formation is recorded.

Reagents and their stability when opened

CaCl ₂	2x2 ml	1 week 1 month	15-25°C 2-8°C
APTT reagent	2x4 ml	1 week 1 month	15-25°C 2-8°C
APC/CaCl ₂	2x2 ml	8 hours 5 days 3 months	15-25°C 2-8°C -20°C

Number of determinations

Automated methods 40-80

Article number: 82 2916 63



APC RESISTANCE DETERMINATION DUE TO FACTOR V LEIDEN MUTATION

LARGE KIT
WITH
CONTROLS

COATEST® APC™ RESISTANCE V

An APTT-based kit for screening of factor V-related APC resistance. The high sensitivity and specificity of the test for the factor V:Q⁵⁰⁶ mutation is obtained by prediluting the sample plasma with an excess of V-DEF Plasma. The test design makes it possible to discriminate between heterozygous and homozygous factor V genotypes. It also allows for analysis of plasma from patients on heparin or oral anticoagulant therapy.

Measurement principle

One volume of plasma is prediluted with four volumes of V-DEF Plasma. The dilution is then incubated with the APTT reagent for a standard period of time. Coagulation is triggered by the addition of CaCl₂ in the absence and presence of exogenous APC and the time for clot formation is recorded.

Reagents and their stability when opened

V-DEF Plasma	4x4 ml	8 hours 24 hours 3 months	15-25°C 2-8°C -20°C
CaCl ₂	1x8 ml	1 week 1 month	15-25°C 2-8°C
APTT reagent	1x16 ml	1 week 1 month	15-25°C 2-8°C
APC/CaCl ₂	4x2 ml	8 hours 5 days 3 months	15-25°C 2-8°C -20°C
Control Plasma Level 1	1x1 ml	6 hours 3 months	2-25°C -20°C
Control Plasma Level 2	1x1 ml	6 hours 3 months	2-25°C -20°C

Number of determinations 80-160

Article number: 82 3120 63

SMALL KIT
WITH
CONTROLS

COATEST® APC™ RESISTANCE V-S

An APTT-based kit for screening of factor V-related APC resistance. The high sensitivity and specificity of the test for the factor V:Q⁵⁰⁶ mutation is obtained by prediluting the sample plasma with an excess of V-DEF Plasma. The test design makes it possible to discriminate between heterozygous and homozygous factor V genotypes. It also allows for analysis of plasma from patients on heparin or oral anticoagulant therapy.

Measurement principle

One volume of plasma is prediluted with four volumes of V-DEF Plasma. The dilution is then incubated with the APTT reagent for a standard period of time. Coagulation is triggered by the addition of CaCl₂ in the absence and presence of exogenous APC and the time for clot formation is recorded.

Reagents and their stability when opened

V-DEF Plasma	2x4 ml	8 hours 24 hours 3 months	15-25°C 2-8°C -20°C
CaCl ₂	2x2 ml	1 week 1 month	15-25°C 2-8°C
APTT reagent	2x4 ml	1 week 1 month	15-25°C 2-8°C
APC/CaCl ₂	2x2 ml	8 hours 5 days 3 months	15-25°C 2-8°C -20°C
Control Plasma Level 1	1x1 ml	6 hours 3 months	2-25°C -20°C
Control Plasma Level 2	1x1 ml	6 hours 3 months	2-25°C -20°C

Number of determinations 40-80

Article number: 82 3138 63

V-DEF PLASMA

V-DEF Plasma is a prediluent of sample plasma to be used in conjunction with the use of Coatest® APC™ Resistance V, and Coatest® APC™ Resistance V-S. It strongly reduces the influence of plasma handling and storage, and provides a high discrimination for factor V- related APC resistance. In addition, V-DEF Plasma allows for analysis of plasma from patients on oral anticoagulant therapy or on heparin treatment (≤ 1IU/ml). V-DEF Plasma has a sufficiently high factor VIII activity to provide essentially normal APTT values.

Reagent

The product contains 5x4 ml of human factor V deficient plasma, stabilized and lyophilized together with the heparin antagonist Polybrene®.

Reagents and their stability when opened

V-DEF Plasma	5x4 ml	8 hours 24 hours 3 months	15-25°C 2-8°C -20°C
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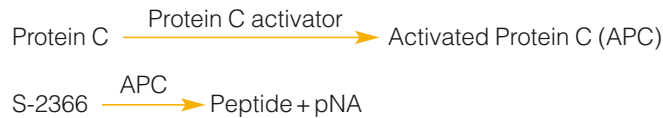
Article number: 82 3146 63

High discrimination between genotypes with 100% sensitivity for FV:Q⁵⁰⁶. Reduces need for PCR determination. Applicable to anticoagulant treated patients.

COAMATIC® PROTEIN C

A chromogenic kit for the determination of protein C activity in human plasma. No influence of heparin levels up to 3 IU/ml.

Measurement principle



Reagents and their stability when opened

Substrate S-2366	2 vials	3 months	2-8°C
Protein C activator	2 vials	3 months	2-8°C

Number of determinations

Test tube method	72
Microplate method	288
Automated methods	up to 180

Article number: 82 2098 63

Simple and rapid performance gives optimal user convenience. Excellent accuracy and precision as well as reagent stability. Validated and documented protocols for a wide range of instruments. Suitable for both large and small laboratories.

COALIZA® PROTEIN S - FREE

A kit for the quantitative determination of free protein S antigen in plasma.

Measurement principle

The Coaliza Protein S – Free kit method is based on a procedure described by Dahlbäck and colleagues (see Reference). The microplate wells are precoated with C4b-binding protein (C4BP), which has a very high affinity for binding free protein S (FPS) antigen in plasma. A monoclonal antibody (HPS 54) conjugated with the enzyme horseradish peroxidase (HRP) is added together with the plasma sample. After the sample and conjugate incubation, unbound material is washed away and bound protein S, in complex with C4BP is detected with the addition of a substrate-chromogen.

The amount of colour in wells is directly proportional to the amount of free protein S antigen in the plasma sample. Reference: Tusar Kanti Giri, Andreas Hillarp, Yiva Härdig, Bengt Zöller, Björn Dahlbäck. A new direct, fast and quantitative enzyme-linked ligandsorbent assay for measurement of free protein S antigen. Thromb Haemost 79, 767-72 (1998).

Reagents and their stability when opened

Microwell Strips	12 x 8	exp. Date	2-8°C
Conjugate concentrate	1 vial	exp. Date	2-8°C
Conjugate and Sample Diluent	2 vials	exp. Date	2-8°C
Washing Buffer, Concentrate	2 vials	exp. Date	2-8°C
Substrate Buffer	2 vials	exp. Date	2-8°C
Chromogen TMB	1 vial	exp. Date	2-8°C
Calibration Plasma	2 vials	exp. Date	2-8°C
Normal Control Plasma	1 vial	exp. Date	2-8°C
Stopping Solution	1 bottle	exp. Date	2-8°C

Number of determinations

96

Article number: 82 3567 63

COMATIC® PROTEIN S - FREE

Automated latex ligand immunoassay for the quantitative determination of free Protein S (PS) in human citrated plasma on automated instruments. Two forms of Protein S are present in plasma: free Protein S (40%), and Protein S linked to the complement C4b-binding protein (C4BP) (60%). Only free Protein S has functional cofactor activity.

Measurement principle

The presence of free Protein S in the sample is measured as the increase of turbidity produced by the agglutination of two latex reagents. Purified C4BP adsorbed onto the first latex reagent reacts with a high affinity for free Protein S of patient plasma in the presence of Ca²⁺ ions. The free Protein S adsorbed on the C4BP latex triggers the agglutination reaction with the second latex reagent, which is sensitized with a monoclonal antibody directed against human Protein S. The degree of agglutination will be directly proportional to the free Protein S concentration in the test sample and is determined by measuring the decrease of the transmitted light at 405 nm caused by the aggregates.

Reagents and their stability when opened

C4BP Buffer	3 vials	exp. date	2-8°C
C4BP Latex	3 vials	1 month	2-8°C
Anti PS MAb Latex	3 vials	1 month	2-8°C

Determinations/Kit

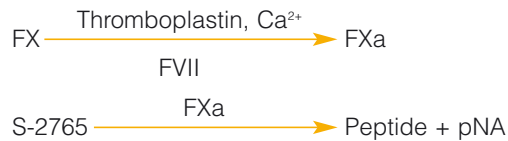
Approximately 75 Tests

Article number: 82 4003 63

**COASET®
FACTOR VII**

A chromogenic kit for the determination of factor VII activity in human plasma. Not affected by preactivation of factor VII.

Measurement principle



Reagents and their stability when opened

Substrate S-2765	1 vial	6 months	2-8°C
BSA (Bovine Serum Albumin)	1 vial	1 week	2-8°C
Buffer	1 vial	3 months	-20°C
Bovine FX	1 vial	2 months	2-8°C
		1 week	2-8°C
		3 months	-70°C
CaCl ₂	1 vial	exp.date	2-8°C
Thromboplastin	1 vial	1 month	2-8°C

Number of determinations

Test tube method	30
Microplate method	120
Automated methods	up to 120

Article number: 82 1900 63

Unaffected by preactivation of factor VII.
Coaset Factor VII is in compliance with the European Pharmacopoeia requirements. Refer to page 87 of this catalogue for more information.

**COAMATIC®
VON WILLEBRAND FACTOR**

Automated latex enhanced immunoassay for the quantitative determination of von Willebrand Factor Antigen (vWF:Ag) in human citrated plasma.

Measurement principle

When a plasma containing vWF:Ag is mixed with the Latex Reagent and the Reaction Buffer the coated latex particles agglutinate. The degree of agglutination is directly proportional to the concentration of vWF:Ag in the sample and is determined by measuring the decrease of transmitted light at 405 nm caused by the aggregates.

Reagents and their stability when opened

Latex Reagent	2 vials	3 months	2-8°C
Reaction Buffer	2 vials	3 months	2-8°C

Number of determinations

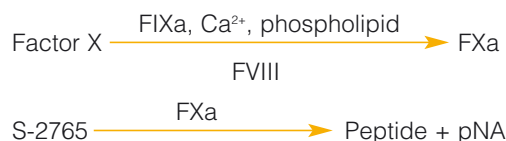
Approximately 60 Test

Article number: 82 4029 63

COAMATIC® FACTOR VIII

A chromogenic kit for the determination of factor VIII activity in human plasma, blood fractions and purified preparations. Fulfills the requirements of the European Pharmacopoeia for factor VIII concentrate testing.

Measurement principle



Reagents and their stability when opened

S-2765+I-2581	1 vial	1 month	2-8°C
Factor reagent	2 vials	1 day	2-8°C
		1 month	-70°C
Buffer	1 vial	1 month	2-8°C

Number of determinations

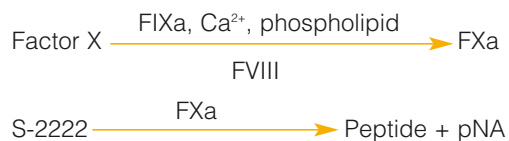
Test tube method	30
Microplate method	120
Automated methods	up to 100

Article number: 82 2585 63

COATEST® FACTOR VIII

The classic chromogenic kit for the determination of factor VIII activity in human plasma, blood fractions and purified preparations.

Measurement principle



Reagents and their stability when opened

S-2222+I-2581	1 vial	6 months	2-8°C
FIXa+FX	1 vial	12 hours	2-8°C
		1 month	-20°C
CaCl ₂	1 vial	exp. date	2-8°C
Buffer	1 vial	1 month	2-8°C
Phospholipid	1 vial	1 month	2-8°C

Number of determinations

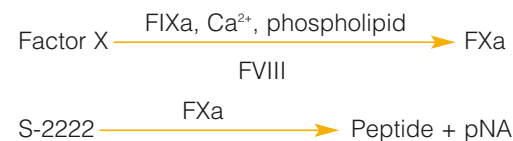
Test tube method	50
Microplate method	200
Automated methods	up to 200

Article number: 82 1033 63

COATEST® VIII:C/4

A chromogenic kit for the determination of factor VIII activity in human plasma, blood fractions and purified preparations. Suitable for low volume testing.

Measurement principle



Reagents and their stability when opened

S-2222+I-2581	1 vial	6 months	2-8°C
FIXa+FX	4 vial	12 hours	2-8°C
		1 month	-20°C
CaCl ₂	1 vial	exp. date	2-8°C
Buffer	1 vial	1 month	2-8°C
Phospholipid	1 vial	1 month	2-8°C

Number of determinations

Test tube method	4x15
Microplate method	200
Automated methods	up to 200

Article number: 82 1918 63

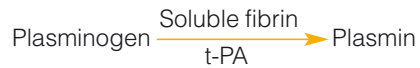
Co-lyophilisation of reagents for convenient handling.
Reliable tool for haemophilia classification.
No interference from heparin levels up to 1.5 IU/ml.

The Factor VIII kits have two measuring ranges. Validated and documented protocols for a wide range of instruments.
All the Factor VIII kits can be used for the potency estimation of Factor VIII according to the European Pharmacopoeia requirements.
For more information refer to page 88 of this catalogue.

COATEST® SOLUBLE FIBRIN

A novel chromogenic kit for the determination of soluble fibrin in human plasma. Utilizes the ability of soluble fibrin to stimulate the t-PA-catalyzed conversion of plasminogen to plasmin. The key reagents are co-lyophilised into microwell strips.

Measurement principle



Reagents and their stability when opened

Microwell strips co-lyophilised with plasminogen, t-PA and S-2403	3x8	2 months	2-8°C
Plasmin Inhibitor antibody solution	1 x1.5 ml	2 months	2-8°C
Assay buffer	1 x25 ml	2 months	2-8°C
Soluble fibrin control (lyophilized human plasma)	1 vial	1 hour	R.T.
		2 months	-20°C

Number of determinations

24

Article number: 82 2965 63

COAMATIC® D-DIMER

Automated latex enhanced immunoassay for the quantitative determination of D-Dimer in human citrated.

Measurement principle

D-Dimer Latex Reagent is a suspension of polystyrene latex particles of uniform size coated with a monoclonal antibody highly specific for the D-Dimer domain included in fibrin soluble derivatives. When a plasma containing D-Dimer is mixed with the Latex Reagent and the Reaction Buffer, the coated latex particles agglutinate.

The degree of agglutination is directly proportional to the concentration of D-Dimer in the sample and is determined by measuring the decrease of the transmitted light at 405 nm caused by the aggregates.

Reagents and their stability when opened

Latex Reagent	4 vials	1 month	2-8°C
Reaction Buffer	3 vials	1 month	2-8°C
D-Dimer Calibrator	2 vials	1 month	2-8°C

Number of determinations

Approximately 115 Tests

Article number: 82 4011 63

COAMATIC® HEPARIN

A chromogenic kit for the determination of heparin and low molecular weight (LMW) heparin in human plasma. One-stage assay optimized for a wide range of instruments.

Measurement principle



Reagents and their stability when opened

Substrate S-2732	2 vials	6 months	2-8°C
Factor Xa	2 vials	3 months	2-8°C

Number of determinations

Test tube method	50
Microplate method	200
Automated methods	120

Article number: 82 3393 63

Optimal user convenience by simple performance and few components. No sample dilution required. Validated and documented protocols for a wide range of instruments. Optimized reaction system yields very consistent activity levels regardless of type of heparin. Independent of the antithrombin level of the patient. No addition of exogenous antithrombin required.

COATEST® LMW HEPARIN/HEPARIN

A chromogenic kit for the determination of heparin and low molecular weight (LMW) heparin in human plasma. One-stage assay mainly intended for non-automated laboratories.

Measurement principle



Reagents and their stability when opened

Substrate S-2732	1 vial	6 months	2-8°C
Factor Xa	1 vial	1 month	2-8°C
Buffer	1 vial	2 months	2-8°C
LMWH standard	1 vial	6 months	2-8°C

Number of determinations

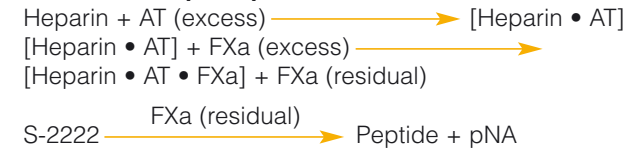
Test tube method	50
Microplate method	125

Article number: 82 1363 63

COATEST® HEPARIN

A chromogenic kit for the determination of heparin and low molecular weight heparin in human plasma.

Measurement principle



Reagents and their stability when opened

Substrate S-2222	1 vial	6 months	2-8°C
Factor Xa	1 vial	1 month	2-8°C
		6 months	-20°C
Antithrombin	1 vial	1 month	2-8°C
		6 months	-20°C
Buffer	1 vial	2 months	2-8°C
Normal plasma	4 vials	2 weeks	2-8°C
		1 month	-20°C

Number of determinations

Test tube method	100
Microplate method	400
Automated methods	up to 285

Article number: 25 5539 63

The heparin kits determine the antiFXa activity of LMW heparin and UF heparin. Excellent reagent stability. Suitable for both large and small laboratories. For Pharmacopoeia procedures see pages 85 and 91 of this catalogue.

COALIZA® PAI-1

An ELISA kit for the quantitative determination of PAI-1 antigen in human plasma or cell culture supernatant.

Measurement principle

Microplate wells coated with a monoclonal anti-PAI-1 antibody are used to capture PAI-1 present in the sample. A second antibody, conjugated to horse-radish peroxidase is added. After incubation and removal of unreacted material, enzyme substrate is added. The amount of colour produced in the wells is proportional to the amount of PAI-1 antigen originally present in the sample.

Reagents and their stability when opened

Microplate	96 wells	8 weeks	2-8°C
Conjugate	1 vial	exp. date	2-8°C
Conjugate diluent	1 vial	exp. date	2-8°C
Sample diluent	2 vials	exp. date	2-8°C
PAI-1 standards	6 stds.	exp. date	2-8°C
Substrate	1 vial	exp. date	2-8°C
Substrate buffer	1 vial	exp. date	2-8°C
Washing buffer	2 vials	exp. date	2-8°C

Number of determinations

96

Article number: 82 1975 63

COATEST® PAI

A chromogenic kit for the determination of plasminogen activator inhibitor-1 (PAI-1) activity in human plasma.

Measurement principle

PAI-1 + t-PA (excess) → [PAI-1 • t-PA] + t-PA (residual)

Plasminogen $\xrightarrow{\text{t-PA (residual), stimulator}}$ Plasmin

S-2403 $\xrightarrow{\text{Plasmin}}$ Peptide + pNA

Reagents and their stability when opened

Substrate S-2403	1 vial	6 months	2-8°C
t-PA	1 vial	1 month	2-8°C
Plasminogen	1 vial	8 hours	2-8°C
		1 month	-20°C
Stimulator	1 vial	1 month	2-8°C
Tris•HAc buffer	1 vial	exp. date	2-8°C
Acetate buffer	1 vial	2 months	2-8°C
PAI-1 depleted plasma	1 vial	8 hours	2-8°C
		3 months	-20°C

Number of determinations

Test tube method	50
Microplate method	100

Article number: 82 1686 63

COALIZA® t-PA

An ELISA kit for the quantitative determination of tissue plasminogen activator antigen in biological fluids (such as plasma, tissue extract and cell culture supernatant).

Measurement principle

Microplate wells coated with a monoclonal anti-t-PA antibody are used to capture any t-PA present in the sample.

A second antibody, conjugated to horse-radish peroxidase (HRP), is added. After incubation and removal of unreacted conjugate, enzyme substrate is added. Incubation with enzyme substrate produces a blue colour which turns yellow when the reaction is stopped with sulphuric acid.

The amount of colour produced in the wells is proportional to the amount of t-PA antigen originally present in the sample.

Reagents and their stability when opened

Microplate	96 wells	8 weeks	2-8°C
Conjugate	1 vial	exp. date	2-8°C
Conjugate diluent	1 vial	exp. date	2-8°C
Sample diluent	2 vials	exp. date	2-8°C
t-PA standards	6 stds.	exp. date	2-8°C
Substrate	1 vial	exp. date	2-8°C
Substrate buffer	1 vial	exp. date	2-8°C
Washing buffer	2 vials	exp. date	2-8°C

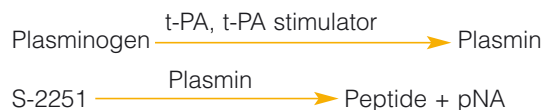
Number of determinations 96

Article number: 82 1538 63

COASET® t-PA

A chromogenic kit for the determination of tissue plasminogen activator activity in human plasma.

Measurement principle



Reagents and their stability when opened

Tris buffer	1 vial	2 months	2-8°C
Acetate buffer	1 vial	2 months	2-8°C
t-PA	1 vial	1 month	2-8°C
t-PA/PAI-1 depleted plasma	1 vial	8 hours 3 months	2-8°C -20°C
Substrate S-2251	1 vial	6 months	2-8°C
Plasminogen	1 vial	8 hours 1 month	2-8°C -20°C
t-PA stimulator	1 vial	1 month	2-8°C

Number of determinations

Test tube method	50
Microplate method	100

Article number: 82 1447 63

New

COALIZA® TAFI

Thrombin activatable fibrinolysis inhibitor (TAFI) or plasma procarboxypeptidase U (proCPU, EC 3.4.17.20) is synthesized in the liver and circulates in plasma as a zymogen, partly bound to plasminogen. It is most efficiently activated by the thrombin-thrombomodulin complex to TAFIa but it is also activated by thrombin alone and by plasmin. TAFIa down-regulates fibrinolysis by removing C-terminal lysines from fibrin, thereby lowering the capacity of fibrin to bind t-PA and plasminogen. Elevated concentrations of TAFI have recently been shown to be associated with an increased risk for venous thrombosis.

Measurement principle

COALIZA® TAFI provides a complete immunoassay for determination of TAFI or TAFIa antigen in plasma. Microwells are coated with a monoclonal antibody specific for human TAFI and TAFIa. After incubation with diluted plasma, TAFI (TAFIa) present in the sample is captured onto the solid phase. After a washing step, a horseradish peroxidase (HRP)-tagged goat polyclonal TAFI antibody is added which binds to free epitopes of immobilized TAFI. After a second washing step the peroxidase substrate is introduced and colour is generated. The amount of colour developed is proportional to the concentration of human TAFI present in the sample.

Reagent and their stability when opened

Precoated microwell-strips	96 wells	4 weeks	2-8°C
Sample Diluent	2 vials	4 weeks	2-8°C
Plasma TAFI Calibration (lyophilised)	3 vials	8 hours	R. T.
TAFI Control 1 (High)	1 vial	24 hours	2-8°C
TAFI Control (Low)	1 vial	24 hours	2-8°C
Conjugate concentrate (lyophilised)	3 vials	72 hours	2-8°C
Conjugate Diluent	1 vial	4 weeks	2-8°C
Substrate TMB	1 vial	4 weeks	2-8°C
Stopping solution	1 vial	exp. date	2-8°C
Wash solution (20x concentrate)	1 vial	4 weeks	2-8°C

Number of determinations 96

Article number: 82 4045 63

COALIZA®
ANTI-CARDIOLIPIN IgG and IgM

An ELISA kit for the determination of IgG and IgM anti-cardiolipin (aCL) antibodies in human serum or plasma.

Measurement principle

The microplate strips are coated with cardiolipin used to capture aCL antibodies present in samples, controls and standards. Subsequently, antibodies specific for human IgG or IgM, labelled with horse-radish peroxidase (HRP), are added to the wells. These bind to any solid-phase cardiolipin bound antibody complex previously formed. Two enzyme-conjugated antibody solutions are provided – one for human IgG antibodies and one for human IgM antibodies.

Concentrations of IgG aCL antibodies and IgM aCL antibodies must be determined separately. The bound enzyme-antibody conjugate is detected by the addition of tetramethyl-benzidine (TMB) and hydrogen peroxide (H₂O₂). Colour develops in proportion to the concentration of aCL antibodies.

Reagents and their stability when opened

Microwell strips	12x8	exp. date	2-8°C
Sample diluent	1 vial	exp. date	2-8°C
GPL calibrator serum	1 vial	exp. date	2-8°C
MPL calibrator serum	1 vial	exp. date	2-8°C
GPL aCL positive control serum	1 vial	exp. date	2-8°C
MPL aCL positive control serum	1 vial	exp. date	2-8°C
Normal control serum	1 vial	exp. date	2-8°C
Conjugate anti-human IgG	1 vial	exp. date	2-8°C
Conjugate anti-human IgM	1 vial	exp. date	2-8°C
Substrate solution	1 vial	exp. date	2-8°C
Phosphate buffered saline (PBS)	1 vial	exp. date	2-8°C
Stopping solution	1 vial	exp. date	2-8°C

Number of determinations 96

Article number: 82 3377 63

COALIZA®
ANTI β2GPI-IgG

ELISA kit for the determination of antibodies anti β2GPI IgG Isotype in human plasma.

New
Coming this year

COALIZA®
ANTI β2GPI-IgM

ELISA kit for the determination of antibodies anti β2GPI IgM Isotype in human plasma.

New
Coming this year

**COAMATIC®
PLASMIN INHIBITOR**

A novel chromogenic kit for the determination of plasmin inhibitor (α_2 -antiplasmin) activity in human plasma. No interference from α_2 -macroglobulin in the assay system.

Measurement principle

Plasmin Inhibitor + Plasmin (excess) \longrightarrow
(Plasmin Inhibitor • Plasmin) + Plasmin (residual)

S-2403 $\xrightarrow{\text{Plasmin (residual)}}$ Peptide + pNA

Reagents and their stability when opened

Substrate S-2403	1 vial	6 months	2-8°C
Plasmin solvent	1 vial	exp. date	
Buffer stock solution	2 vials	exp. date	
Plasmin	1 vial	1 month	2-8°C

Number of determinations

Microplate method	200
Test tube method	50
Automated methods	125-200

Article number: 82 3187 63

No interference from other plasmin inhibitors. Validated and documented protocols for a wide range of instruments.

**COAMATIC®
PLASMINOGEN**

A chromogenic kit for the determination of plasminogen activity in human plasma. Addition of plasminogen-free fibrinogen to the streptokinase reagent overcomes the over estimation of plasminogen which may arise in patients with elevated levels of FDP or fibrinogen.

Measurement principle

Plg + Sk/Fib \longrightarrow [Plg • Sk/Fib]

S-2403 $\xrightarrow{[\text{Plg} \bullet \text{Sk/Fib}]}$ Peptide + pNA

Reagents and their stability when opened

Substrate S-2403	2 vials	6 months	2-8°C
Streptokinase/fibrinogen	2 vials	1 month	2-8°C

Number of determinations

Test tube method	50
Microplate method	200
Automated methods	up to 200

Article number: 82 2452 63

Not sensitive to elevated concentrations of FDP or fibrinogen in plasma. Validated and documented protocols for a wide range of instruments.

ANTITHROMBIN (RAPID SINGLE TEST) HEPARIN (RAPID SINGLE TEST)

COACUTE® ANTITHROMBIN R

A chromogenic kit for the determination of antithrombin activity in human plasma. The use of factor Xa in preference to thrombin eliminates interference from heparin cofactor II. Co-lyophilised reagents in ready-to-use cuvettes.

Measurement principle

AT + Heparin (excess) → [AT • Heparin]

[AT • Heparin] + FXa → [FXa • AT • Heparin]
S-2772 → Peptide + pNA

Reagents and their stability when opened

FXa and substrate S-2772	2x5 cuvettes	1 month	2-8°C
Buffer with heparin	10 tubes	exp. date	2-8°C
Stopper solution	1 vial	exp. date	2-8°C

Number of determinations

10

Article number: 82 2122 63

Method principle based on FXa inhibition. Allows accurate determination of antithrombin activity in patients receiving heparin therapy.

COACUTE® HEPARIN

A chromogenic kit for the determination of heparin and low molecular weight (LMW) heparin in human plasma. Co-lyophilised reagents in ready-to-use cuvettes.

Measurement principle

Heparin + AT (excess) → [AT • Heparin]

[AT • Heparin] + FXa → [FXa • AT • Heparin]
S-2732 → Peptide + pNA

Reagents and their stability when opened

FXa, antithrombin and substrate S-2732	2x5 cuvettes	1 month	2-8°C
Buffer	10 tubes	exp. date	2-8°C
Stopper solution	1 vial	exp. date	2-8°C

Number of determinations

10

Article number: 82 1660 63

Anti-FXa method. Flexible kit for laboratories monitoring all types of heparins.

Ideal for
STAT
testing

BACK-UP ASSAY

Used by laboratories as a backup assay.

CONFIRMATORY ASSAY

Used by laboratories as a confirmatory assay when the results from their routine assay are uninterpretable or erroneous.

REMOTE SITE ASSAY

Used by laboratories in remote sites.

LOW VOLUME ASSAY

For laboratories performing only a small number of tests.

PRE-CALIBRATED ASSAYS MONOTEST

No reagent waste
Calibration curve indicated in the kit package

Coacute®
Keep one in the refrigerator at all times, you never know when you will need it.

CALIBRATION PLASMA
Composition

Lyophilised human plasma prepared from a pool of citrated plasma collected from healthy donors.

Application

For construction of calibration curves using the assigned value for each analyte (batch specific values provided). The following analytes are determined using chromogenic assays: antithrombin, plasmin inhibitor, plasminogen, protein C, factor X, factor VIII. The following analytes are determined using clot assays: Factor II, V, VII, VIII, IX, X, XI and XII. Protein S-free and VWF are determined using latex assay. Values for all analytes are within the normal range.

Package 10x1 ml

Article number: 82 3534 63

CONTROL PLASMA - NORMAL
Composition

Lyophilised human plasma prepared from a pool of citrated plasma collected from healthy donors.

Application

For quality control of the following parameters measured with chromogenic assays: antithrombin, plasmin inhibitor, plasminogen, protein C, factor X, factor VIII. For quality control of the following clot based assays: Factor II, V, VII, VIII, IX, X, XI and XII. Protein S-free is determined with latex assay. Values for all analytes are within the normal range.

Package 10x1 ml

Article number: 82 3542 63

CONTROL PLASMA - ABNORMAL LEVEL 1 & 2
Composition

Lyophilised human plasma prepared from a pool of citrated plasma collected from healthy donors.

Application

For quality control of the following parameters measured with chromogenic assays: antithrombin, plasmin inhibitor, plasminogen, protein C, factor X, factor VIII. Protein S-free and VWF are determined with latex assay. Values for Level 1 and Level 2 are in ranges of 50-60% and 20-30% of normal values, respectively.

Package	Level 1	5x0.5 ml
	Level 2	5x0.5 ml

Article number: 82 3559 63

CALIBRATION PLASMA-LMW HEPARIN

Composition

Lyophilised human plasma, prepared by addition of heparin. Calibrated against the 1st International WHO standard (LMW heparin).

Application

For construction of calibration curves for use in chromogenic heparin assays.

Package	calibrator 1	4x1 ml
	calibrator 2	4x1 ml
	calibrator 3	4x1 ml

Article number: 82 3500 63

CONTROL PLASMA-LMW HEPARIN

Composition

Lyophilised human plasma prepared by addition of LMW heparin. Calibrated against the 1st International WHO standard (LMW heparin) .

Application

Quality control of chromogenic heparin assays.

Package	Low	4x1 ml
	High	4x1 ml

Article number: 82 3492 63

D-DIMER CONTROLS

Composition

Low and High D-Dimer Controls are prepared by means of a dedicated process and contain different concentration of partially purified D-Dimer obtained by digestion of Factor XIIIa cross-linked human fibrin with human plasmin.

Application

For quality control (precision and accuracy) of D-Dimer assay performed with the COAMATIC® D-Dimer kit. Control at border-line and abnormal levels.

Package	Low	5 x 0.5 ml
	High	5 x 0.5 ml

Article number: 82 4037 63

CONTROL PLASMA LEVEL 1 COATEST® APC™ RESISTANCE

Composition

Lyophilised citrated, stabilised human plasma prepared from pools of plasma collected from healthy donors.

Application

Quality control of Coatest® APC™ Resistance and Coatest® APC™ Resistance V.

Package	5x1 ml
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Article number: 82 2650 63

CONTROL PLASMA LEVEL 2 COATEST® APC™ RESISTANCE

Composition

Lyophilised citrated, stabilised human plasma prepared from pools of plasma collected from donors carrying the Factor V:Q⁵⁰⁶ mutation.

Application

Quality control of Coatest® APC™ Resistance and Coatest® APC™ Resistance V.

Package	5x1 ml
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Article number: 82 2668 63

ANTITHROMBIN 10 IU

Composition and purity

Lyophilised powder prepared from human plasma after affinity chromatography on heparin-Sepharose gel. Contains human albumin as a stabiliser.

Included in Coatest® Heparin

Package 10x10 IU

Note: This preparation is not a standard.

Article number: 82 0720 39

ANTITHROMBIN 25 IU

Composition and purity

Lyophilised powder prepared from human plasma after affinity chromatography on heparin-Sepharose gel. Pure preparation, does not contain stabiliser.

Package 1x25 IU

Note: This preparation is not a standard.

Article number: 81 0796 39

FX

Composition and purity

Lyophilised powder prepared from bovine plasma and purified by barium citrate adsorption and liquid chromatography. Contains buffer salts.

Application

For use in chromogenic factor VII assays and for other studies where a highly purified factor X is needed.

Package 1x2 U

1 unit corresponds to the normal content in 1 ml bovine plasma.

Article number: 82 2239 63

PLASMIN

Composition and purity

Lyophilised powder of human plasmin prepared by activation of plasminogen with a matrix bound activator. Contains phosphate buffer salts, lysine and ϵ -aminocaproic acid.

Specific activity

24-40 CU/vial (batch specific insert enclosed).

Application

Fibrinolytic research reagent, including determination of plasmin inhibitor.

Package 1x2 mg

Article number: 81 0655 39

FXa

Composition and purity

Lyophilised powder prepared from bovine plasma and purified by barium citrate adsorption and liquid chromatography. Activation is performed by matrix bound activator from Russell's Viper Venom. Contains buffer salts, albumin and polyethyleneglycol. The activity (71 nkat) is determined with the substrate S-2222.

Included in Coatest® Heparin

Package 10x71 nkat

Article number: 82 0985 39

PROTHROMBIN ACTIVATOR DILUENT

Composition

0.05 mol/l Tris•HCl pH 7.6, I=0.15 with NaCl, bovine serum albumin, polyethylene glycol and a fibrin polymerization inhibitor.

Application

For use in prothrombin methods.

Package 1x20 ml

Article number: 82 3526 63

PLASMINOGEN

Composition and purity

Lyophilised powder of human plasminogen prepared from Cohn supernatant by affinity chromatography. At least 95% of the plasminogen content is Glu-plasminogen. Contains lysine, sodium chloride and glucose.

Specific activity

20-40 CU/vial (batch specific).

Application

Determination of streptokinase, tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI) and soluble fibrin.

Package 1x1.5 mg

Article number: 81 0663 39

TISSUE PLASMINOGEN ACTIVATOR (t-PA)

Composition and purity

Lyophilised powder of 10 µg (at least 5,000 IU) tissue plasminogen activator. Prepared from human melanoma cell culture. More than 95% of the enzyme is in the native, one-chain form. The fibrinolytic activity is expressed in International Units (IU) assessed by calibration against the International Standard for t-PA, National Institute for Biological Standards and Control, London, UK.

Application

As a standard in the methods for the determination of t-PA activity and as a reagent in the methods for the determination of plasminogen activator inhibitor 1 (PAI-1) and soluble fibrin.

Package 1x10 µg

Article number: 82 1157 39

t-PA STIMULATOR

Composition and purity

Lyophilised powder of cyanogen bromide digested human fibrin(ogen).

Application

As a reagent for use in t-PA dependent plasminogen activation.

Package 1x3 mg

Article number: 82 2130 63

THROMBIN

Composition and purity

Thrombin prepared from bovine plasma. Contains buffer salts and bovine albumin. The activity (53 nkat) is determined with the substrate S-2238.

Package 10x53 nkat

Note: This preparation is not a standard.

Article number: 82 0712 63

TRIS BSA BUFFER**Composition**

0.5 mol/l Tris•HCl pH 7.3, I=2.0 with NaCl and 2% bovine serum albumin.

Application

Sample diluent buffer.

Package 1x20 ml (concentrate)

Article number: 82 3518 63

TRIS EDTA BUFFER**Composition**

0.5 mol/l Tris pH 8.4, 1.5 mol/l NaCl, 70 mmol/l disodium - EDTA.

Application

Buffer for chromogenic determination of heparin, FX, thrombin inhibitors, FXa inhibitors.

Package 1x10 ml (concentrate)

Article number: 82 3666 63

I-2882

Fibrin polymerization Inhibitor.

Application

TFPI Research Method.

Package 1x40 mg

Article number: 82 3815 10



S-2222™

Chromogenic substrate for factor Xa.

Package 25 mg

Article number: 82 0316 39

S-2238™

Chromogenic substrate for thrombin.

Package 25 mg

Article number: 82 0324 39

S-2251™

Chromogenic substrate for plasmin and streptokinase-activated plasminogen.

Package 25 mg

Article number: 82 0332 39

S-2266™

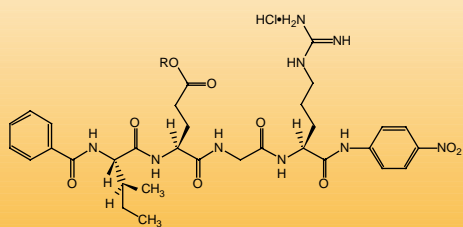
Chromogenic substrate for glandular kallikreins and factor XIa.

Package 25 mg

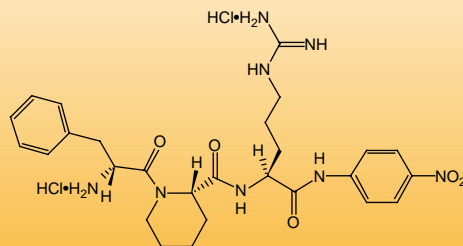
Article number: 82 0480 39


S-2222

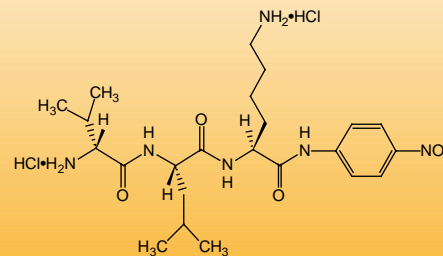
Formula: Bz-Ile-Glu(γ -OR)-Gly-Arg-pNA•HCl
 R=H (50%) and R=CH₃ (50%)
 Molecular weight: 741.3


S-2238

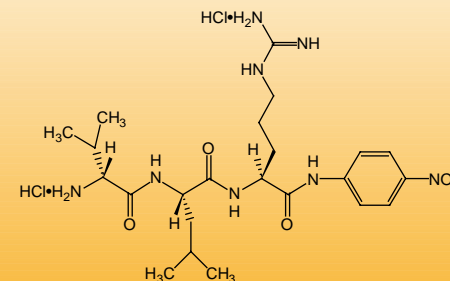
Formula: H-D-Phe-Pip-Arg-pNA•2HCl
 Molecular weight: 625.6


S-2251

Formula: H-D-Val-Leu-Lys-pNA•2HCl
 Molecular weight: 551.6


S-2266

Formula: H-D-Val-Leu-Arg-pNA•2HCl
 Molecular weight: 579.6



S-2288™

Chromogenic substrate for t-PA and a broad spectrum of other serine proteases.

Package 25 mg

Article number: 82 0852 39

S-2302™

Chromogenic substrate for plasma kallikrein and factor XIIa.

Package 25 mg

Article number: 82 0340 39

S-2314™

Chromogenic substrate for C1s.

Available on request.

S-2366™

Chromogenic substrate for activated protein C and factor XIa.

Package 25 mg

Article number: 82 1090 39


S-2288

Formula: H-D-Ile-Pro-Arg-pNA•2HCl
Molecular weight: 577.6


S-2302

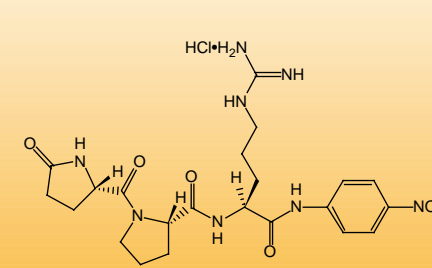
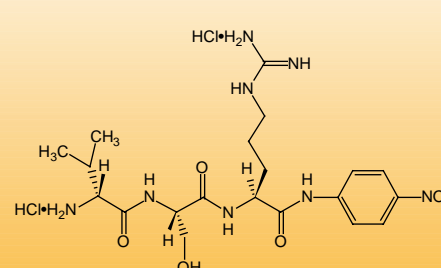
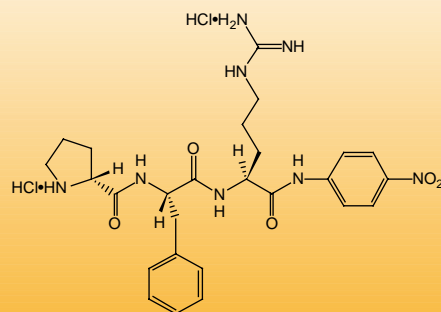
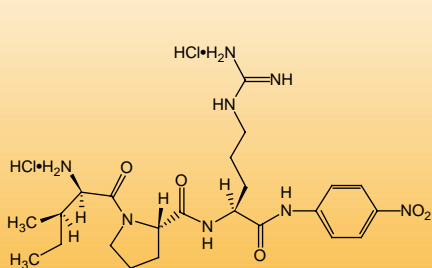
Formula: H-D-Pro-Phe-Arg-pNA•2HCl
Molecular weight: 611.6


S-2314

Formula: H-D-Val-Ser-Arg-pNA•2HCl
Molecular weight: 553.5


S-2366

Formula: pyroGlu-Pro-Arg-pNA•HCl
Molecular weight: 539.0



S-2390™

Chromogenic substrate for plasmin.

Available on request.

S-2403™

Chromogenic substrate for plasmin and streptokinase-activated plasminogen.

Package 25 mg

Article number: 82 2254 39

S-2406™

Chromogenic substrate for plasmin and streptokinase-activated plasminogen.

Available on request.

S-2444™

Chromogenic substrate for urokinase.

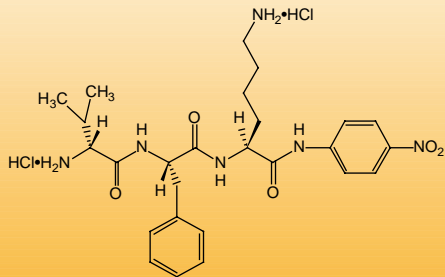
Package 25 mg

Article number: 82 0357 39



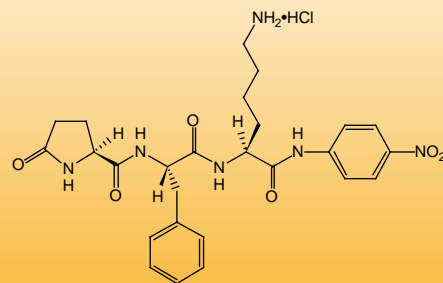
S-2390

Formula: H-D-Val-Phe-Lys-pNA•2HCl
Molecular weight: 585.6



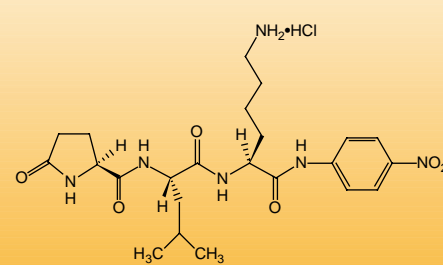
S-2403

Formula: pyroGlu-Phe-Lys-pNA•HCl
Molecular weight: 561.0



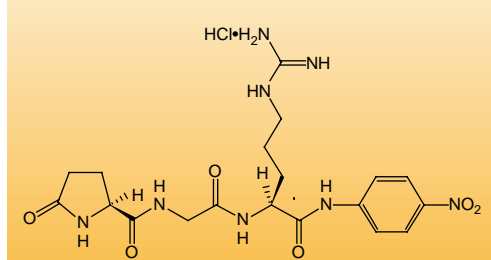
S-2406

Formula: pyroGlu-Leu-Lys-pNA•HCl
Molecular weight: 527.0



S-2444

Formula: pyroGlu-Gly-Arg-pNA•HCl
Molecular weight: 498.9



S-2468™

Chromogenic substrate for plasmin and streptokinase-activated plasminogen.

Available on request.

S-2484™

Chromogenic substrate for granulocyte elastase.

Package 25 mg

Article number: 82 0886 10

S-2586™

Chromogenic substrate for chymotrypsin.

Package 25 mg

Article number: 82 0894 39

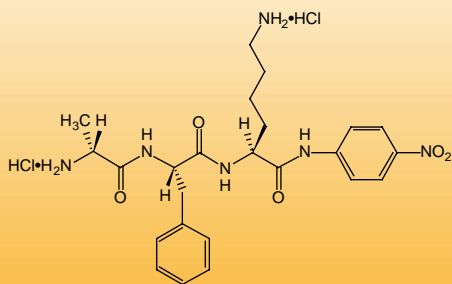
S-2732™

Chromogenic substrate for factor Xa.

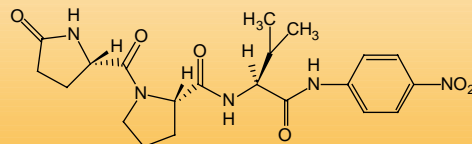
Available on request.


S-2468

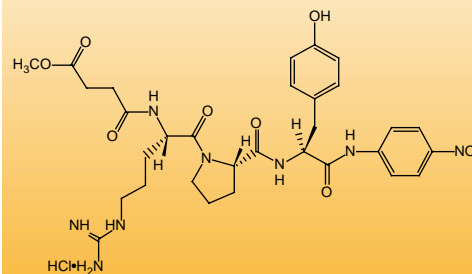
Formula: H-D-Ala-Phe-Lys-pNA•HCl
Molecular weight: 557.5


S-2484

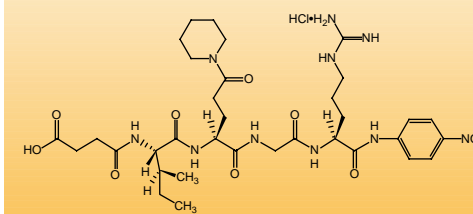
Formula: pyroGlu-Pro-Val-pNA
Molecular weight: 445.5


S-2586

Formula: MeO-Suc-Arg-Pro-Tyr-pNA•HCl
Molecular weight: 705.3


S-2732

Formula: Suc-Ile-Glu(γ-Pip)-Gly-Arg-pNA•HCl
Molecular weight: 797.3



S-2765™

Chromogenic substrate for factor Xa.

Package 25 mg

Article number: 82 1413 39

S-2767™

Chromogenic substrate for factor Xa.

Available on request.

S-2772™

Chromogenic substrate for factor Xa.

Package 26 mg

Article number: 82 3013 63

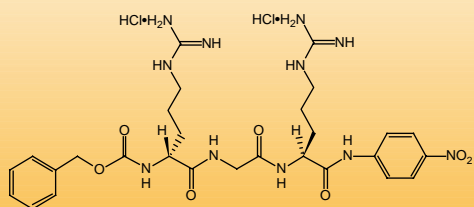
S-2782™

Chromogenic substrate for factor Xa.

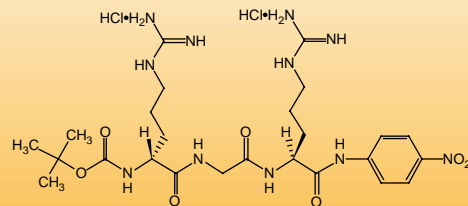
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S-2765

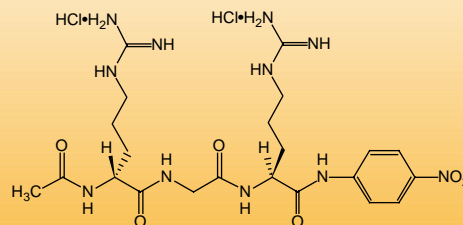
Formula: Z-D-Arg-Gly-Arg-pNA•2HCl
Molecular weight: 714.6


S-2767

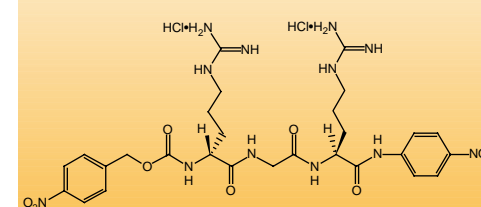
Formula: Boc-D-Arg-Gly-Arg-pNA•2HCl
Molecular weight: 680.6


S-2772

Formula: Ac-D-Arg-Gly-Arg-pNA•2HCl
Molecular weight: 622.5


S-2782

Formula: 4-Nz-D-Arg-Gly-Arg-pNA•2HCl
Molecular weight: 759.6



S-2787™

Chromogenic substrate for factor Xa.

Available on request.

S-2846™

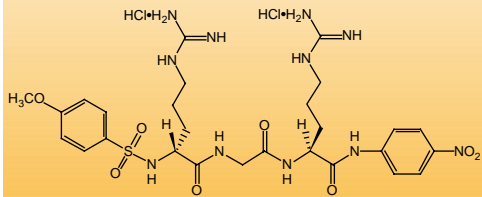
Chromogenic substrate for activated protein C.

Available on request.



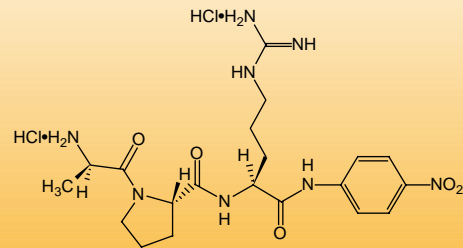
S-2787

Formula: 4-Mbs-D-Arg-Gly-Arg-pNA•2HCl
Molecular weight: 750.7

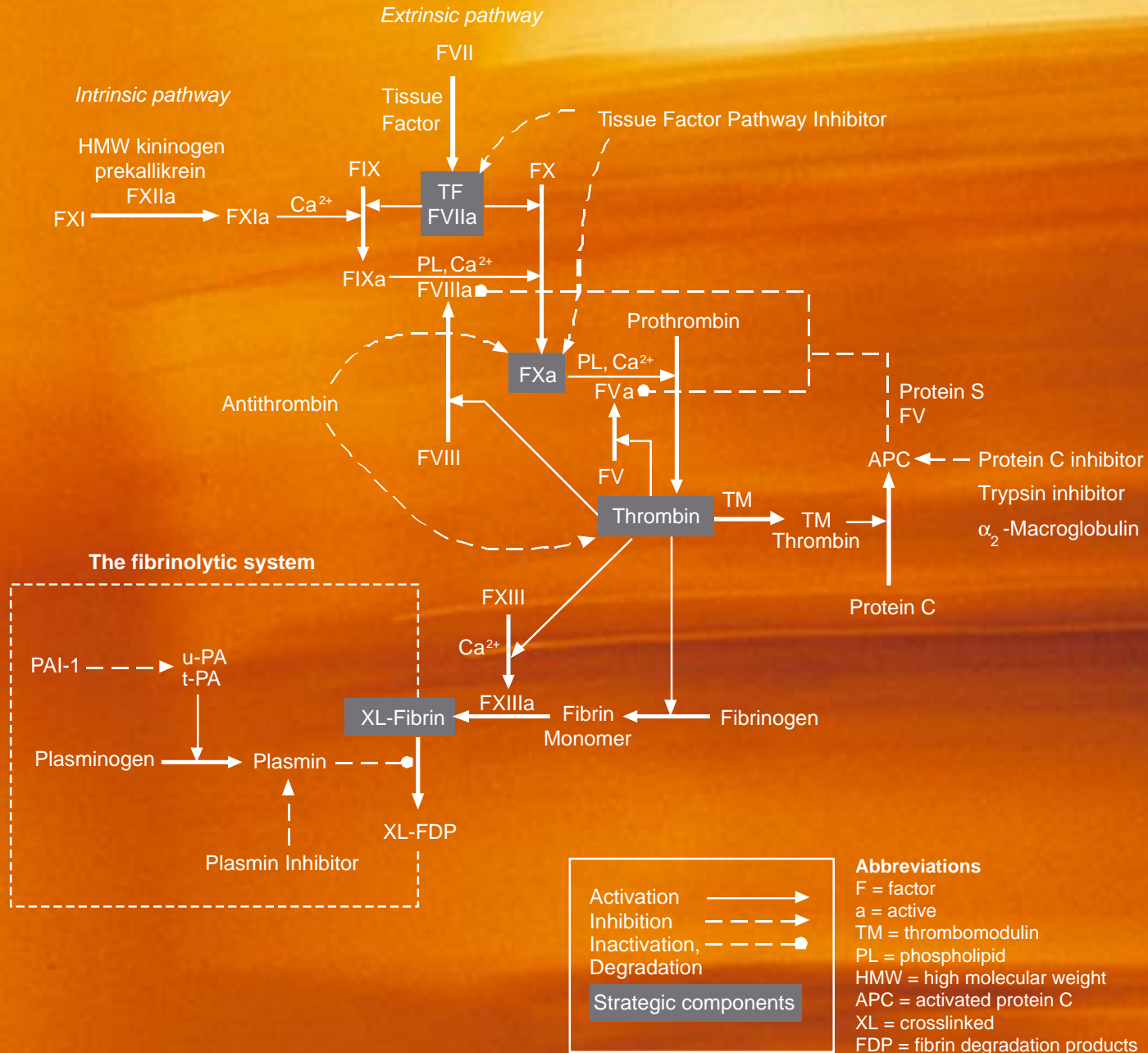


S-2846

Formula: H-D-Ala-Pro-Arg-pNA•2HCl
Molecular weight: 535.4



The coagulation cascade



WHAT IS A CHROMOGENIC SUBSTRATE?

Enzymes are proteins that catalyze most of the chemical reactions that take place in the body. They make it possible for chemical reactions to occur at neutral pH and body temperature. The chemical compound upon which the enzyme exerts its catalytic activity is called a substrate.

Proteolytic enzymes act on their natural substrates, proteins and peptides by hydrolyzing one or more peptide bond(s). This process is usually highly specific in the sense that only peptide bonds adjacent to certain amino acids are cleaved.

THE COLOR CHANGE IS PROPORTIONAL TO THE PROTEOLYTIC ACTIVITY

Chromogenic substrates are peptides that react with proteolytic enzymes under the formation of color. They are made synthetically and are designed to possess a selectivity similar to that of the natural substrate for the enzyme. Attached to the peptide part of the chromogenic substrate is a chemical group which when released after the enzyme cleavage gives rise to color. The color change can be followed spectrophotometrically and is proportional to the proteolytic activity.

The chromogenic substrate technology was developed in the early 1970s, and has since then become a tool of substantial importance in basic research.

The majority of chromogenic substrate applications are found in various clinical fields.

In particular they have been used to generate fundamental knowledge of the mechanisms regulating blood coagulation and fibrinolysis. Furthermore, products based on chromogenic substrate technology have brought a new generation of diagnostics into the clinical laboratory.

“Bells are ringing for the stop watch - electronic timers are taking over in sports, and the automated spectrophotometers in coagulation and fibrinolysis. The recent and rapid progress in the development of artificial chromogenic substrates is no less than a revolution in the laboratory evaluation of these systems.”
Helge Stormorken, 1976.¹

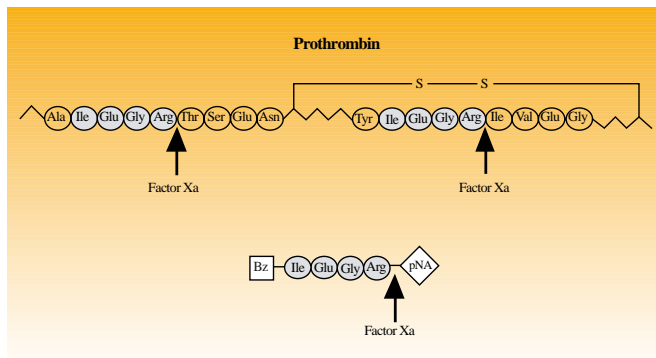


Figure 1. Prothrombin, the natural substrate of Factor Xa, is cleaved by Factor Xa at two positions, each preceded by the same four amino acid sequence. Factor Xa activity can be determined by the chromogenic substrate S-2222 which is composed of the same amino acids coupled to a chromophore.

PROTEOLYTIC ENZYMES

In the living organism, proteolytic enzymes (proteases) are produced to degrade and modify proteins. A main task for proteolytic enzymes is to degrade proteins into peptides or amino acids to be used either as an energy source or as building blocks for resynthesis of proteins. Furthermore, proteolytic enzymes modify cellular environments and facilitate cell migration in connection with wound repair and cancer, ovulation and implantation of the fertilized egg, embryonic morphogenesis, and involution of mammary glands after lactation.² Another important function of the proteases is their role as regulators in processes such as inflammation, infection and blood clotting. Most proteolytic enzymes are highly specific for their substrates. The classification of proteases, however, is not based on their choice of substrate but on their mechanism of action.³ Four different groups of proteolytic enzymes, named after

- ASPARTIC PROTEASES
- CYSTEINE PROTEASES
- SERINE PROTEASES
- METALLO PROTEASES

the active site amino acid residue responsible for the catalytic activity, are generally distinguished: the aspartic proteases (e.g. pepsin), the cysteine proteases (e.g. cathepsin B and cathepsin H), the serine proteases (e.g. trypsin, thrombin and plasmin) and metalloproteases (e.g. collagenases and gelatinases). Although the members of each group of proteolytic enzymes may have very diverse biological functions, amino acid analysis often shows a high degree of structural similarity between them. Detailed knowledge of the structure and mechanism of action of one enzyme can in many cases reveal an understanding of the structure and functions of other enzymes within the same group.

Classes of proteases	
name	active site
serine proteases	Ser His Asp*
cysteine proteases	Cys His Asp*
aspartic proteases	Asp Asp
metallo proteases	His His Zn ²⁺

*Asp not always present

Serine proteases

The most extensively studied group of proteolytic enzymes comprises the serine proteases.⁴ As indicated by the name each member of this group have a reactive seryl amino acid residue in its active site.

TRYPSINS SUBTILISINS

The serine proteases are divided into two families: the trypsin and the subtilisins. The trypsin family is the largest and contains, among others, trypsin and chymotrypsin, elastase, mast cell tryptase, and many of the factors regulating blood coagulation and fibrinolysis. The trypsin type of enzymes have a highly similar amino acid content. They are found in vertebrates and other animals, as well as in fungi and prokaryotic cells. In contrast, the subtilisins are only found in bacteria. Members of the trypsin family are classified according to the type of amino acid that occurs at the preferred cleavage site.



Elastase and chymotrypsin cleave after hydrophobic and aromatic amino acids, while other trypsin-like proteases cleave only at the C-terminal side of the basic amino acids arginine or lysine.

The amino acid sequence and thus also the three-dimensional structure differ completely between the trypsins and the subtilisins. The catalytically active domains of trypsin and subtilisin have therefore most probably evolved independently, converging from two different genes.

However, since the three amino acids of functional importance at the active sites, serine (Ser), aspartic acid (Asp) and histidine (His), are arranged in the same geometrical relationship in all members of the two families the proteolytic mechanisms are very similar.³

This fact may lead to the suggestion that the arrangement of the three catalytically active amino acids at the active site is very efficient for hydrolysis of peptide bonds.

Mammalian serine proteases are usually synthesized as inactive proenzymes, zymogens, consisting of a single peptide chain. Activation occurs when the zymogen is cleaved at one or several specific sites.

Most commonly such cleavage is accomplished by the action of another protease.

Most serine proteases contain two functionally distinct parts.⁵ The region where the catalytically active amino acids are found is very similar in trypsin and chymotrypsin as well as in the serine proteases involved in blood coagulation.

FOUR MAIN TYPES OF STRUCTURAL DOMAINS

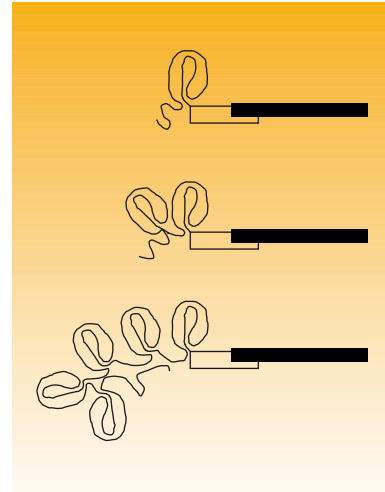
The other region is located in the exterior parts of the enzyme.

This region is of considerable size in the serine proteases regulating

blood coagulation and fibrinolysis and four main types of structures can be distinguished: kringle domains, growth factor domains, vitamin K dependent carboxylated calcium binding domains, and domains homologous to the finger structure of fibronectin.⁶⁻⁸ All four domain types are not present in all groups of serine proteases.

Figure 2. Schematic representation of mammalian serine proteases.

The catalytically active part (black bar in the figure) is homologous to trypsin and highly conserved in all mammalian serine proteases. The number of kringle modules varies between different serine proteases, e.g. one kringle in urokinase, two kringles in prothrombin, five kringles in plasminogen.



The proteolytic mechanism of serine proteases

The bond-breaking reaction exerted by a serine protease on its substrate is the result of an interaction between the substrate and the charge relay network of the enzyme.^{10,11} This network, which is present in the active site of all serine proteases, is known as the catalytic triad. It is built up from the side-chains of three specific amino acids (the hydroxy group of serine, the imidazole group of histidine and the carboxylic acid group of aspartic acid) that interact with each other through an array of hydrogen bonds.

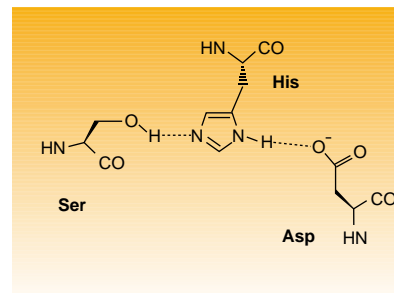


Figure 3. Charge relay network of serine proteases.

The proteolytic action of a serine protease on its substrate comprises several steps starting with the formation of a non-covalent complex between the enzyme and the substrate. A nucleophilic attack by the serine hydroxyl group on the amide carbonyl carbon atom in the substrate results in cleavage of the amide bond and the formation of an acyl-enzyme intermediate.^{12,13}

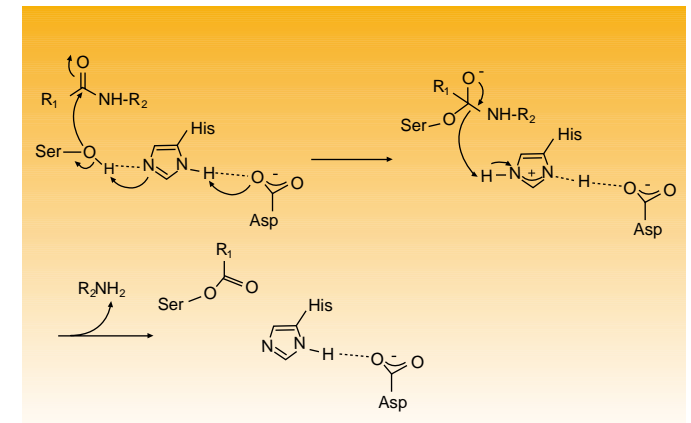


Figure 4. Formation of an acyl-enzyme intermediate.

The acyl-enzyme ester bond is then hydrolysed in the rate limiting step and the enzyme is now free to catalyze the cleavage of another substrate molecule.

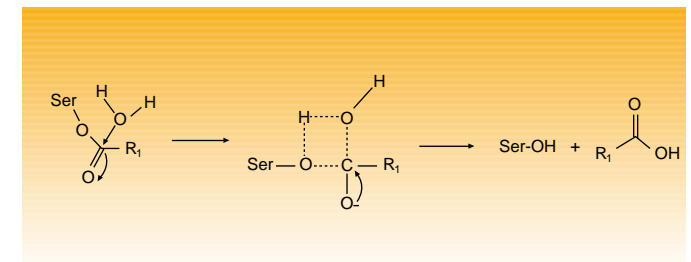


Figure 5. Hydrolysis of the acyl-enzyme intermediate.

Enzyme specificity and substrate selectivity

Specificity is a property of the enzyme and describes how restrictive the enzyme is in its choice of substrate; a completely specific enzyme would have only one substrate.¹⁴

The specificity of the serine proteases is usually not very high since they have similar active sites and act through the same proteolytic mechanism. Consequently, a single serine protease may act on various substrates although at different rates. How the substrate fits the active site of the enzyme is of crucial importance to the outcome of the enzyme-substrate reaction.

The bond to be cleaved must have a specific orientation relative to the amino acid side chains of the catalytic triad.

The most important factor governing the fit of a substrate for an enzyme is the amino acid sequence around the bond to be cleaved. Trypsin cleaves amides and esters of the basic amino acids arginine and lysine. Thrombin has a similar preference, but is more specific for arginine than for lysine.

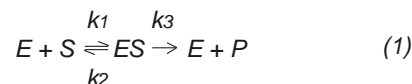
Selectivity is a property of the substrate and indicates the degree to which the substrate is bound to and cleaved by different enzymes. The best measure for selectivity is given by the ratio k_{cat}/K_m (see below).

Synthetic substrates are considerably smaller than the natural substrates and can usually be cleaved by more than one enzyme, i. e. synthetic substrates are not completely selective. The explanation for this is that large substrates such as fibrinogen not only interact with the active site but also with exterior domains of the enzyme. Such interactions allow substrates to discriminate between different serine proteases and fibrinogen thus becomes highly selective for thrombin.

Enzyme kinetics

An enzyme acts as a catalyst for a certain chemical reaction. This means that the enzyme decreases the activation energy and reaction time for the reaction without changing the equilibrium. The enzyme is neither consumed nor modified in the reaction. An enzyme-catalyzed reaction starts with the formation of an enzyme-substrate complex, ES.

The complex has two possible outcomes. It can dissociate to free enzyme (E) and substrate (S) with a rate constant k_2 , or it can proceed to form product (P) with a rate constant k_3 . At a fixed concentration of the enzyme the product is formed at a rate linearly proportional to the substrate concentration. However, after saturation of the active site of each enzyme molecule with its substrate the rate of product formation is independent of the substrate concentration, [S]. The equilibrium in (1) can be pushed towards product formation by increasing the substrate concentration.



Abbreviations: E; enzyme, S; substrate, ES; enzyme-substrate complex, P; product, V; rate of formation of product, k_n ($n = 1, 2, 3$); rate constant, [N]; concentration of reactant N.

The rate of product formation depends on the concentration of enzyme-substrate complex.

$$V = k_3[ES] \quad (2)$$

The rates of formation and consumption of ES can be written as:

$$\text{rate of formation of ES} = k_1[E][S] \quad (3)$$

$$\text{rate of consumption of ES} = (k_2 + k_3)[ES] \quad (4)$$

At steady state, [ES] is constant and the rates in (3) and (4) are equal:

$$k_1[E][S] = (k_2 + k_3)[ES] \quad (5)$$

Rearrangement of (5) gives an expression for [ES] at steady state:

$$[ES] = \frac{[E][S]}{\frac{k_2 + k_3}{k_1}} \quad (6)$$

The Michaelis constant, K_m , is defined below:

$$K_m = \frac{k_2 + k_3}{k_1} \quad (7)$$

The concentration of free enzyme [E] equals the total enzyme concentration minus the concentration of enzyme-substrate complex:

$$[E] = [E_{tot}] - [ES] \quad (8)$$

Substitution of (7) and (8) into (6) gives:

$$[ES] = \frac{([E_{tot}] - [ES])[S]}{K_m} = [E_{tot}] \frac{[S]}{[S] + K_m} \quad (9)$$

Substituting this expression for [ES] in (2) gives:

$$V = k_3[E_{tot}] \frac{[S]}{[S] + K_m} \quad (10)$$

The reaction reaches its maximal rate, V_{max} , when all active sites of the enzyme are occupied, i.e. when [S] is much larger than K_m , which means that $\frac{[S]}{[S] + K_m}$ approaches 1.

Thus,

$$V_{max} = k_3[E_{tot}] \quad (11)$$

Under these conditions product molecules are formed at a constant and maximal velocity. Equation (11) can also be written as:

$$k_3 = \frac{V_{max}}{[E_{tot}]} = k_{cat} \quad (12)$$

k_3 reflects the turnover number, which is the maximal number of substrate molecules that can be converted to product per time unit, dimension time^{-1} .

The Michaelis-Menten equation (13) is obtained from (10) and (11) and explains the relationship between reaction rate and substrate concentration as shown in Figure 6.

$$V = V_{max} \frac{[S]}{[S] + K_m} \quad (13)$$

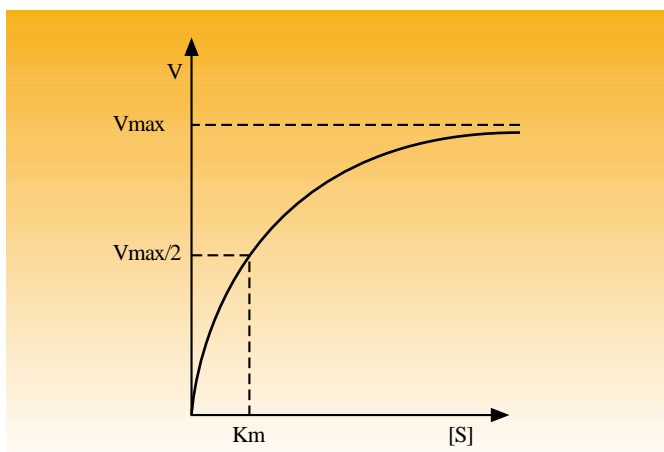


Figure 6. Reaction rate V versus substrate concentration $[S]$ for an enzyme-catalyzed reaction.

At a low substrate concentration ($[S] \ll K_m$), then

$$V = V_{max} \frac{[S]}{K_m} \quad (14)$$

i.e. the rate is directly proportional to $[S]$. This means that an increase in substrate concentration will cause an increase in the reaction rate.

On the other hand, at a high substrate concentration ($[S] \gg K_m$), then

$$V = V_{max} \quad (15)$$

i.e. the reaction rate is independent of $[S]$.

A special situation can be identified when the substrate concentration equals K_m ($[S] = K_m$); then

$$V = \frac{V_{max}}{2} \quad (16)$$

which means that the Michaelis constant K_m is equal to the substrate concentration at which the reaction rate is exactly half the maximum rate or $V_{max}/2$.

The Michaelis-Menten equation (13) is transformed into an equation giving a straight line plot by taking the reciprocal of both sides of the equation.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} [S]} \quad (17)$$

A plot of $1/V$ versus $1/[S]$ is called the Lineweaver-Burk plot or double-reciprocal plot, and has the intercept $1/V_{max}$ and the slope K_m/V_{max} . Thus, the kinetic parameters K_m and V_{max} are readily derived by measuring the rate of catalysis at different substrate concentrations.

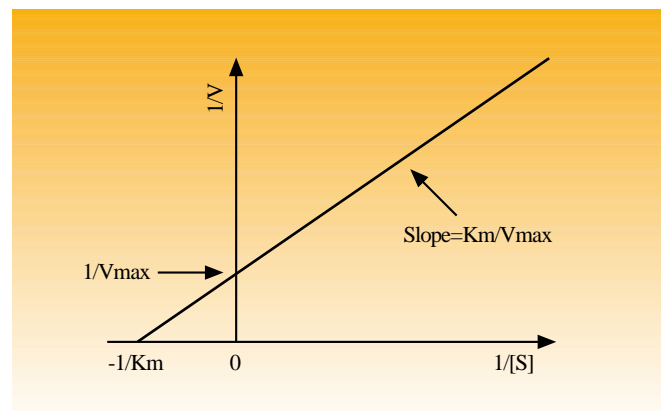


Figure 7. The Lineweaver-Burk plot.

Multiplication of both sides of equation (17) with the substrate concentration $[S]$ gives

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \quad (18)$$

The plot of $[S]/V$ versus $[S]$ is sometimes referred to as the Hanes plot. The advantage of the Hanes plot over the Lineweaver-Burk plot is that the experimental errors in V give in the former a more or less constant contribution over a wide range of $[S]$ values (15). Consequently, more accurate results are obtained using the Hanes procedure for data treatment.

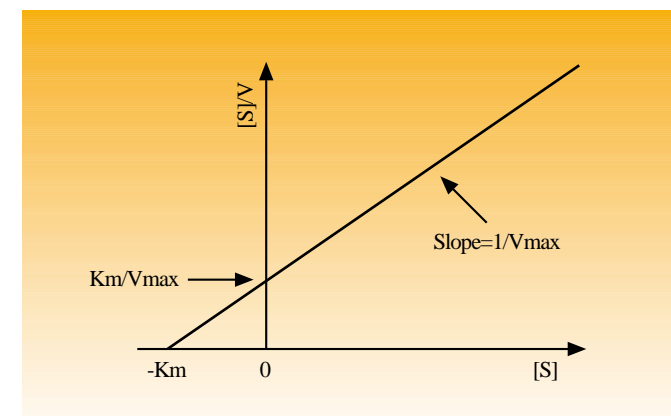


Figure 8. The Hanes plot.



CHROMOGENIC SUBSTRATES IN PRACTICE

Measurements made using chromogenic substrates reflect enzyme activity. Often it is more important to have knowledge about the activity of an enzyme than of the amount or mass - the quantity recorded in an immunological assay.

Synthetic substrates are very sensitive, i.e. they can detect very low enzyme activities. They are in fact often more sensitive than a corresponding natural substrate.

This ability of chromogenic substrates to detect low enzyme concentrations makes them useful in, for example, the search for the presence of certain enzyme activities either in research or in quality control procedures. Sometimes there is a lack in correspondence between a natural and a chromogenic substrate in their responses to a certain enzyme preparation. For example, thrombin that has been partly degraded through autohydrolysis (β -thrombin) reacts just as well with its chromogenic substrate as does the native form of thrombin (α -thrombin) while only native thrombin reacts with the natural substrate fibrinogen.

SETTING UP CHROMOGENIC SUBSTRATE ASSAYS

A chromogenic substrate is less selective, i.e. it has less discrimination in its reactivity towards related enzymes compared to the natural substrate. However, this lack of absolute selectivity can be compensated for when setting up chromogenic substrate assays. This is done by the proper selection of type of buffer, pH, relative concentrations of sample and reagents, addition of inhibitors, and/or choice of activator or incubation times. When presented with the opportunity of using one or more chromogenic substrates in a particular experimental setting for which there is no existing method, there are a few considerations that are worthwhile to make.

Substrate: If the specificity of the enzymatic activity to be measured is known then comprehensive overviews such as the Tables 1 and 3 on page 42-45 will serve as a guide in selecting a proper substrate. The local distributor of Chromogenix products may also be contacted for advice on the choice of substrate(s). If the specificity of the enzyme is unknown, a screening

procedure can be applied. When different substrates are available, such screening of the enzyme specificity can be carried out by comparing the rate of hydrolysis or pNA-generation obtained with the different substrates.

SCREENING PROCEDURE

Unless certain experience is available to the investigators it is usually advisable to discuss the plan and/or the result with Chromogenix. Advice on the next step can thus be given concerning either continued screening or the selection of a particular substrate that is suitable in the planned investigation.

Contaminating enzymes: If the sample to be tested with a chromogenic substrate contains more than one enzyme that may react with the same substrate, there are a number of measures that can be taken in order to eliminate the interfering/contaminating activity. A natural or synthetic inhibitor can be introduced, the sample can be further diluted or conditions can be found (different pH and/or buffer) where the relative activities of the present enzymes are optimized. Such considerations can be based on the information below concerning temperature, pH, buffer and ionic strength. Furthermore, for a selection of serine proteases from the four cascade systems in blood, their reactivities with different substrates under different conditions are shown in Table 4.

Temperature: The rate by which the chromogenic substrate is cleaved is highly dependent on the temperature. It is therefore important to know at what temperature(s) a particular method is applicable - it may be at room (ambient) temperature, 25, 30, or 37 °C.

An increase in temperature of 1 °C causes an increase in the reaction velocity of 2.5-7.5%. The temperature thus must be kept constant during the measurement and if results from different experiments are to be compared they must be performed at the same temperature. It is advisable to run the reactions in thermostated cuvettes and to use preheated stock solutions.

pH: Both K_m and k_{cat} are dependent on the pH. This means that kinetic calculations can only be made using results obtained at the same pH.

Usually, the enzyme activity is measured at the pH optimum for the proteolytic activity of the enzyme. However, when several proteases are present in the same solution, as, e.g. when the sample is from plasma, it is not always advantageous to search for the pH that gives the maximum reactivity of the enzyme under investigation. Instead it is better to choose a pH where other serine proteases that may compete for the substrate have relatively lower levels of activity.

Buffers: The buffer medium and the concentration of buffer substances must be well defined.

Usually Tris-HCl is used since the pKa of Tris buffer is 8.1 (25 °C), which makes it suitable for measurements at pH values between 7.3-9.3, where most of the serine proteases show maximal activities. Furthermore, this buffer is stable - it can even be autoclaved. Tris-imidazole has also been used, but is not to be recommended as imidazole is known to slightly inhibit certain proteases such as trypsin and plasmin.

Ionic strength and other additives: The appropriate ionic strength is usually obtained by adjusting the concentration of NaCl. Further substances that it may be necessary to add are $CaCl_2$ (when Ca-dependent enzymes are studied), NaN_3 (or other bactericidal agents) to prevent bacterial growth and polyethylene glycol or Tween 80 to prevent adsorption of the enzymes to the reaction vessel walls.

Substrate handling: The substrate solution is usually prepared by adding sterile water to the dry powder. Substrates with low solubility in water can be dissolved in DMSO (dimethyl sulfoxide) and then diluted in water. The final DMSO concentration should preferably not exceed 10% in the reaction mixture.

STABILITY OF SUBSTRATE SOLUTIONS

Substrates dissolved in sterile water are stable for more than 6 months in the refrigerator (2 - 8 °C) and for several weeks at room temperature (25 °C). The stability is considerably reduced in alkaline buffers. Furthermore, contamination by microorganisms and exposure to light for longer periods should be avoided. The substrate concentration should be chosen so that linear kinetics is obtained. A substrate concentration of twice the K_m ($2 \times K_m$) is usually appropriate.

Table 1. Substrate selectivity. The selectivity data of the table have been compiled to permit the investigator to understand how a contaminating enzyme would influence the enzyme-substrate reaction under study. Another way of expressing this is to say that the table shows the relative reactivities of two or more enzymes on one particular substrate. The table should be read horizontally. Each row represents the reactivity of a substrate designated for use with a particular enzyme, indicated to the left, relative to other relevant enzymes.

Example: The set of data in the top row shows the relative reactivity of the thrombin substrate S-2238 with various enzymes. All the experiments were performed using the same buffer, i.e. the one most appropriate for the reaction between thrombin and S-2238. In addition, the substrate concentration was always the same, or 2 x Km for the reaction of S-2238 with thrombin. The concentrations of the different enzymes are given in Table 2 and are related to the plasma concentration of the corresponding zymogen. The reactivity of S-2238 with thrombin, measured as the time-dependent increase in absorbance ($\Delta A/\text{min}$), is given the value 100% (the actual value of $\Delta A/\text{min}$ is given in brackets). The reactivities of S-2238 with the enzymes FXa, FXIa, APC, plasmin, single chain t-PA, plasma kallikrein, and C1s have then been related to the reactivity of S-2238 with thrombin, and proved to be 5, 5, 40, 5, 5, 60, and 2%, respectively. For information on buffers and Km values, see table 3.

Enzyme	Substrate	Thrombin (B)	FXa (B)	FXIa (H)	APC (H)	Plasmin (H)	Single chain t-PA (H)	Plasma Kallikrein (H)	C1s (H)	Buffer		Substrate conc 2xKm (mM)
										50 mM pH	Tris HCl NaCl (mM)	
Thrombin	S-2238	100 (0.11)	5	5	40	5	5	60	2	8.3	130	0.20*
	S-2366	100 (0.14)	5	35	80	70	3	130	2			0.29
	S-2846	100 (0.078)	3	5	30	5	1	30	2			0.090
FXa	S-2222	1	100 (0.34)	2	0	2	2	5	1	8.3	130	0.80
	S-2337	1	100 (0.37)	1	0	2	2	3	1			0.60
	S-2732	1	100 (0.51)	1	0	2	1	3	0			0.70
	S-2765	0	100 (0.61)	1	2	1	5	15	1			0.22
	S-2767	1	100 (0.53)	1	2	1	5	3	1			0.44
	S-2772	1	100 (0.32)	1	2	1	4	4	5			1.4
	S-2782	0	100 (0.63)	2	1	1	10	10	1			0.30
	S-2787	0	100 (0.45)	1	1	1	10	10	2			0.28
FXIa	S-2288	130	290	100 (0.077)	-	-	-	760	75	8.3	130	1.8
	S-2366	150	35	100 (0.14)	-	-	-	360	10			2.4
APC	S-2288	80	30	25	100 (0.13)	15	-	170	-	8.3**	-	0.32
	S-2366	75	4	30	100 (0.19)	60	-	110	-			0.40
	S-2846	70	4	15	100 (0.16)	20	-	80	-			0.70

B=bovine H=human

* Substrate conc 20xKm
** Buffer see table 2



Enzyme	Substrate	Thrombin (B)	FXa (B)	FXIa (H)	APC (H)	Plasmin (H)	Single chain t-PA (H)	Plasma Kallikrein (H)	C1s (H)	Buffer		Substrate conc 2xKm (mM)
										50 mM Tris HCl pH	NaCl (mM)	
Plasmin	S-2251	4	3	-	2	100 (0.050)	2	-	-	7.4	110	0.80
	S-2302	15	140	-	40	100 (0.071)	1	-	-			1.0
	S-2366	80	10	-	70	100 (0.17)	4	-	-			0.80
	S-2390	1	2	-	2	100 (0.039)	0	-	-			0.15
	S-2403	0	1	-	0	100 (0.14)	0	-	-			0.70
	S-2406	0	1	-	1	100 (0.23)	0	-	-			1.7
	S-2468	0	1	-	0	100 (0.024)	0	-	-			0.16
Single chain t-PA	S-2288	170	430	-	-	125	100 (0.058)	1100	-	8.3	130	2.0
	S-2765	3	590	-	-	15	100 (0.14)	110	-			1.8
	S-2782	2	490	-	-	3	100 (0.16)	45	-			2.4
Plasma Kallikrein	S-2266	5	3	2	15	-	-	100 (0.32)	0	7.8	16	1.0
	S-2288	35	20	10	25	-	-	100 (0.50)	2			1.2
	S-2302	3	10	0	5	-	-	100 (0.48)	5			0.44
	S-2366	20	5	25	40	-	-	100 (0.37)	10			1.2
C1s	S-2314	20	25	20	50	-	-	180	100 (0.11)	8.0	-	1.4
	S-2765	10	2300	60	15	-	-	610	100 (0.025)			1.0

B=bovine H=human

* Substrate conc 20xKm



Enzyme	Used conc. of enzyme, mg/l	Plasma conc. of zymogen, mg/l
Thrombin	0.040	150
FX_a	0.40	8
FXI_a	0.20	5.0
APC	0.16	4.0
Plasmin	0.50	200
Single chain t-PA	1.0	0.005
Plasma Kallikrein	0.80	50
C1s	2.0	50

Table 2. Enzyme concentrations used in the determination of relative reactivities. The enzyme concentrations used were in most cases 1/25 of the plasma concentrations of the corresponding zymogens.

Table 3. Kinetic data for the chromogenic substrates available from Chromogenix. Suitable chromogenic substrates are listed for a number of serine proteases, most of them part of the cascade systems in blood. Some of the substrates are cleaved by more than one enzyme although at different rates. The kinetic analyses of the enzymatic cleavage of pNA from the substrates were performed under strictly standardized conditions using the clinical chemistry analyser Cobas Mira S. A stable, well-defined temperature is vital for all enzyme kinetic studies and in this study all reactions were performed at 37 °C. A suitable buffer was chosen for each enzyme and the pH value given in the compilation is the value to which it was adjusted at 25°C. Note that the pH value of Tris buffers decreases as the temperature increases, at the rate of approximately 0.1 unit per °C (50 mM Tris-HCl). The kinetics of the reaction was followed spectrophotometrically by measuring the change in absorbance over time, ΔA/min. To ensure the highest precision, ΔA/min was measured at four different substrate concentrations. Insertion of the ΔA/min values into Eq. 18 on page 40, followed by linear regression gave K_m , k_{cat} and V_{max} for the reaction.

	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM·s)) · 10 ³	Enzyme concentration (mg/l) for ΔA/min=0.05 at [S]=2·K _m
Thrombin, human Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2238	0.0070	180	26	0.03
S-2366	0.15	330	2.2	0.02
S-2846	0.043	190	4.4	0.03
Thrombin, bovine Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2238	0.010	200	20	0.02
S-2366	0.15	295	2.0	0.02
S-2846	0.045	200	4.4	0.03
FXa, human Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl, 0.5% BSA				
S-2222	1.1	100	0.090	0.06
S-2337	0.67	110	0.16	0.05
S-2732	1.5	230	0.15	0.03
S-2765	0.26	240	0.92	0.02
S-2767	0.60	210	0.35	0.03
S-2772	1.5	120	0.080	0.05
S-2782	0.29	210	0.72	0.03
S-2787	0.38	170	0.45	0.03
FXa, bovine Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2222	0.40	100	0.25	0.06
S-2337	0.30	110	0.37	0.05
S-2732	0.35	130	0.37	0.05
S-2765	0.11	195	1.8	0.03
S-2767	0.22	160	0.73	0.04
S-2772	0.70	100	0.14	0.06
S-2782	0.15	190	1.3	0.03
S-2787	0.14	120	0.86	0.05



	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM·s)) *10 ⁻³	Enzyme concentration (mg/l) for $\Delta A/\text{min}=0.05$ at $[S]=2 \cdot K_m$
FXIa, human Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl and 0.5% BSA S-2288 S-2366	0.90 1.2	190 340	0.21 0.28	0.06 0.03
FXIa, bovine Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl and 0.5% BSA S-2266 S-2288 S-2302 S-2366	0.63 0.70 0.17 0.73	12 21 17 16	0.019 0.030 0.10 0.022	0.9 0.5 0.6 0.7
APC, human Buffer: 100 mM Tris HCl, pH 8.3, 260 mM CsCl, 4 mM CaCl ₂ and 0.2 % BSA S-2288 S-2366 S-2846	0.16 0.20 0.35	110 190 130	0.69 0.95 0.37	0.07 0.04 0.06
Plasmin, human Buffer: 50 mM Tris HCl, pH 7.4, 110 mM NaCl S-2251 S-2302 S-2366 S-2390 S-2403 S-2406 S-2468	0.40 0.50 0.40 0.075 0.35 0.85 0.080	20 26 60 15 60 70 7.4	0.050 0.052 0.15 0.20 0.17 0.082 0.092	0.4 0.3 0.1 0.5 0.1 0.1 1
One-chain t-PA, human Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl and 0.01% Tween 80 S-2288 S-2765 S-2782	1.0 0.9 1.2	10 25 30	0.010 0.028 0.025	0.8 0.3 0.3
Plasma Kallikrein, human Buffer: 50 mM Tris HCl, pH 7.8, 16mM NaCl (I = 0.05) S-2266 S-2288 S-2302 S-2366	0.50 0.60 0.22 0.60	85 130 150 80	0.17 0.22 0.68 0.13	0.2 0.1 0.08 0.2

	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM·s)) *10 ⁻³	Enzyme concentration (mg/l) for $\Delta A/\text{min}=0.05$ at $[S]=2 \cdot K_m$
C1s, human Buffer: 50 mM Tris HCl, pH 8.0, - S-2314 S-2765	0.70 0.50	10 3	0.014 0.006	1 4
Urine Kallikrein, human Buffer: 50 mM Tris HCl, pH 9.0, 16 mM NaCl (I = 0.05) S-2266	0.030	1	0.033	6
Glandular Kallikrein, bovine Buffer: 50 mM Tris HCl, pH 9.0, 16 mM NaCl (I = 0.05) S-2266	0.050	8	0.16	0.4
Granulocyte elastase, human Buffer: 50 mM Tris HCl, pH 8.3, 280 mM NaCl, 8% DMSO S-2484	0.50	15	0.030	0.3
u-PA, human Buffer: 50 mM Tris HCl, pH 8.8, 43 mM NaCl, 0.5% BSA S-2444	0.080	11	0.14	0.6
Trypsin, bovine Buffer: 50 mM Tris HCl, pH 9.0, 245 mM NaCl S-2222 S-2765	0.020 0.010	240 170	12 17	0.01 0.02
Chymotrypsin, bovine Buffer: 50 mM Tris HCl, pH 8.3, 380 mM NaCl, 3 mM CaCl ₂ S-2586	0.080	120	1.5	0.03

PROTEIN CONCENTRATIONS IN PLASMA

Component	Molecular Weight kDa	Plasma Concentration mg/l	Plasma Concentration $\mu\text{mol/l}$
Fibrinogen	330	3000	9
Prothrombin	72	150	2
Factor V	330	20	0.05
Factor VII	50	0.5	0.01
Factor VIII	330	0.1	0.0003
Factor IX	56	5	0.09
Factor X	59	8	0.13
Factor XI	160	5	0.03
Factor XII	80	30	0.4
Factor XIII	320	10	0.03
Protein C	62	4	0.06
Protein S	70	10 (free)	0.14
Protein Z	62	2	0.03
Prekallikrein	86	50	0.6
HMW kininogen	120	70	0.6
Fibronectin	450	300	0.7
Plasminogen	92	200	2
t-PA	60	0.005	0.0001
Urokinase	53	0.004	0.0001
Antithrombin	58	145	2.5
Heparin Cofactor II	66	80	1.2
Plasmin Inhibitor	63	60	1
Protein C Inhibitor	57	4	0.07
α2-Macroglobulin	725	2000	3

Table 4. Source-Review of current literature.

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THEORETICAL BASIS FOR CALCULATION

The hydrolysis of the chromogenic peptide substrate by the proteolytic enzyme follows in general the Michaelis-Menten kinetics. This means that, if the substrate is present at a sufficiently high concentration or if a comparatively small fraction of the substrate is hydrolysed, the rate of product (colour) formation is proportional to the activity of the enzyme. The rate of pNA formation, i.e. the increase in absorbance per second, is measured photometrically at 405 nm. At this wavelength the extinction coefficient of pNA is $9600 \text{ mol}^{-1} \cdot \text{l} \cdot \text{cm}^{-1}$.

The enzymatic activity can be quantified in two ways:

1. By comparing the activity of an enzyme with that of a standard preparation, which is defined in terms of a specified number of units set by an international or national authority or society (WHO, NIH etc.), or by the activity present in 1 ml of activated pooled normal plasma (Plasma Equivalent Unit = PEU). The standardisation is performed by using a standard curve obtained with at least five different concentrations, each performed in duplicate. The standard material must be of the same kind and of the same quality as the sample which is to be measured. This may be still more important for a secondary or domestic standard.
2. By measuring the amount (mol) of substrate split, or rather product formed per unit time (absolute activity).

One unit of enzymatic activity, katal (kat) is defined as the amount of activity that converts one mole of substrate per second under standardised conditions.

Such conditions as type of substrate, substrate concentration, buffer, pH, ionic strength and temperature should be given along with unit.

Thus, 1 nkat gives a conversion rate of:

$$1 \times 10^{-9} \text{ mol/sec} = 60 \times 10^{-9} \text{ mol/min}$$

If the total (measuring) volume used is V (ml), the increase in concentration per minute caused by 1 nkat is

$$C = \frac{60}{V} \times 10^{-6} \text{ mol/l}$$

If the absorbance is measured at 405 nm, in a 1 cm cuvette the difference in extinction coefficient is

$$\epsilon = 9600 \text{ mol}^{-1} \cdot \text{l}.$$

The increase in absorbance/min can then be calculated by using Lambert-Beer's law:

$$A = \epsilon \times C$$

Thus, 1 nkat gives:

$$\Delta A/\text{min} = 9600 \times \frac{60}{V} \times 10^{-6} = \frac{0.576}{V}$$

or

$$\text{nkat} = \frac{V}{0.576} \times \Delta A/\text{min}$$

By using a sample volume v (ml):

$$\text{nkat/ml} = \mu\text{kat/l} = \frac{V}{0.576 \times v} \times \Delta A/\text{min}$$

$$\mu\text{kat/l} = 1.74 \times \frac{V}{v} \times \Delta A/\text{min} \text{ (rate method)}$$

For the end-point method, the incubation time t (min) with substrate is taken into account by the following formula:

$$\mu\text{kat/l} = 1.74 \times \frac{V}{v \times t} \times A$$

According to nomenclature, one unit (U) is the amount of enzyme activity that converts one mol of substrate per minute under standardised conditions. By using the above formulas the units are:

$$U/l = 104 \times \frac{V}{v} \times \Delta A/\text{min} = 104 \times \frac{V}{v \times t} \times A$$

INTERNATIONAL UNITS AND ENZYME ACTIVITY

The enzyme activity expressed as katal is related to the type of substrate, to the nature of the enzyme and to the experimental conditions. In the following paragraphs, a tentative correlation between the different types of units and the enzyme activity with Chromogenix substrates is reported. The purpose is to help the researchers in developing chromogenic methods, by using such information as a starting point.

The conversion factors, have been taken from journal articles or from laboratory results within our R&D department, thus the consultation of such references or of the assay conditions reported in the kinetic tables is recommended.

FACTOR Xa and X

Factor Xa, which has a molecular weight of 44 KDa, is the activated form of Factor X (MW: 59 KDa). The International Units of Factor X correspond to the amount of Factor X contained in 1 ml of normal plasma. This is about 8 mg/l or 0.13 $\mu\text{mol/l}$. Since there is no WHO standard for FXa, one would assume that if all the Factor X in normal plasma was converted to the activated form, the Factor Xa concentration would be approximately 5.7 mg/l. The activity of human Factor Xa as calculated from the kinetic tables is 1.5 $\text{nkcat}/\mu\text{g}$ with the substrate S-2222, and 4.4 $\mu\text{kat}/\mu\text{g}$ with the substrate S-2765. The activity of 1 μg of Factor Xa as determined by Friberger¹ is 1.9 $\text{nkcat}_{\text{S-2222}}$. Thus, 1 plasma equivalent unit of Factor X would correspond to 15.2 $\text{nkcat}_{\text{S-2222}}$.

1. FRIBERGER P et al. Synthetic peptide substrate assays and fibrinolysis and their application on automates. In: Seminars in Thrombosis and Haemostasis, Vol. 9, 281-300 (1983).

PLASMINOGEN and PLASMIN

Plasminogen (MW: 92 KDa) is the zymogen form of plasmin (83 KDa). The activation of plasminogen is accomplished by t-PA, urokinase or streptokinase. It is not the purpose of this text to describe the mechanisms of activation of plasminogen and its inhibition, but it is important to underline that they can affect the reaction of the active enzyme with chromogenic substrates, casein or fibrin. For more information refer to Gaffney¹. 1 ml of normal plasma contains about 180 μg or 2 nmol of plasminogen. The activity of plasminogen can be expressed in casein units (CU) e.g. for example to Sgouris et al.². Friberger³ found that the amount of plasminogen in 1 ml of normal plasma corresponds to 3.8 CU, as determined by the chromogenic method described (streptokinase activated plasminogen). The Chromogenix Plasminogen Reagent (Art. No. 81 06 63), when activated with streptokinase shows an activity of 7.3 $\text{nkcat}_{\text{S-2251}}$ per CU. In 1983 Friberger⁴ reported that 1 μg of plasmin corresponds to 0.20 $\text{nkcat}_{\text{S-2251}}$, or to 0.024 CU or to 0.028 CTA-U. In 1975, the first standard for plasmin was established⁵ and the international unit was defined equivalent to CTA-U⁵⁻⁶. This contained a purified human plasmin in 50% glycerol. When tested by Friberger et al.⁷, it showed about the same activity as the Kabi preparation in terms of μmol of substrate hydrolysed per minute per unit of enzyme. Since then the different aspects of caseinolytic, amidolytic and fibrinolytic activity have been explored in details and, the discrepancy between amidolytic and fibrinolytic activity in reference preparations was underlined as in the case of the 2nd International Reference Preparation of plasmin in 1983⁸. The current 3rd International Standard for plasmin, 97/536 was established in 1998, and the results presented at the Fibrinolysis Subcommittee of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis (Ljubljana, Slovenia, June 1998).

The 2nd International Reference Preparation was used as reference material and a chromogenic assay was the method used. There is no established WHO standard for plasminogen, but the National Institute for Biological Standards and Control, established the 1st British Reference Preparation, 78/646⁹. It is a preparation of Glu-plasminogen, assayed by fibrinolytic and chromogenic methods against the 2nd International Standard for plasmin. Complete activation of plasminogen was achieved by both urokinase or streptokinase and the activity was comparable to that of the reference preparation of plasmin.

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PROTEIN C

Protein C, a 62,000-dalton glycoprotein with approximately 28% carbohydrates, is synthesised in the liver. Activated protein C (APC) is a key anticoagulant enzyme in the down-regulation of coagulation. Protein C consists of two polypeptide chains with a heavy chain linked by a disulfide bond to a light chain. The heavy chain contains the serine active site and the activation peptide and it also contains proposed binding sites for factors Va and VIIIa. The light chain contains a region of γ -carboxyglutamic acid residues (calcium ion and phospholipid binding region) and the epidermal growth factor region (proposed protein S-binding region).

The average concentration of protein C in human plasma is 4 $\mu\text{g/mL}$ and its half-life in plasma is 7 to 9 hours.

One Unit of Protein C corresponds to the amount of Protein C contained in one mL of freshly pooled normal plasma. In order to facilitate comparison of results from analysis of protein C in plasma, an international standard has been prepared and with the assigned potency expressed in international units (IU). Thus 1 IU corresponds to about 4 μg of functional protein C. A freeze-dried human plasma (denoted 86/622) has been established by the WHO Expert Committee on Biological Standardization as the 1st International Standard for protein C in plasma, which is available from NIBSC.¹

The assigned functional activity of this plasma is 0.82 IU/ampoule. Protein C in plasma may be activated by the thrombin/thrombomodulin complex or, more conveniently, by a specific venom enzyme from the snake Agkistrodon contortrix contortrix (Southern Copperhead Snake).²

The most suitable chromogenic substrate for the assay of APC described so far is S-2366.^{3,5}

The Chromogenix Protein C Reagent (Art. No 82 20 98), which contains a purified preparation of the snake venom enzyme allows activation of protein C without interference from other coagulation factors.

The amount of activated protein C is determined by the rate of hydrolysis of the chromogenic substrate S-2366.

The activity of human APC, derived from the rate of hydrolysis of S-2366 in a purified system under properly standardized conditions (0.1 mol/L Tris-HCl pH 8.3, 0.26 mol/L CsCl, 4 mmol/L CaCl₂ and with 0.2% BSA as bulking agent) is about 37 nkat/ μg of active enzyme.

The corresponding activity obtained in a plasma containing system after snake venom activation, using Coamatic Protein C, is about 25 nkat/ μg of active enzyme.

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THROMBIN

The current International Standard for thrombin is the Human α -thrombin 89/588 available from NIBSC. This is a high purity preparation of α -thrombin prepared from Cohn fraction III and assayed by a clotting time method against the first International Standard for thrombin, 75/157¹.

The National Institute of Health standard (Lot J) is also commonly used for calibration and a study conducted by Gaffney PJ et al.² was focused on the relationship between the two standards, and between the International Units and the NIH Units.

As a result of this study, based both on a clotting and a

chromogenic assay (with the substrate S-2238), 1 NIH-U corresponds to 1.15 IU.

In an article³ it was shown that bovine thrombin has a higher amidolytic activity than human thrombin when the same NIH-U are compared.

It was also underlined that the influence of β and γ -forms, that were probably contaminating the bovine enzyme, might be the reason for this discrepancy.

In the same article it was concluded that 1 NIH-U bovine thrombin was equivalent to 3.4 nkat_{S-2238}, and that 1 NIH-U of human thrombin was equivalent to 2.7 nkat_{S-2238}.

From an earlier publication⁴ 1 NIH-U of human thrombin corresponded to 2.5 nkat_{S-2238}. The correspondence between NIH-U or IU of thrombin and the enzyme activity expressed in nkat, depends on the substrate, the enzyme preparation (content of α -, β - and γ -thrombin) and the assay conditions. From the article of Friberger⁴, 1 μg thrombin corresponds to 2.2 NIH-U or 5.5 nkat_{S-2238} or to 0.02 plasma equivalent units. In another study⁵, 1 μg trombin corresponds to 3.1 NIH-U. In the experiments done in Chromogenix (see table 3 of the catalogue) 1 μg thrombin was equivalent to 3 nkat_{S-2238} (human) or 4.4 nkat_{S-2238} (bovine). It might also be added that if all prothombin is activated in 1 ml of human plasma, about 1.5 nanomoles or 17.5 NIH-U of thrombin are formed⁵.

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TISSUE-PLASMINOGEN ACTIVATOR (t-PA)

Tissue plasminogen activator (t-PA) exists in two forms: as single-chain (sct-PA) and as two-chain t-PA (tct-PA), the latter form being generated from sct-PA through proteolytic cleavage by plasmin. They both activate plasminogen to plasmin (plasminogenolytic activity; refer to the Coaset t-PA kit). Also they both hydrolyse chromogenic substrates (amidolytic activity; refer to the Research Method for t-PA in purified preparations). A quantitative analysis for the composition of a mixture of one-chain and two-chain t-PA by amidolytic assay has been proposed¹. The first International Standard for t-PA, 83/517, was established in 1985² by using a melanoma extract. The potency assignment was done with a fibrin clot lysis method. In 1987, the second International Standard for t-PA, 86/670, was established³. It was again a purified preparation from a cultured melanoma cell supernatant containing about 98% of single-chain t-PA. Chromogenic t-PA reagent (Art. No. 82 11 57), is composed mainly of single-chain t-PA (>95%) with a specific fibrinolytic activity of about 500,000 IU/mg of enzyme (batch-specific reagent) assessed against the second international standard. The enzyme activity in terms of nkat with the substrate S-2288 is 0.087 nkat with 400 IU (see kinetic tables).

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UROKINASE

Urinary type plasminogen activator (u-PA) is present either as a single chain proenzyme form (scu-PA) with a very low plasminogen activating activity or as an activated two-chain form (tcu-PA). tcu-PA occurs as both high molecular weight (HMW tcu-PA) or low molecular weight (LMW tcu-PA) enzyme species. HMW tcu-PA is 2-3 times more potent than LMW tcu-PA in biological assays such as the clot lysis method but their amidolytic activity on the chromogenic substrate S-2444 is equivalent¹. The International Reference Preparation (IRP) of urokinase (66/46) was established by the Expert Committee on Biological Standardisation of the WHO in 1968². Until then urokinase was expressed in casein Ploug (Leo standard) or CTA units (CTA standard). These units were related to the amount of urokinase capable to hydrolyse a certain amount of casein in a defined time. 1 CTA-U was assumed to be identical to 1 International Unit (IU). Although there was no official numerical relationship between Ploug units and IU, 1.5 IU has been accepted to be equivalent to 1 Ploug unit. The IRP 66/46 is a mixture of 66% LMW and 34% HMW forms of tcu-PA³. Following the need of a HMW International Standard, a new international standard (87/594) was established in 1989⁴. Due to the complexity of u-PA it is difficult to determine a direct relationship between IU, CTA-U, Plough-U and nkat_{S-2444}. In 1983 Friberger⁵ reported that 0.34 nkat_{S-2444} correspond to 110 Plough-U, 160 CTA-U or about 160 IU. In an earlier work by Paar D and Marhulm D, on urokinase purified from urine⁶, 100 CTA-U corresponded to 0.27 nkat_{S-2444}.

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RESEARCH METHODS

ANTITHROMBIN
CHYMOTRYPSIN
FACTOR
GRANULOCYTE ELASTASE
HEPARIN
KALLIKREIN
PREKALLIKREIN
PROTEOLYTIC
t-PA
TRYPSIN
UROKINASE



INTRODUCTION

Historically, the development of a new chromogenic substrate for a specific protease has always been accompanied by the release of Method Sheets where the application and the methodology for a particular use were described in detail. Some methods are simple chromogenic assays where a buffer and the substrate are the only reagents to be used (i.e. proteolytic activity). Other methods instead require the use of additional compounds, which are or have been commercially available from Chromogenix or elsewhere, and consist of more reaction steps.

These protocols were validated in our laboratories according to the equipment and the reagents available at the time. In several cases they have been adopted in research, quality control, or routine laboratories and some of them later became Chromogenix kits now present in our product range. During the last 20 years, the so-called Method Sheets have been taken as the starting point by several scientists, for the development of assays for particular applications, or studies. In some cases the experimental conditions have been changed according to the particular need of the investigation being done.

These methods are now presented in a different form: "Research Methods". The intention here is to provide assay protocols that are not available as kits, but complementary to our product range. In the following pages, you can find the methods developed by Chromogenix for several assays. They have to be considered as general guidelines or basic tools for the development of your own assays, some of them requiring a validation within your laboratory with respect to the reagents and equipment used.

For each method, you can find an updated Bibliography with references, where the method has been used, like as originally described or with modifications. This information should facilitate and accelerate the development of the best test protocol. If you do not have that specific journal issue in your laboratory, you can visit our web site www.chromogenix.com. Here you can find easy access to MEDLINE (through LINKS). You can search for particular articles, print the abstract and order the original copy through LOANSOME DOC (and receive the document through your local library). At the same time, on our web site you have the possibility to be updated on the new products from Chromogenix.

Research Methods

- Antithrombin (anti-FIIa)
- Chymotrypsin
- Factor X
- Granulocyte elastase
- Heparin (anti-FIIa)
- Kallikrein inhibitor activity
- Kallikrein, urine
- Kallikrein-like activity, plasma
- Prekallikrein activator
- Prekallikrein, plasma
- Proteolytic activity
- t-PA
- Trypsin
- Urokinase
- Prothrombin activity
- Factor VIII activity elevated
- Hirudin
- TFPI

New

ANTITHROMBIN (anti-FIIa)

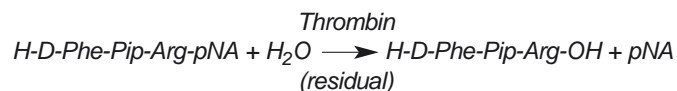
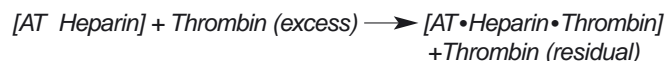
Determination of antithrombin activity in plasma with S-2238.

Measurement Principle

The antithrombin activity in plasma is measured after addition of an excess of heparin, to form an AT•Heparin complex. An excess of thrombin is then added and allowed to react quantitatively in a 1:1 stoichiometric relationship with the AT Heparin complex present.

The residual thrombin splits off p-nitroaniline (pNA) from the substrate H-D-Phe-Pip-Arg-pNA (S-2238). The rate at which pNA is released is measured photometrically at 405 nm.

This can be followed on a recorder (initial rate method) or read after stopping the hydrolysis with acid (acid stopped method). The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) or absorbance (A) and the AT activity is linear and inversely proportional in the 5-125% range of normal plasma.



Reagents

1. **S-2238, 25 mg** Art. No. 82 03 24
Reconstitute the substrate S-2238 (MW: 625.6) in 53 ml of distilled water.
Note: Polybrene® can be added to the substrate solution at a final concentration of 0.33 mg/ml.

2. **Thrombin, 53 nkat** Art. No. 82 07 12
Reconstitute with 1.5 ml sterile water. The solution is stable for 4 weeks at 2-8°C.

4. **Tris/Heparin Buffer, pH 8.4 (25°C)**

Tris	6.1 g	(50 mmol/l)
NaCl	10.2 g	(175 mmol/l)
Na ₂ EDTA - 2H ₂ O	2.8 g	(7.5 mmol/l)
Distilled water	800 ml	

Adjust the pH to 8.4 at 25°C by adding an appropriate amount (approx. 22 ml) of 1 mol/l HCl. Add 3000 IU of heparin. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for two months at 2-8°C.

5. **Acetic acid 20%**
Acetic acid is used in the acid-stopped method.

Equipment

1. Spectro- or filter-photometer, 405 nm
 2. Semi-microcuvettes, 1 cm.
 3. Thermostat, 37°C
 4. Stop watch
 5. Disposable plastic tubes
- Additional equipment for the initial rate method:
6. Photometer with cuvette housing, thermostated at 37°C.

Specimen collection

Nine parts of freshly drawn venous blood are collected into one part trisodium citrate.
Centrifugation: 2000 x g for 10-20 min at 20-25°C.

Standard curve

Normal plasma has an antithrombin activity of 100%. Two standards (e.g. 25% and 100%) made up fresh should be included in each test run. Check whether $\Delta A/\text{min}$ or A for the two standards correspond with the stored standard curve. The tolerance limit is ± 0.1 Absorbance units. Prepare the standards according to the table below:

Antithrombin %	Normal plasma μl	Tris/Heparin buffer μl
0	-	400
25	100	300
50	200	200
75	300	100
100	400	-

Method

Dilute samples and standards as follows:

Tris/Heparin Buffer	3000 μl
Test plasma or standard	50 μl

Initial rate method	
Diluted test plasma or standard	400 μl
Incubate at 37°C	3-6 min
Thrombin (20-25°C)	100 μl
Mix and incubate at 37°C	30 sec
Substrate (37°C)	300 μl

Transfer immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C, calculate $\Delta A/\text{min}$.

Acid stopped method	
Diluted test plasma or standard	400 μl
Incubate at 37°C	3-6 min
Thrombin (20-25°C)	100 μl
Mix and incubate at 37°C	30 sec
Substrate (37°C)	300 μl
Incubate at 37°C	30 sec
Acetic acid 20%	300 μl

Read the absorbance (A) of the sample against distilled water at 405 nm within 4 hours.

Limitations of the procedure

In some pathological states (DIC, sepsis) plasma alone may hydrolyse the substrate S-2238. This interfering reaction may be determined by assay of a test sample in the absence of added thrombin.

This activity rarely corresponds to more than 1% of that of the added thrombin. To improve the validity of the assay the value obtained in the absence of added thrombin can be subtracted from the sample value.

Bilirubin, haemoglobin and plasma from hyperlipaemic patients interfere in absorbance reading.

Patients plasma blanks are necessary in these instances for the acid stopped method only.

At concentrations below 25% AT it is recommended to double the plasma concentration (100 µl plasma + 3 ml buffer).

The result is then divided by two.

Calculation

Plot A or $\Delta A/\text{min}$ for the standards against their known antithrombin activity. Percent of normal AT activity is determined by plotting the A or $\Delta A/\text{min}$ for the test sample on the standard curve and reading the corresponding AT value.

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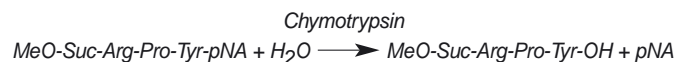
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CHYMOTRYPSIN

Determination of chymotrypsin with S-2586.

Measurement Principle

The chymotrypsin activity is determined by its amidolytic effect on the substrate MeO-Suc-Arg-Pro-Tyr-pNA (S-2586). The rate at which p-nitroaniline (pNA) is released is measured photometrically at 405 nm. This can be followed on a recorder (initial rate method) or read after stopping the reaction with acetic acid (acid stopped method). The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) or absorbance (A) and the chymotrypsin activity is linear in the 0.05-1.0 $\mu\text{kat/l}$ or 3-60 U/l range. The amidolytic activity of different chymotrypsin preparations does not necessarily parallel the protease activity.



Reagents

- S-2586, 25 mg Art. No. 82 08 94
Reconstitute the substrate S-2586 (MW: 705.3) with 60 ml of distilled water.
- Tris/Calcium Buffer, pH 8.3 (25°C)
Tris 12.1 g (100 mmol/l)
NaCl 56.2 g (960 mmol/l)
Distilled water 800 ml

Adjust the pH to 8.3 at 25°C by adding approximately 50 ml of 1 mol/l HCl. Add 10 ml of 1 mol/l CaCl_2 solution. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for two months at 2-8°C.

- Acetic acid, 20%
Acetic acid is used in the acid-stopped method.

Equipment

- Spectro- or filter photometer, 405 nm
- Semi-microcuvettes, 1 cm.
- Thermostat, 37°C
- Stop watch
- Disposable plastic tubes
- Photometer with cuvette housing, thermostated at 37°C (for the initial rate method)

Sample

The sample containing chymotrypsin is dissolved in or diluted with 1 mmol/l HCl to a concentration of 0.1 g/l. This stock solution is stable for more than two weeks at 2-8°C. Before assay, the solution is diluted 1:200 with 1 mmol/l HCl. If the sample is a pure protein, it is advisable to use 0.1% Carbowax 6000 (Union Carbide, NY) or 1% albumin (previously checked for amidolytic activity) to avoid adsorption to surfaces.

Method

Initial rate method	
Buffer	200 μl
Incubate at 37°C	3-4 min
Chymotrypsin sample	200 μl
Mix and incubate at 37°C	2-3 min
Substrate (37°C)	200 μl
Mix	

Transfer the sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C. Calculate $\Delta A/\text{min}$.

Acid stopped method	Sample	Blank
Buffer	200 μl	200 μl
Incubate at 37°C	3-4 min	-
Chymotrypsin sample	200 μl	200 μl
Mix and incubate at 37°C	2-3 min	-
Substrate (37°C)	200 μl	-
Mix and incubate at 37°C	3 min	-
Acetic acid 20%	200 μl	200 μl
Mix	yes	-
Substrate (37°C)	-	200 μl
Mix	-	yes

Read the absorbance (A) of the sample against a water or sample blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

Calculate the chymotrypsin activity of the stock solution from the following formulas:

$$\begin{aligned} \text{Initial rate method: } \mu\text{kat/l} &= 5.19 \times \Delta A/\text{min} \times 200 \\ \text{U/l} &= 311 \times \Delta A/\text{min} \times 200 \end{aligned}$$

$$\begin{aligned} \text{Acid stopped method: } \mu\text{kat/l} &= 2.31 \times A \times 200 \\ \text{U/l} &= 138 \times A \times 200 \end{aligned}$$

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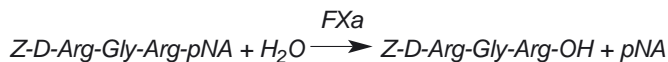
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FACTOR X

Determination of factor X in plasma with S-2765.

Measurement Principle

The method is based on a two-stage principle. In stage one, Factor X is activated in the presence of calcium to Factor Xa (FXa) using the activator Russell's Viper venom (RVV). In stage two, the generated FXa hydrolyses the chromogenic substrate Z-D-Arg-Gly-Arg-pNA (S-2765), thus liberating the chromophoric group pNA (p-nitroaniline). The colour is then read photometrically at 405 nm. The generated FXa (and thus the intensity of colour) is proportional to the FX activity of the sample.



Reagents

- S-2765, 25 mg** Art. No. 82 14 13
Reconstitute the substrate S-2765 (MW: 714.6) with 20 ml sterile water.
- Russell's Viper Venom (RVV)**
Prepare a solution of Russell's Viper Venom at a concentration of 0.087 mg/ml.
- CaCl₂**
0.1 mol/l calcium chloride solution.
- Tris EDTA Buffer** Art. No. 82 36 66
Dilute the buffer 1:10 with distilled water according to the insert sheet instructions.

5. Normal Plasma

Calibrated, lyophilised or fresh frozen human plasma is used for the standardisation of the assay. A pooled normal plasma can be prepared by taking samples from 20 healthy donors. 10-30 ml citrate blood (9 vol blood and 1 vol 0.1 mol/l sodium citrate) from each donor is centrifuged at 2000 x g for 20 minutes at 15-25°C. The plasma is pooled and subsequently dispensed in small volumes, which are frozen rapidly at -20°C or below. To avoid low temperature activation of prekallikrein the plasma is kept at 15-25°C before use or freezing. Thawing of plasma should be performed at 37°C and then kept at 15-25°C until used.

6. RVV + CaCl₂

Before use, mix 1 volume of RVV with 1 volume of CaCl₂. The mixture is stable for 48 hours at 2-8°C

7. Acetic acid 20%

Acetic acid is used as a stopper solution in the end-point method.

Equipment

- Thermostated microplate reader or spectrophotometer
- Photometer with cuvette housing, thermostated at 37°C (for the initial rate method)
- Microplates or semimicro cuvettes, 1 cm
- Centrifuge
- Thermostat 37°C
- Stopwatch

Specimen collection

Blood (9 vol) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 20-25°C. Storage: 1 week at 2-8°C or 3-4 months at -20°C.

Standard curve

FX %	Predilution		Final dilution	
	Normal Plasma μ l	Buffer μ l	Prediluted Plasma μ l	Buffer μ l
0	-	-	-	1000
25	25	75	50	1000
50	50	50	50	1000
75	75	25	50	1000
100	-	-	50	1000
124	-	-	50	800

Method

Sample dilution	
Buffer	1000 μ l
Test plasma or standard	50 μ l
Mix	

Acid stopped method	Test Tube	Microplate
Diluted sample	200 μ l	50 μ l
Incubate at 37°C	3-4 min	3-4 min
Substrate (37°C)	200 μ l	50 μ l
Mix and add within 30 sec		
RVV+CaCl ₂ (20-25°C)	200 μ l	50 μ l
Mix and incubate at 37°C	3 min	3 min
Acetic acid 20%	200 μ l	50 μ l

Sample blank activities should be determined and subtracted when analysing strongly coloured plasma, e.g. lipemic and hemolytic. The sample blanks are prepared by mixing the diluted sample, acetic acid 20% and water instead of the reagents (400 μ l for test tubes and 100 μ l for microplates). Read the absorbance of the samples and blanks at 405 nm. The colour is stable for at least four hours.

When possible, use a dual wavelength mode with 490 nm as the reference wavelength.

Initial rate method: When performing the initial rate method, transfer the microplate to a microplate reader immediately after the addition of RVV+CaCl₂ and read the change in A/min. The microplate reader must be pre-incubated at 37°C.

Calculation

Plot A or $\Delta A/\text{min}$ for the standards against their concentration of Factor X. Read the Factor X value for the corresponding A or $\Delta A/\text{min}$ of the unknown test sample from the standard curve.

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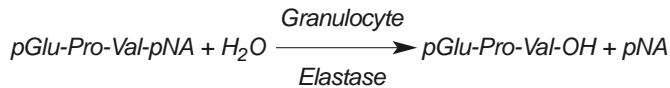
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GRANULOCYTE ELASTASE

Determination of granulocyte elastase activity with S-2484.

Measurement Principle

The elastase activity is determined by its amidolytic effect on the substrate pyro-Glu-Pro-Val-pNA (S-2484). The rate at which p-nitroaniline (pNA) is released is measured photometrically at 405 nm. This can be followed on a recorder (initial rate method) or read after stopping the reaction with acetic acid (acid stopped method). The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) or absorbance (A) and the granulocyte elastase activity is linear in the 0.1-1.5 $\mu\text{kat/l}$ or 6-90 U/l range. The amidolytic activity does not necessarily parallel the elastolytic activity for different elastase preparations.



Reagents

1. S-2484, 25 mg Art. No. 82 08 86
Reconstitute the substrate S-2484 (MW: 445.5) with 7 ml of DMSO. One volume of this stock solution is diluted with 3 volumes of distilled water.

2. Tris Buffer, pH 8.3 (25°C)
Tris 12.1 g (100 mmol/l)
NaCl 52.6 g (960 mmol/l)
Distilled water 800 ml

Adjust the pH to 8.3 at 25°C by adding about 50 ml of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for two months at 2 to 8°C.

3. Acetic acid 20%
Acetic acid is used in the acid-stopped method.

Equipment

1. Spectro- or filter photometer, 405 nm
2. Semi-microcuvettes, 1 cm.
3. Thermostat, 37°C
4. Stop watch
5. Disposable plastic tubes
7. Photometer with cuvette housing, thermostated at 37°C (for the initial rate method).

Sample

The sample containing granulocyte elastase is dissolved in or diluted with distilled water, saline or buffer to an activity of 0.1-1.5 $\mu\text{kat/l}$ which approximately corresponds to a concentration of 0.5-7.5 mg/l of a rather pure enzyme. If the sample is a pure protein, it is advisable to use 0.1% Carbowax 6000 (Union Carbide, NY) or 1% albumin (previously checked for amidolytic activity) to avoid adsorption to surfaces.

Method

Initial rate method	
Buffer	200 μl
Incubate at 37°C	3-4 min
Elastase sample	200 μl
Mix and incubate at 37°C	2-3 min
Substrate (37°C)	200 μl

Transfer the sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C. Calculate $\Delta A/\text{min}$.

Acid stopped method	Sample	Blank
Buffer	200 μl	200 μl
Incubate at 37°C	3-4 min	-
Elastase sample	200 μl	200 μl
Mix and incubate at 37°C	2-3 min	-
Substrate (37°C)	200 μl	-
Mix and incubate at 37°C	3 min	-
Acetic acid 20%	200 μl	200 μl
Mix	yes	-
Substrate (37°C)	-	200 μl
Mix	-	yes

Read the absorbance (A) of the sample against a water or sample blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

Calculate the elastase activity of the sample from the formulas:

Initial rate method: $\mu\text{kat/l} = 5.19 \times \Delta A/\text{min}$
 $\text{U/l} = 311 \times \Delta A/\text{min}$
 Acid stopped method: $\mu\text{kat/l} = 2.31 \times A$
 $\text{U/l} = 138 \times A$

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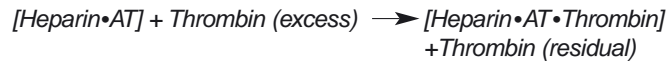
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HEPARIN (anti-FIIa)

Determination of heparin in plasma with S-2238.

Measurement Principle

Heparin is analysed as a complex with antithrombin (AT) present in the sample. The concentration of this complex is dependent on the availability of AT. In order to obtain a more constant concentration of AT, purified AT is added to the test plasma. Thrombin in excess is neutralized in proportion to the amount of heparin, which determines the amount of heparin-AT complex. The remaining amount of thrombin hydrolyses the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S-2238) thus liberating the chromophoric group, pNA. The colour is then read photometrically at 405 nm.



Reagents

1. S-2238, 25 mg Art. No. 82 03 24
Reconstitute the substrate S-2238 (MW: 625.6) with 40 ml of distilled water.

2. Thrombin

Human thrombin or bovine thrombin can be used in 0.15 mol/l NaCl solution. The activity of the solution should be 14 nkat/l (about 6 NIH-U/ml).

If bovine thrombin 53 nkat from Chromogenix (Art. No. 82 07 12) is used, dissolve the content of one vial with 3.8 ml saline.

3. Antithrombin, 10 IU Art No. 82 07 20

Reconstitute with 5 ml water to obtain a concentration of 2 IU/ml.

4. Tris Buffer, pH 8.4 (25°C)

Tris	6.1 g	(50 mmol/l)
NaCl	10.2 g	(175 mmol/l)
Na ₂ EDTA H ₂ O	2.8 g	(7.5 mmol/l)
Distilled water	800 ml	

Adjust the pH to 8.4 at 25°C by adding an appropriate amount (approx. 22 ml) of 1 mol/l HCl.

5. Normal plasma

Blood should be taken from normal donors. 10-30 ml of citrated blood (9 vol blood and 1 vol 0.1 mol/l sodium citrate) are taken from each donor. The first ml of blood is discarded and the tube is kept in an ice bath. Plasma is prepared by centrifugation at 2000 x g for 20 minutes at 4°C.

Equal amounts of plasma from the donors are mixed and dispensed in small volumes. The normal plasma is stable for 3 months at -20°C or below. Thaw at 37°C and then keep on ice.

6. Acetic acid 20%

Acetic acid is used in the acid-stopped method.

Equipment

- Spectro- or filter photometer, 405 nm
 - Semi-microcuvettes, 1 cm.
 - Thermostat, 37°C
 - Stop watch
 - Disposable plastic tubes
 - Photometer with cuvette housing, thermostated at 37°C.
- Additional equipment for the initial rate method:

Specimen collection

Blood (9 vol) is mixed with sodium citrate (1 vol) cooled to 0°C with ice and centrifuged at 2000 x g for 20 min at 4°C. Dilute plasma 1:5 with Tris Buffer pH 8.4.

Standard curve

The same heparin as is used for the patient is diluted to 1 IU/ml with saline 0.9%. Then 100 µl dilution is further diluted with 1.9 ml buffer to obtain a concentration of 0.05 IU/ml.

Standard IU/ml	Buffer µl	AT µl	Normal plasma dil 1:5 µl	Heparin 0.05 IU/ml µl
0.00	800	100	100	0
0.25	700	100	100	100
0.50	600	100	100	200
0.75	500	100	100	300
1.00	400	100	100	400

Method

Initial rate method		Tube No. 1
Buffer		800 µl
AT		100 µl
Test plasma		100 µl
Mix		
		Tube No. 2
Standard or Tube No.1		200 µl
Incubate at 37°C		3-4 min
Thrombin		100 µl
Incubate at 37°C		30 sec
Substrate (37°C)		200 µl
Mix		

Transfer sample immediately to a 1 cm micro-cuvette (preheated at 37°C) for measurement of the absorbance change at 405 nm. Calculate ΔA/min.

Read the absorbance against a normal plasma blank in a photometer at 405 nm.

Acid stopped method		Tube No. 1
Buffer		800 µl
AT		100 µl
Test plasma		100 µl
Mix		
		Tube No. 2
Standard or Tube No.1		200 µl
Incubate at 37°C		3-4 min
Thrombin		100 µl
Incubate at 37°C		30 sec
Substrate (37°C)		200 µl
Incubate at 37°C		60 sec
Acetic acid 20 %		300 µl
Mix		

Blanks for acid stopped method	Normal plasma blank	Test Plasma blank
Standard 0 IU/ml	200 µl	-
Sample from Tube No.1	-	200 µl
Acetic acid 20%	300 µl	300 µl
Mix		
Distilled water	300 µl	300 µl
Mix		

Note: As a rule a normal plasma blank or even water is used as a blank. If bilirubin exceeds 100 µmol/l or the test plasma is opaque, read the test plasma sample against its own blank.

Calculation

Plot A or ΔA/min for the standards against their known heparin concentration.

Heparin concentration is determined by plotting the A or ΔA/min for the test sample on the standard curve and read the corresponding heparin value.

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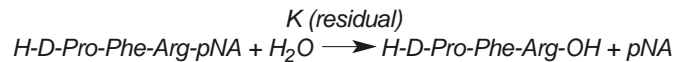
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KALLIKREIN INHIBITOR ACTIVITY

Determination of kallikrein inhibitor activity in plasma, with S-2302.

Measurement Principle

Plasma is incubated with a purified plasma kallikrein preparation. The amount of kallikrein inhibited is proportional to the activity of the kallikrein inhibitor present in the plasma. The remaining amount of kallikrein activity is then determined by using the substrate H-D-Pro-Phe-Arg-pNA (S-2302). The rate at which p-nitroaniline (pNA) is released is measured photometrically at 405 nm. This can be followed on a recorder (initial rate method) or read after stopping the reaction with acetic acid (acid stopped method).



Reagents

1. S-2302, 25 mg Art. No. 82 03 40
Reconstitute the substrate S-2302 (MW: 611.6) with 20 ml of distilled water.

2. Plasma Kallikrein

Use purified human plasma kallikrein (refer to Gallimore MJ et al., 1978). Prepare a solution of 1 nkat_{S-2302}/ml human plasma kallikrein in Tris buffer pH 7.8.

1 nkat_{S-2302} corresponds to 0.06 U or 0.017 PEU (refer to Friberger P et al. 1979).

3. Tris Buffer, pH 7.8 (25°C)
Tris 6.1 g (50 mmol/l)
NaCl 21.1 g (361 mmol/l)
Polybrene 20 mg
Distilled water 800 ml

Adjust the pH to 7.8 at 25°C by adding an appropriate amount (approx. 38 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for six months at 2 to 8°C.

4. Normal plasma

Blood samples are taken from at least 10 healthy donors. For the preparation of the samples, refer to the Specimen collection section.

5. Acetic acid, 20%

Acetic acid is used in the acid stopped method.

Equipment

1. Spectro- or filter photometer, 405 nm
 2. Siliconised semi-microcuvettes, 1 cm
 3. Centrifuge
 4. Thermostat, 37°C
 5. Stop watch
 6. Disposable plastic tubes
- Additional equipment for the initial rate method
7. Photometer with cuvette housing, thermostated at 37°C.

Specimen collection

Blood (9 vol) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 15-25°C. In order to avoid low-temperature activation of plasma kallikrein inhibitor the plasma should be kept at 15-25°C for not more than a few hours or immediately frozen at -20°C or below. After thawing at 37°C the plasma should be kept at 15-25°C and used as soon as possible. Frozen plasma may lose some plasma kallikrein inhibitor activity on freezing or thawing, but is stable for several months at -20°C or below.

Standard curve

Normal plasma has a kallikrein inhibitor concentration of 100% and is diluted according to the table below (see Note 1).

K-Inhibitor %	Normal plasma μ l	Buffer μ l
25	100	300
50	200	200
75	300	100
100	400	-

Method

Sample dilution	Tube No. 1
Buffer	1900 μ l
Test plasma or standard (see note 1)	100 μ l
Mix	

Initial rate method	Tube No. 2
Sample from tube No. 1	200 μ l
Incubate at 37°C	3-4 min
Plasma kallikrein	200 μ l
Mix and incubate at 37°C	5 min
Substrate (37°C)	200 μ l
Mix	

Transfer sample immediately to a 1 cm siliconised semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C. Calculate $\Delta A/\text{min}$.

Acid stopped method	Tube No. 2
Sample from tube No. 1	200 μ l
Incubate at 37°C	3-4 min
Plasma kallikrein	200 μ l
Mix and incubate at 37°C	5 min
Substrate (37°C)	200 μ l
Mix and incubate at 37°C	4 min
Acetic acid 20%	200 μ l
Mix	

Plasma blanks are prepared by adding the reagents in reverse order without incubation. Read the absorbance (A) of the sample against its blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

Plasma Kallikrein inhibitor in percentage of normal plasma

Plot A or $\Delta A/\text{min}$ for the standards against their concentration of kallikrein inhibitor on log-lin graph paper. Read the kallikrein inhibitor value for the corresponding A or $\Delta A/\text{min}$ of the unknown test sample from the standard curve.

Plasma Kallikrein inhibitor in enzyme activity units

In each test series a kallikrein activity determination with buffer instead of sample dilution must be performed. The difference between this activity and the sample activity is then calculated.

Initial rate method:

$$\begin{aligned} \mu\text{kat/l} &= (\Delta A/\text{min buffer} - \Delta A/\text{min sample}) \times 104 \\ \text{U/l} &= (\Delta A/\text{min buffer} - \Delta A/\text{min sample}) \times 6\,250 \end{aligned}$$

Acid stopped method:

$$\begin{aligned} \mu\text{kat/l} &= (A_{\text{buffer}} - A_{\text{sample}}) \times 34.7 \\ \text{U/l} &= (A_{\text{buffer}} - A_{\text{sample}}) \times 2\,080 \end{aligned}$$

Notes

1. A 150% standard is prepared by diluting 300 μl normal plasma with 3700 μl buffer. A 200% standard is prepared by diluting 100 μl normal plasma with 900 μl buffer. For 0% use the buffer only (note that the adsorbance to surfaces can result in lower readings when plasma is absent).
2. It is suggested, that the spontaneous kallikrein activity (α 2-M complex) should be determined in patients in whom the kallikrein system is suspected to be activated. See Determination of Kallikrein-like Activity in Plasma.

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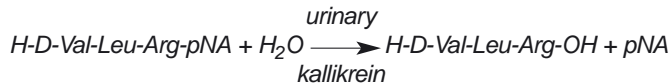
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URINE KALLIKREIN

Determination of kallikrein in urine with S-2266

Measurement Principle

Kallikrein in urine hydrolyses the substrate H-D-Val-Leu-Arg-pNA (S-2266) and the rate of p-nitroaniline (pNA) formation increases linearly with increasing concentration of kallikrein up to 30 nkat/l. (See note). By adding aprotinin, a potent inhibitor of glandular kallikrein, to the sample blank, protease activities not inhibited by aprotinin as well as the colour from the urine itself can be subtracted.



Reagents

- S-2266, 25 mg Art. No. 82 04 80
Reconstitute the substrate S-2266 (MW: 579.6) with 28.8 ml of distilled water.
- Tris Buffer, pH 8.2 (25°C)
Tris (200 mmol/l)
24.4 g
Distilled water 800 ml

Adjust the pH to 8.2 at 25°C by adding an appropriate amount of 1 mol/l HCl (approximately 100 ml with distilled water. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, is stable for two months at 2 -8°C.

3. Trasylol® buffer

Trasylol (lyophilized aprotinin) is added to the buffer (Reagent 2) to a concentration of 20 KIU/ml.

4. Acetic acid, 50%

Equipment

- Spectro- or filter photometer, 405 nm
- Semi-microcuvettes, 1 cm
- Centrifuge
- Thermostat, 37°C

- Stop-watch
- Disposable plastic tubes

Specimen collection

As the kallikrein concentration may vary during the day, the total volume collected during 24 hours should be pooled. No drugs should be taken on the day of the sampling unless it is the aim to evaluate the influence of the drug in the kallikrein secretion. After mixing the urine pool a portion is transferred into a disposable plastic tube and kept at 2-8°C (less than 24 hours) or below -20°C. Just before the analysis, the urine sample is centrifuged and the supernatant is used.

Method

Acid stopped method	Sample	Blank
Buffer	500 µl	-
Trasylol	-	500 µl
Incubate at 37°C	5-10 min	5-10 min
Urine	400 µl	400 µl
Mix and incubate at 37°C	2-5 min	2-5 min
Substrate (37°C)	100 µl	100 µl
Mix and incubate at 37°C	30 min	30 min
Acetic acid 50%	100 µl	100 µl

Read the absorbance (A) of the sample against its blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

The activity of kallikrein per litre of urine or excrete during 24 hours is calculated from the formula:

$$\begin{aligned} \text{nkat/l} &= 159 \times A & \text{U/l} &= 9.55 \times A \\ \text{nkat/24hr} &= 159 \times v \times A & \text{U/24hrs} &= 9.55 \times v \times A \end{aligned}$$

A = absorbance

v = total volume in litre of urine collected during 24 hours.

Note: If the kallikrein activity exceeds 30 nkat/l the urine should be diluted with the same volume of buffer and the result multiplied with 2.

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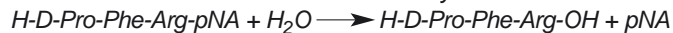
PLASMA KALLIKREIN-LIKE ACTIVITY

Determination of kallikrein-like activity in plasma, with S-2302.

Measurement Principle

The plasma kallikrein-like activity catalyses the splitting of p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA (S-2302). The rate at which the pNA is released is measured photometrically at 405 nm. This can conveniently be read after stopping the reaction with acetic acid (acid stopped method). The activity measured is mainly the kallikrein- α 2-macroglobulin complex.

Kallikrein-like activity



Reagents

- | | |
|--|--------------------|
| 1. S-2302, 25 mg | Art. No. 82 03 40 |
| Reconstitute the substrate S-2302 (MW: 611.6) with 20 ml of distilled water. | |
| 2. Tris Buffer, pH 7.8 (25°C) | |
| Tris | 6.1 g (50 mmol/l) |
| NaCl | 6.6 g (113 mmol/l) |
| Distilled Water | 800 ml |

Adjust the pH to 7.8 at 25°C by adding an appropriate amount (approx. 38 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for six months at 2 to 8°C.

- Acetic acid, 20%

Equipment

- Spectro- or filter photometer, 405 nm
- Semi-microcuvettes, 1 cm
- Centrifuge
- Thermostat, 37°C
- Stop watch
- Disposable plastic tubes

Specimen collection

Blood (9 vol) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 15-25°C. In order to avoid low-temperature activation of prekallikrein the plasma should be kept at 15-25°C for not more than a few hours or immediately frozen at -20°C or below. After thawing at 37°C the plasma should be kept at 15 to 25°C and used as soon as possible. Frozen plasma may lose some kallikrein-like activity on freezing or thawing, but is stable for several months at -20°C or below.

Method

Sample dilution	Tube No. 1
Buffer	1000 μ l
Test plasma	100 μ l
Mix	

Acid stopped method	Tube No. 2
Sample from tube No. 1	200 μ l
Incubate at 37°C	3-4 min
Substrate	200 μ l
Mix and incubate at 37°C	10 min
Acetic acid, 20%	200 μ l
Mix	

Plasma blanks are prepared by adding the reagents in reverse order without incubation. Read the absorbance (A) of the sample against its blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

Plasma kallikrein-like activity in enzyme activity units:
 μ kat/l = A x 5.73
 U/l = A x 344

Notes

- The substrate S-2302 is also sensitive to plasmin. By testing with 2 mmol/l S-2251 it is possible to check whether plasmin is present in the sample. The substrate S-2251 is not sensitive to kallikrein.
- If the method is to be used for subtraction of blank activities in the prekallikrein assay, it may be preferable to dilute the plasma as indicated for that assay.

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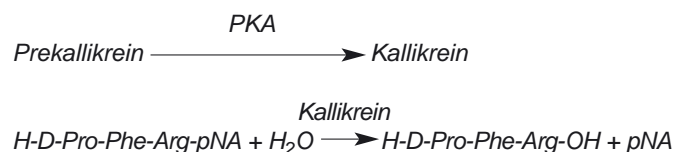
PREKALLIKREIN ACTIVATOR (PKA)

Determination of PKA in albumin and immunoglobulin preparations with S-2302.

Measurement Principle

Prekallikrein (prekininogenase) is activated to kallikrein by prekallikrein activator (PKA). The kallikrein formed catalyses the splitting of p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA (S-2302). The rate at which pNA is released is measured photometrically at 405 nm and can be followed on a recorder (initial rate method).

The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) and the prekallikrein activator concentration is linear between 0 and 51 IU/ml of prekallikrein activator. The concentration of prekallikrein activator is calculated using an international standard.



Reagents

1. S-2302, 25 mg Art. No. 82 03 40
Reconstitute the substrate S-2302 (MW: 611.6) with 6.8 ml of distilled water. Working solution: dilute one volume of the stock solution with nine volumes of the buffer (Reagent 2). The working solution is stable for 8 hours at 20-25°C.

2. Tris Buffer, pH 7.8 (25°C)

Tris	6.1 g	(50 mmol/l)
NaCl	0.7 g	(12 mmol/l)
Distilled water	800 ml	

Adjust the pH to 7.8 at 25°C by adding approximately 38 ml of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for six months at 2-8°C.

3. Prekallikrein Activator

The 1st International Standard 1984 (NIBSC, 82/530) contains 85 International Units per ampoule. Reconstitute with 1 ml of distilled water. Refer to PJ Kerry et al., Br J Haematol, 345-352, (1985) for more information on the standardisation of PKA.

4. Prekallikrein fraction

A prekallikrein fraction is prepared according to the chromatography procedure described in the appendix. Check the quality of the prekallikrein according to paragraph J in the appendix before each test run. The prekallikrein solution is stable for at least one year at -70°C.

Equipment

1. Spectro- or filter photometer, 405 nm
2. Semi-microcuvettes, 1 cm. (plastic is recommended.)
3. Thermostat, 37°C
4. Stop watch
5. Disposable plastic tubes
6. Photometer with cuvette housing, thermostated at 37°C

Sample

Albumin and immunoglobulin preparations. Dilute the sample to a corresponding prekallikrein activator concentration of 10-40 IU/ml.

Standard curve

The 1st International Standard has a PKA concentration of 85 IU/ml and is diluted as indicated in the table below.

Prekallikrein activator IU/ml	International Standard μl	Buffer μl
0.0	-	1000
10.2	120	880
20.0	235	765
34.9	410	590
50.2	590	410

Method

Initial rate method	
Step A for sample and standard	Sample Tube No. 1
Sample or standard	25 μl
Prekallikrein	100 μl
Mix and incubate at 37°C in capped tubes	45 min
Step B for sample and standard	Sample Tube No. 2
Substrate (37°C)	1000 μl
Mixture from Tube No. 1	25 μl
Mix	

Transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change for at least two minutes in a photometer at 405 nm and at 37°C. Immunoglobulin may occasionally contain significant kallikrein activities and thus a blank reading is necessary.

Step A for immunoglobulin blank	Blank Tube No. 1
Immunoglobulin	25 μl
Buffer (37°C)	100 μl
Mix	
Step B for immunoglobulin blank	Blank Tube No. 2
Substrate (37°C)	1000 μl
Mixture from Tube No. 1	25 μl
Mix	

Transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change for at least two minutes in a photometer at 405 nm and at 37°C.

Calculation

Calculate $\Delta A/\text{min}$.
Perform the following calculation for the assay of prekallikrein activator in Immunoglobulin preparations:
 $\Delta A/\text{min sample} - \Delta A/\text{min blank}$
Plot $\Delta A/\text{min}$ for the standards against their prekallikrein activator concentration. Calculate the prekallikrein activator concentration of the sample from the established standard curve.

Appendix

PREPARATION OF PLASMA PREKALLIKREIN FRACTION

The blood and plasma must only come into contact with plastic materials or siliconised glass. No delays are permissible during the preparation. The dispensing of the pool and freezing of the aliquot should be carried out the same day as the chromatographic run is performed.

EQUIPMENT

1. Plastic centrifuge tubes, 50 ml
2. Dialysis tubing 20x30 nm
3. Chromatographic column K26/40 (Plastic or siliconized glass).
4. UV-detector with recorder.
5. Fraction collector

REAGENTS

- I. Sodium citrate 3.8% with Polybrene 1 mg/ml

Tri-sodium citrate (C ₆ H ₅ Na ₃ O ₇ •2H ₂ O)	3.8 g
Polybrene	0.1 g
Distilled water to	100 ml
- II. Buffer pH 8.0 (25°C)

Tris	60.55 g	(50 mmol/l)
Sodium Chloride	11.7 g	(20 mmol/l)
Polybrene	0.5 g	(50 mg/l)
Distilled water	8 l	

Adjust the pH to 8.0 at 25°C by adding approximately 50 ml of 6 mol/l HCl. Fill up to 10 l with distilled water.

- III. DE-52 cellulose (diethylaminoethyl cellulose, Whatman).
Let 75 g of DE-52 cellulose swell in 500 ml of buffer (Reagent II). After six hours decant the buffer and resuspend the gel in 500 ml of buffer (Reagent II). Repeat this step.
- IV. Silicon emulsion 35%

Silicon oil	182 g
Triton X-100	5.4 g
Distilled Water to	313 g

PREPARATION

- A. Add 1.4 ml of silicon emulsion 35% (Reagent IV) to 500 ml of distilled water and mix. Place the glassware in the diluted silicon solution for five minutes and then heat it for two hours at 180°C.
- B. Pack the columns with about 200 ml of preswollen DE-52 cellulose. Equilibrate the column by passing 100 ml of buffer (Reagent II) at a flow rate of about 30 ml/hour.
- C. Boil the dialysis tubing in distilled water for one hour and rinse the tubing several times with distilled water.
- D. Draw 27 ml of whole blood into a 50 ml plastic centrifuge tube containing 3.0 ml of sodium citrate - Polybrene solution (Reagent I).

Note: To avoid contamination with activated clotting factors, discard the first 5.0 ml of blood before collection.

- E. Centrifuge the blood at 2500 rpm (755 x g) for 10 minutes at 20 to 25°C. Decant the plasma.
- F. Dialyse the plasma against buffer (Reagent II) for 24 hours at 20 to 25°C with several changes of buffer. Measure the volume of the dialyzed plasma in a graduated plastic cylinder.
- G. Apply the dialysed plasma to the column and connect the outlet through the UV-detector to the fraction collector.
- H. Elute the column with buffer (Reagent II), about 30 ml/hour and collect 5 ml per fraction.
- I. Pool the peak fractions with A₂₈₀ exceeding 3 to give about the same volume as the dialysed plasma. A₂₈₀ for the pool of fractions must be about 6.
- J. Test the pool for kallikrein activity by mixing 25 µl of the pool with 1000 µl of S-2302 (37°C) in a cuvette and record the absorbance change in a photometer at 405 nm and at 37°C for at least two minutes. Calculate ΔA/min. Requirement: ΔA/min <0.001.
- K. Mix 25 µl of Prekallikrein Activator Standard with 100 µl of the prekallikrein pool and incubate for exactly 45 minutes at 37°C.

- L. Mix 25 µl of the standard-prekallikrein mixture with 1000 µl of S-2302 (37°C). Transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change for at least two minutes in a photometer at 405 nm and at 37°C.
- M. Calculate ΔA/min. Requirement: ΔA/min should be about 0.22.
- N. If the requirements in J and M are fulfilled, freeze the preparation in aliquot and store at -70°C.

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PLASMA PREKALLIKREIN

Determination of prekallikrein in plasma with S-2302.

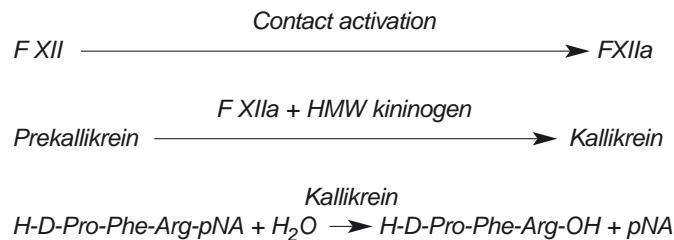
Measurement Principle

The activation of plasma prekallikrein is mediated by the Hageman factor on negatively charged surfaces and in presence of HMW kininogen. A number of methods have been described for the activation of prekallikrein.

This method is related to the use of a prekallikrein activator composed by ellagic acid, phospholipid, Hageman factor and HMW kininogen, which was commercially available from Chromogenix. Different batches of this prekallikrein activator, not available any more, were assayed against the first PKA international standard and resulted in an activity of about 100-120 IU per vial. In the "Reagents" section the main characteristics of the prekallikrein activators have been reported. However, the assay should be validated with respect to the particular activator used.

Following the activation, the plasma kallikrein formed catalyses the hydrolysis of p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA (S-2302). The rate at which pNA is released is measured photometrically at 405 nm. This can be followed on a recorder (initial rate method) or read after stopping the reaction with acetic acid (acid stopped method).

The concentration of prekallikrein is calculated by using standards prepared from normal plasma.



Reagents

1. **S-2302, 25 mg** Art. No. 82 03 40
 Reconstitute the substrate S-2302 (MW: 611.6) with 20 ml of distilled water.

2. **Plasma Prekallikrein Activator**
 Prepare a solution of plasma prekallikrein activator (PKA) with an activity of 20-24 IU/ml. This component should contain ellagic acid, phospholipid and a plasma fraction composed by Hageman factor and high molecular weight kininogen. (Meier H et al. 1977; Wiggins RC et al. 1977; Friberger P et al. 1979; Kerry PJ et al. 1985).

In alternative refer to Claeson G et al. 1978; Friberger P et al. 1979; Shibuya Y et al. 1991, to use other prekallikrein activators.

3. **Tris Buffer, pH 7.8 (25°C)**
 Tris 6.1 g (50 mmol/l)
 Distilled water 800 ml

Adjust the pH to 7.8 at 25°C by adding an appropriate amount (approximately 38 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for six months at 2-8°C.

4. **Normal plasma**
 Blood should be taken from at least 20 healthy donors. 10-30 ml of citrated blood (9 vol blood and 1 vol 0.1 mol/l sodium citrate) are taken from each donor (the first ml of blood is discarded). Plasma is prepared by centrifugation at 2000 x g for 20 minutes at 15-25°C. Equal amounts of plasma from the donors are mixed and dispensed in small volumes. In order to avoid low-temperature activation of prekallikrein the plasma should be kept at 15-25°C and used as soon as possible or quickly frozen at -20°C or below. After thawing at +37°C the plasma should be kept at 15-25°C and used as soon as possible. Frozen plasma is stable for three months at -20°C or below. Avoid refreezing.

5. **Acetic acid, 20%**
 Acetic acid is used in the acid-stopped method.
Equipment

1. Spectrophotometer or microplate reader
2. Semimicrocuvettes, 1 cm or microplates
3. Centrifuge
4. Thermostat, 37°C
5. Stop watch
6. Disposable plastic tubes
8. Photometer with cuvette housing, thermostated at 37°C (for the initial rate method)

Specimen collection

Blood (9 vol) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 15-25°C. In order to avoid low-temperature activation of prekallikrein plasma should be kept at 15-25°C for not more than 24 hours or immediately frozen at -20°C or below. After thawing at 37°C the plasma should be kept at 15-25°C and used as soon as possible. Frozen plasma may lose some prekallikrein on freezing or thawing, but will remain stable for three months at -20°C or below. Avoid refreezing.

Standard curve

The normal plasma has a prekallikrein concentration of 100% and is diluted according to the table below.

Prekallikrein %	Normal plasma μ l	Buffer μ l
25	100	300
50	200	200
75	300	100
100	-	-
125	see below	-

Method

Sample dilution	Tube No. 1
Buffer	3000 µl
Test plasma or standard	50 µl
Mix	

To obtain the 125% standard, mix 125 µl normal plasma with 6 ml buffer.

The test tube method or the Microplate method can be performed by the acid-stopped or the initial rate methods.

Acid stopped method	Test tube	Microplate
Prekall. Activator	200 µl	50 µl
Incubate at 37°C	3-4 min	3-4 min
Sample from tube No. 1	200 µl	50 µl
Mix and incubate at 37°C	2 min*	2 min*
Substrate (37°C)	200 µl	50 µl
Mix and incubate at 37°C or read the initial rate	2 min	2 min
Acetic acid 20%	200 µl	50 µl

**The incubation time depends from the prekallikrein activator used. 2 min is the incubation time with a PKA with the characteristics described in the reagent section.*

For the acid-stopped method: read the absorbance at 405 nm within 4 hours. If the plasma is icteric, hemolytic or lipemic, plasma blanks should be determined. Plasma blank is prepared by adding the reagents in reverse order starting with the acetic acid, without incubation. Subtract the absorbance of the blank from the absorbance of the corresponding sample. For the initial rate method in test tubes: transfer sample immediately after addition of the substrate to a 1 cm semi-microcuvette (preheated at 37°C) for measurement of the absorbance change at 405 nm.

Calculation

Plot A or $\Delta A/\text{min}$ for the standards against their concentration of prekallikrein on linear graph paper. Read the prekallikrein value for the corresponding A or $\Delta A/\text{min}$ for the unknown test sample from the standard curve.

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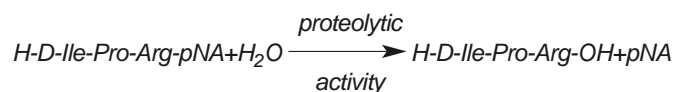
PROTEOLYTIC ACTIVITY

Determination of proteolytic activity in plasma, serum or euglobulin fractions with S-2288.

Measurement Principle

Several proteases with arginine specificity readily split the substrate H-D-Ile-Pro-Arg-pNA (S-2288). The proteolytic activity is thus determined by the rate at which p-nitroaniline (pNA) is released. The formation of pNA can be followed spectrophotometrically at 405 nm by using a recorder (initial rate method).

The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) and the proteolytic activity is usually linear in the 0.05 - 0.5 $\mu\text{kat/l}$ or 3 - 30 U/l range. If possible the linearity of the assay should be checked for each individual type of sample. This can be done by serial dilution of the sample. In several instances the proteolytic activity may originate from α 2-macroglobulin enzyme complexes.



Reagents

- S-2288, 25 mg Art. No. 82 08 52

Reconstitute the substrate S-2288 (MW: 577.6) with 7.2 ml of distilled water to obtain a 6 mmol/l solution.

- Tris Buffer, pH 8.4 (25°C)

Tris	12.1 g	(100 mmol/l)
NaCl	6.2 g	(106 mmol/l)
Distilled Water	800 ml	

Adjust the pH to 8.4 at 25°C by adding an appropriate amount (approximately 44 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, is stable for six months at 2-8°C.

Equipment

- Spectro- or filter photometer, 405 nm with cuvette housing, thermostated at 37°C
- Semi-microcuvettes, 1 cm
- Thermostat, 37°C
- Stop watch
- Disposable plastic tubes

Sample

Dilute the plasma, serum or euglobulin fraction with buffer (Reagent 2) to a proteolytic activity of 0.05 - 0.5 $\mu\text{kat/l}$ or 3 - 30 U/l.

Method

Initial rate method	
Buffer	200 μl
Incubate at 37°C	2-4 min
Diluted sample (20-25°C)	200 μl
Mix and incubate at 37°C	2-4 min
Substrate (37°C)	200 μl

Mix and transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C, calculate $\Delta A/\text{min}$.

Calculation

The proteolytic activity in the sample is calculated from the following formulas:

$$\mu\text{kat/l} = \Delta A/\text{min} \times 5.21 \times F$$

$$\text{U/l} = \Delta A/\text{min} \times 313 \times F$$

F = dilution factor (e.g. 10 if the sample is diluted 1:10 before initial rate determination).

Note: For some enzymes with low K_m less substrate can be used.

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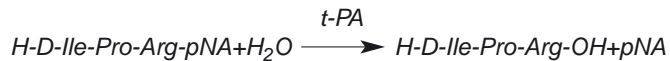
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TISSUE PLASMINOGEN ACTIVATOR (t-PA)

Determination of t-PA in purified preparations with S-2288.

Measurement Principle

Tissue plasminogen activator (t-PA) is a serine proteases, which activates plasminogen by splitting a single Arg-Val bond of the plasminogen molecule. In purified systems these enzymes have been shown to hydrolyse tripeptide chromogenic substrates. The t-PA activity is thus determined by the rate at which p-nitroaniline (pNA) is released. The formation of pNA can be followed spectrophotometrically at 405 nm by using a recorder (initial rate method). The correlation between the change in absorbance per minute ($\Delta A/\text{min}$) and the t-PA activity is linear in the 0.05 - 0.5 $\mu\text{kat/l}$ or 3 - 30 U/l range. The amidolytic activity does not necessarily parallel the fibrinolytic activity for different t-PA preparations.



Reagents

1. S-2288, 25 mg Art. No. 82 08 52
Reconstitute the substrate S-2288 (MW: 577.6) with 8.65 ml (t-PA one-chain) or 43 ml (t-PA two-chain) of distilled water.

2. Tris Buffer, pH 8.4 (25°C)
Tris 12.1 g (100 mmol/l)
NaCl 6.2 g (106 mmol/l)
Distilled water 800 ml

Adjust the pH to 8.4 at 25°C by adding an appropriate amount (approximately 44 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, is stable for six months at 2-8°C.

3. Acetic acid 20%
Acetic acid is used in the acid-stopped method.

Equipment

1. Spectro- or filter photometer, 405 nm with cuvette housing, thermostated at 37°C
2. Semi-microcuvettes, 1 cm
3. Thermostat, 37°C
4. Stop watch
5. Disposable plastic tubes

Sample

Purified tissue plasminogen activator is dissolved in buffer to an enzyme activity of 0.05 - 0.5 $\mu\text{kat/l}$ (3 - 30 U/l). See Note. It has been advised to use a surfactant to avoid adsorption to surfaces. A final concentration of 0.1 g/l of Triton X-100 is recommended.

Method

Initial rate method	
Buffer	200 μl
Incubate at 37°C	2-4 min
Sample (20-25°C)	200 μl
Mix and incubate at 37°C	2-4 min
Substrate (37°C)	200 μl
Mix	

Transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C. Calculate $\Delta A/\text{min}$.

Calculation

The t-PA activity in the prepared tissue plasminogen activator solution is calculated from the following formulas:

$$\begin{aligned} \mu\text{kat/l} &= \Delta A/\text{min} \times 5.21 \\ \text{U/l} &= \Delta A/\text{min} \times 313 \end{aligned}$$

Note: In the test (600 μl) 0.25 μg (100 IU) of the porcine heart tissue plasminogen activator gives:
 $\Delta A/\text{min} \cong 0.012$ (one-chain)
 $\Delta A/\text{min} \cong 0.065$ (two-chain)

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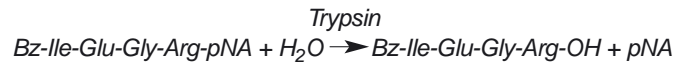
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TRYPSIN

Determination of trypsin in duodenal fluid with S-2222

Measurement Principle

Trypsin catalyses the hydrolysis of p-nitroaniline (pNA) from the substrate Bz-Ile-Glu-(OR)-Gly-Arg-pNA (S-2222). The rate at which pNA is released is followed on a photometer at 405 nm. The reaction rate increases linearly with increasing activities of trypsin up to at least 4.8 $\mu\text{kat/l}$, which corresponds to a trypsin concentration of 2 mg/l.



Reagents

1. S-2222, 25 mg Art. No. 82 03 16
Reconstitute the substrate S-2222 (MW: 741.3) with 34 ml of distilled water.

2. Tris/Calcium Buffer, pH 8.2 (25°C)
Tris 6.1 g (50 mmol/l)
CaCl₂ 2.2 g (20 mmol/l)
Distilled water 800 ml

Adjust the pH to 8.2 at 25°C by adding an appropriate amount of 1 mol/l HCl. Make up to 1000 ml with distilled water. If not contaminated by microorganisms, the buffer is stable for six months at 2 to 8°C.

3. HCl, 1 mmol/l
1 mmol/l HCl is used for dilution of samples.

Equipment

1. Photometer with cuvette housing, thermostated at 37°C
2. Semi-microcuvettes, 1 cm.
3. Water bath or thermostat, 37°C
4. Stop watch
5. Disposable plastic tubes
6. Centrifuge
7. Ice-bath

Sample

A single lumen plastic tube is used (ID:2 mm, OD:4 mm, length: 125 cm) with 4-6 holes cut in the distal 10 cm and a stainless leader at the tip. The position of the tube is checked by X-ray immediately before the test.

Duodenal fluid is collected after stimulating pancreatic secretion with either 300 ml of water, orally, or preferably secretin, intravenously, 1U/kg body weight. Duodenal fluid is collected in 4 x 15 min samples by siphon action in 250 ml plastic bottles and kept on ice (1°C). The samples may be stored at -20°C for not more than a week. Just before analysis, thaw the sample quickly at 37°C. If the fluid is turbid, centrifuge it at 2-8°C and then keep the supernatant on ice.

Determine the pH of the samples. (Note: if the pH of the duodenal fluid is below 5, this indicates the presence of a large amount of gastric juice, which may yield an incorrect value). Dilute the sample at 1:100 or 1:1000 with 1 mmol/l HCl and keep it on ice. At low trypsin activities the sample is assayed undiluted or diluted 1:10.

Method

Initial rate method	
Buffer	800 μl
Incubate at 37°C	5-6 min
Diluted sample	100 μl
Mix and incubate at 37°C	1-2 min
Substrate (37°C)	100 μl
Mix	

Transfer the sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) and measure the change in absorbance in a photometer at 405 nm and at 37°C.

Calculation

Calculate $\Delta A/\text{min}$ for the sample.
The trypsin activity is then calculated from the formula:
 $\mu\text{kat/l} = \Delta A/\text{min} \times 17.36 \times F$
 $\text{U/l} = \Delta A/\text{min} \times 1042 \times F$
 $F = \text{Dilution factor for sample (e.g. 100 when diluted 1:100)}$

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UROKINASE

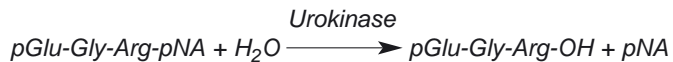
Determination of urokinase activity with S-2444.

Measurement Principle

The urokinase activity is determined by its amidolytic effect on the substrate pyro-Glu-Gly-Arg-pNA (S-2444). The rate at which p-nitroaniline (pNA) is released is measured photometrically at 405 nm.

This can be followed on a recorder (initial rate method) or read after stopping the reaction with acetic acid (acid stopped method).

The correlation between $\Delta A/\text{min}$ (or absorbance) and the urokinase activity is linear in the range 5-40 Ploug or CTA units. The urokinase concentration should preferably be given in units of substrate hydrolysing activity, but may be calculated by using standards prepared from a standard urokinase preparation. The amidolytic activity, however, does not necessarily parallel the fibrinolytic activity for different urokinases.



Reagents

- S-2444, 25 mg** Art. No. 82 03 57
Reconstitute the substrate S-2444 (MW: 498.9) with 16.7 ml of distilled water.
- Urokinase standard**
The urokinase standard is dissolved in or diluted with Solvent (Reagent 3) to a concentration of 400 units/ml (Ploug or CTA units). The dilution is stable for one day at 2-8°C.
- Solvent**
Distilled water containing 5 g/l of Carbowax 6000 (Union Carbide, NY, USA).

- Tris Buffer, pH 8.8 (25°C)**
Tris 6.1 g (50 mmol/l)
NaCl 2.2 g (38 mmol/l)
Distilled Water 800 ml

Adjust the pH to 8.8 at 25°C by adding an appropriate amount (approx. 12 ml) of 1 mol/l HCl. Fill up to 1000 ml with distilled water. The buffer, if not contaminated, will remain stable for two months at 2-8°C.

Note: Although the substrate is quite selective, there may be a risk for influence of other proteases if the preparation is heavily contaminated. The addition of Trasylol (aprotinin), 10 KIU/ml, to the buffer may in such cases be favourable.

- Acetic acid 20%**
Acetic acid is used in the acid-stopped method.

Equipment

- Spectro- or filter photometer, 405 nm
- Semi-microcuvettes, 1 cm.
- Thermostat, 37°C
- Stop watch
- Disposable plastic tubes
- Photometer with cuvette housing, thermostated at 37°C (for the initial rate method)

Sample

The urokinase is dissolved in or diluted with Solvent (Reagent 3) to a concentration of approximately 400 units/ml (Ploug or CTA units) By using commercially available urokinase (Leo or Abbott) it was found that the dilution was stable for at least one day when kept at 2-8°C.

Note: if the urokinase preparation is contaminated with proteolytic enzymes, Trasylol (aprotinin) may be added to a concentration of 10 KIU/ml in order to increase the stability.

Standardisation

40 units: Use the urokinase standard 400 units/ml (Reagent 2).
5 units: Use the urokinase standard 400 units/ml (Reagent 2) diluted 1:8 with buffer (Reagent 4).

Standard curve

The urokinase standard 400 units/ml (Reagent 2) is further diluted according to the table below:

Ploug or CTA Units	Urokinase standard (400 units/ml) μl	Solvent μl
5	100	700
10	100	300
20	200	200
30	300	100
40	400	-

Method

Initial rate method	
Buffer	800 μl
Incubate at 37°C	5-10 min
Urokinase sample/standards	100 μl
Mix and incubate at 37°C	1-2 min
Substrate (37°C)	100 μl

Transfer sample immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C, calculate $\Delta A/\text{min}$.

Acid stopped method	Sample	Sample blank
Buffer	800 µl	800 µl
Incubate at 37°C	5-10 min	-
Urokinase sample/standards	100 µl	100 µl
Mix and incubate at 37°C	1-2 min	-
Substrate (37°C)	100 µl	-
Mix and incubate at 37°C	5 min	-
Acetic acid 20%	100 µl	100 µl
Mix	yes	yes
Substrate (37°C)	-	100 µl
Mix	-	yes

Read the absorbance (A) of the sample against a water or sample blank in a photometer at 405 nm. The colour is stable for at least 4 hours.

Calculation

Plot $\Delta A/\text{min}$ or A for the standards against their known urokinase activity. Calculate the urokinase activity of the sample in Ploug or CTA units. By multiplying the results with 10 the concentration in units/ml is obtained. The urokinase activity can also be calculated from the following formulas:

Initial rate method:

$$\mu\text{kat/l} = \Delta A/\text{min} \times 17.4$$

$$\text{U/l} = \Delta A/\text{min} \times 1042$$

Acid stopped method:

$$\mu\text{kat/l} = A \times 3.8$$

$$\text{U/l} = A \times 229$$

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PROTHROMBIN ACTIVITY

Determination of prothrombin activity in plasma with S-2238.

Background

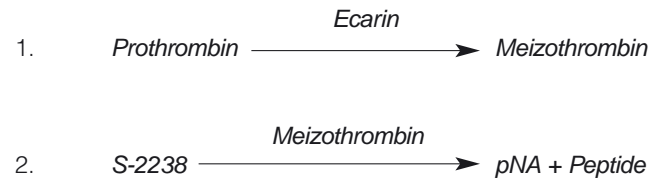
A number of studies during the last few years support the notion that venous thromboembolism (VTE) is a multifactorial disease most often triggered by circumstantial risk factors (trauma, surgery, pregnancy, oral contraceptives, immobilisation and age) in combination with one or more genetic or acquired coagulation disorders (see ref. 1 of a review). Elevated activity of prothrombin in the absence of a known underlying genetic disorder is also associated with an increased thrombotic risk².

A mutation G → A in the untranslated 3'-region of the prothrombin gene at nucleotide position 20210 constitutes a risk factor for VTE with an odds ratio of 3-5²⁻¹⁰. About 90% of the carriers of this mutation have elevated levels (>115%) of prothrombin activity^{2,7,8}. Levels above the upper limit of the normal range (75 – 130%) are commonly hetero- and homozygotes^{2,7-9}.

So far, there is no explanation why a comparatively mild increase of prothrombin activity constitutes a risk factor for thrombosis and this is therefore an area of active clinical and biochemical research. Chromogenic methods for accurate determination of elevated activities of prothrombin and other coagulation factors, such as factor VIII^{11,12} are important tools for assessing the risk for VTE in patients and family members.

Measurement Principle

Prothrombin is activated to meizothrombin by the snake venom enzyme Ecarin from *Echis Carinatus*. After a certain incubation time, the amount of meizothrombin formed is measured with the thrombin selective substrate S-2238, which also is cleaved by meizothrombin. The absorbance recorded at 405 nm is proportional to the prothrombin activity in the sample.



Reagents

- Tris BSA Buffer** Art. No. 82 35 18
Buffer for sample dilution, containing 0.5 mol/l Tris HCl pH 7.3, I= 2.0 with NaCl and 2% bovine serum albumin. Before use, dilute the stock solution 1+9 with sterile water to obtain a buffer working solution. The buffer working solution is prepared and used within the same day.
- Prothrombin Activator Dil.** Art. No. 82 35 26
Buffer for dilution of Ecarin, containing 0.05 mol/l Tris HCl pH 7.6, I=0.15 with NaCl, bovine serum albumin, polyethylene glycol and a fibrin polymerization inhibitor.
- Ecarin (Sigma, E504)**
Reconstitute with sterile water according to the Ecarin package insert. Freeze in suitable aliquots at -20°C or at -70°C. Stable for 3 months at both storage temperatures. Before use, dilute with Prothrombin Activator Diluent to obtain a concentration of 2.4 U/ml. Stable for 8 hours at 20-25°C and for 1 week at 2-8°C.
Note: Echis Carinatus crude venom can also be used. A suitable final concentration of this reagent is approximately 5 µg/ml; however, this may vary between different sources. 10-20% loss of activity may occur upon freezing at -20°C.
- S-2238** Art. No. 82 03 24
Reconstitute with 13 ml of sterile water to obtain a 3 mmol/l solution.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 volume) and centrifuged at 2000 x g for 20 min at 20-25°C. Separate plasma carefully from blood cells. Perform the analysis within 24 hours when plasma is stored at 2-25°C. Alternatively, freeze aliquots ≤ 1ml at -20°C or below. Perform the analysis of frozen samples within two months when stored at -20°C or within one year when stored at -70°C or below. No significant loss of prothrombin activity occurs upon freezing once, provided freezing is made in small aliquots (< 1 ml) and thawing is performed in a water bath or in an electric heater at 25-37°C.

Sample and Standard Dilutions

Standards

Calibrated normal plasma is diluted 1:23 – 1:160 to provide standard concentrations of 25-175%. The following table provides a suggestion of standard dilutions.

Standard Dilution	Prothrombin Activity
1:18	167%
1:22	136%
1:30	100%
1:60	50%
1:120	25%

Samples

Plasma samples are diluted 1:40 in Tris BSA Buffer working solution for application on microplate and diluted 1:80 for application on ACL (see below).

Microplate Assay Procedure

Standard/Sample dilution	50 µl
Incubate at 37°C	2-4 min
Ecarin or Echis Carinatus (37°C)	50 µl
Incubate at 37°C	3 min
Substrate (37°C)	50 µl
Read kinetically or incubate at 37°C	2 min
Acetic acid, 20%	50 µl

Determine the absorbance difference A405nm-490 nm for the standard dilution and the samples. Draw a standard curve from the absorbances obtained for the standard dilutions. Read the prothrombin activity for the samples from the standard curve.

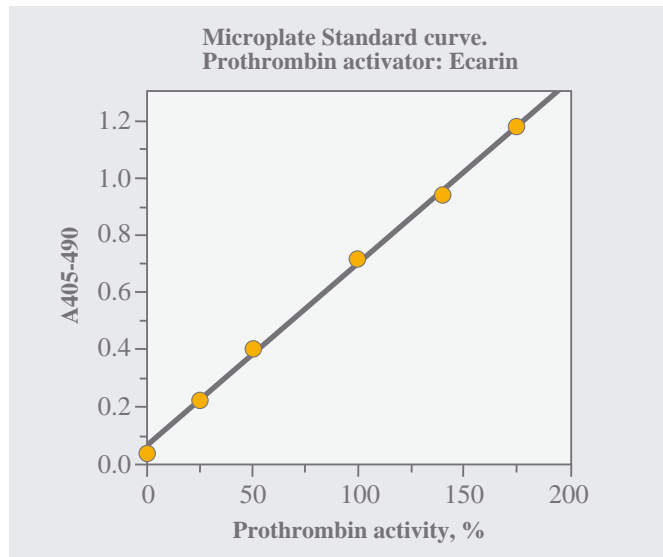


Fig. 1. Standard curve with the microplate method.

Application on ACL

Use the plasminogen channel program. Prepare a standard dilution 1:40, which corresponds to a nominal prothrombin activity of 100% (see above regarding calibration). Standard dilutions corresponding to 25% and 50% are then automatically prepared by the instrument. In order to allow determination of prothrombin activity up to 200%, sample plasma should be diluted 1:80 and the obtained result should be multiplied with two.

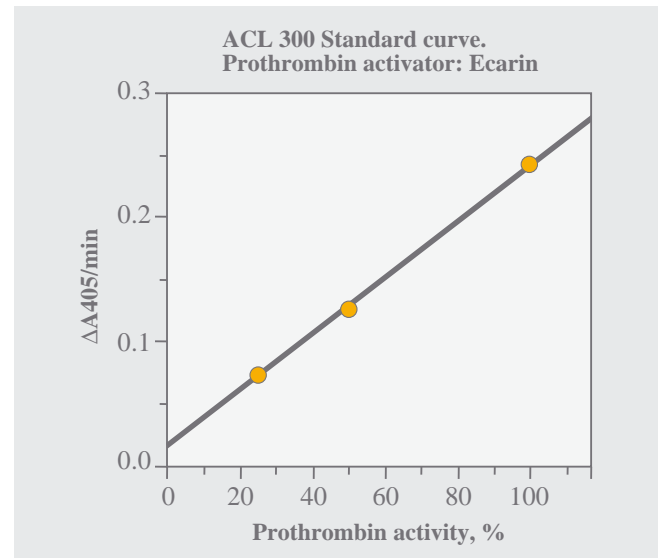


Fig. 2. Standard curve with the ACL method

Expected values¹³

The normal range is 75 – 130% (mean 102% ± 2 SD) as determined from analysis in microplate and on the ACL 300 of 101 healthy individuals (49 men and 52 women; age range 20 – 68 years). Analysis of plasma from 42 carriers of the G20210A mutation, who were not on oral anticoagulant treatment at the time of blood sampling, resulted in an activity range of 94 – 164% (mean 128% ± 2SD).

Interference and Limitations

No influence in the assay is obtained from variation of antithrombin activity in the range 50 - 150% of normal. Since meizothrombin is formed and measured, no influence in the assay is obtained from heparin levels ≤ 1 IU/ml plasma. Since Ecarin also activates decarboxyprothrombin, which is produced during oral anticoagulant therapy with anti-vitamin K drugs, plasma from patients undergoing such treatment should not be analysed with this method.

Repeatability

The imprecision, expressed as CV, within and between series (7 series, 5 replicates in each series) is ≤4% at 50% and 100% prothrombin activity.

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ELEVATED FACTOR VIII

Determination of elevated levels of factor VIII activity in plasma.

Background

Historically, factor VIII (FVIII) has always been associated with bleeding, since the well known pathological condition Haemophilia A is a consequence of very low plasma levels of FVIII. On the other hand, FVIII is also an acute phase reactant, and hence elevated levels are associated with conditions such as trauma, infection and exercise. Common to many other coagulation factors, FVIII also rises during pregnancy. FVIII is a key procoagulant factor and recent studies have shown the association between elevated levels of FVIII activity and an increased risk of venous and seemingly also arterial thrombosis¹⁻⁴. A concomitant increase was also noticed for von Willebrand factor¹⁻³. Importantly, FVIII activity shows a high correlation to FVIII antigen (FVIII:Ag), thereby attributing the increased activity to an increased FVIII synthesis^{2, 5}. So far the increased plasma FVIII:Ag has not been linked to any polymorphism of the FVIII gene promoter⁵, but the search for a genetic contribution is still under investigation. FVIII activity in thrombotic patients is often above 1.5 IU/ml and might reach levels of 4-5 IU/ml², sometimes in connection with highly inflammatory conditions. Therefore, a specific adaptation of Coamatic Factor VIII has been developed to allow accurate determination of elevated FVIII activity. The advantages in using a chromogenic method as compared to one-stage clotting methods are numerous. In particular the chromogenic method is not sensitive to preactivation of FVIII⁶, thereby avoiding overestimation of FVIII activity. Furthermore, due to its linear dose-response, it has a higher resolution at elevated levels and also a high precision. These features make Coamatic Factor VIII ideal as a tool for thrombophilia screening in addition to its established use for diagnosis of hemophilia and FVIII potency estimation of concentrates.

Reagents: Coamatic Factor VIII

Kit configuration

S-2765 + I-2581	1 vial
Factor reagent	2 vials
Buffer, stock solution	1 vial

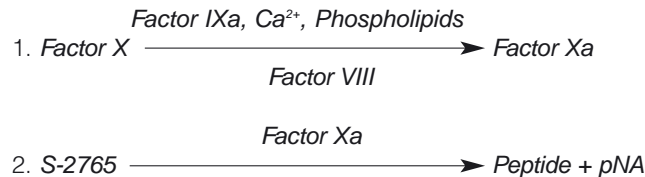
Measurement principle

Factor VIII acts as an enzymatic cofactor of factor IXa during the activation of factor X to factor Xa in the presence of calcium ions and phospholipids.

Factor Xa hydrolyses the chromogenic substrate S-2765 thus liberating the chromophore pNA.

The colour is then read spectrophotometrically at 405 nm. The generated factor Xa and thus the intensity of colour is proportional to the factor VIII activity in the sample.

Thrombin, that is contained in the factor reagent, brings about a rapid and complete activation of the FVIII present in the sample.



Specimen collection

Follow the instructions described in the Coamatic Factor VIII package insert.

Determination of elevated levels of Factor VIII activity

The applications of the Coamatic Factor VIII kit are currently referred to a low assay range and to a normal assay range. The upper measuring limit using the procedure for the normal assay range is 1.42 IU/ml for the microplate method and 1 IU/ml for the ACL method. The determination of FVIII activities higher than these limits, can be performed by pre-diluting the plasma samples 1:4 and assaying the diluted samples following the protocol described for the normal

assay range but restricting this range to 0-1 IU/ml. The results should be multiplied by 4 to obtain the final value of FVIII activity.

- Pre-dilute 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 (microplate procedure) or in the instrument application sheet (automated instruments).

Microplate method

Reagent preparation

Factor reagent:	3.0 ml of sterile water
Substrate:	6.0 ml of sterile water
Buffer:	dilute 1:10 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 FVIII. In case the normal plasma does not contain exactly 1 IU/ml FVIII, the values of the standard must be recalculated accordingly.

FVIII IU/ml	Predilution		Final dilution	
	Plasma μ l	Buffer μ l	Diluted Plasma μ l	Buffer μ l
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) Pre-dilute the sample by mixing 1 vol plasma with 3 vol of Coamatic Factor VIII Buffer
- 2) Dilute further as follows:

Sample	25 μ l
Buffer	2000 μ l

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.

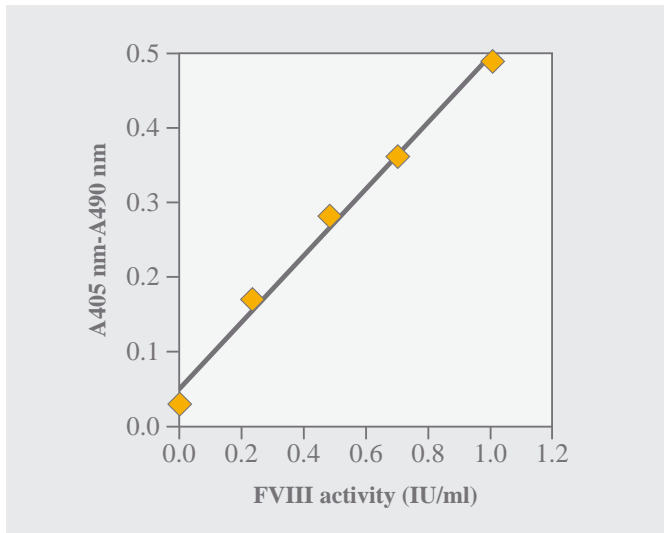


Fig. 1. Standard curve with the microplate method.

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 1:10 with sterile water

Standard curve

The standard curve is prepared by using a human normal plasma calibrated against an International Standard for plasma FVIII.

Dilute the standard as follows: 25 µl plasma + 2000 µl buffer

Sample Dilution

1. Pre-dilute 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.

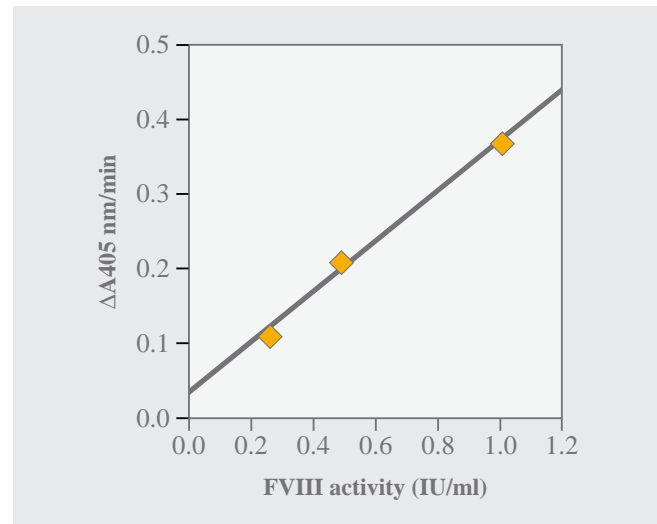


Fig. 2. Standard curve with the ACL method.

Measuring range

With pre-dilution 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 1 and 2 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 diluted 1:4.

The precision of the method has been evaluated by using plasma samples diluted according to the protocol described above.

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

The FVIII activity of 130 patient samples has been determined with Coamatic Factor VIII on ACL, by pre-diluting 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 3):

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

For FVIII activities higher than 1 IU/ml, the samples can be under-estimated if the pre-dilution is not performed.

Coamatic Factor VIII has been compared with a one-stage clotting method on the ACL analyser.

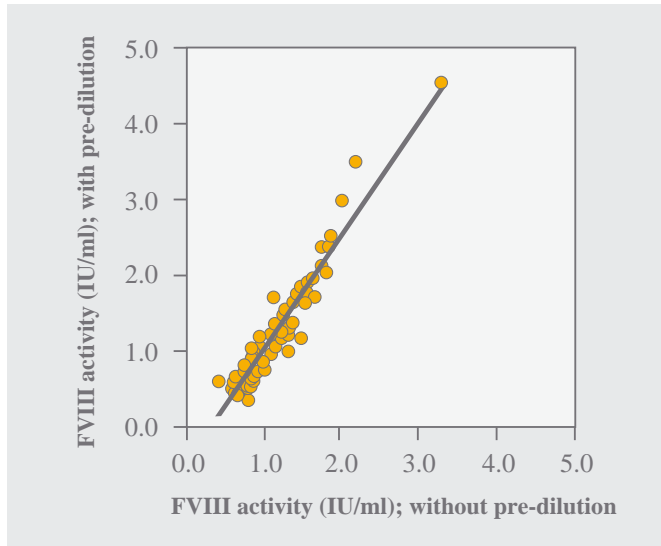


Fig. 3. Comparison of FVIII activities obtained with and without sample pre-dilution using Coamatic Factor VIII.

For the Coamatic Factor VIII assay, the samples were pre-diluted 1:4 as recommended in the protocol described above.

For the clotting method the plasma samples were pre-diluted 1:4 (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 1:5.

71 plasma samples from thrombotic patients were analysed. The results are shown in figure 4.

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 FVIII

Range (y) = 0.18 – 2.51 IU/ml FVIII

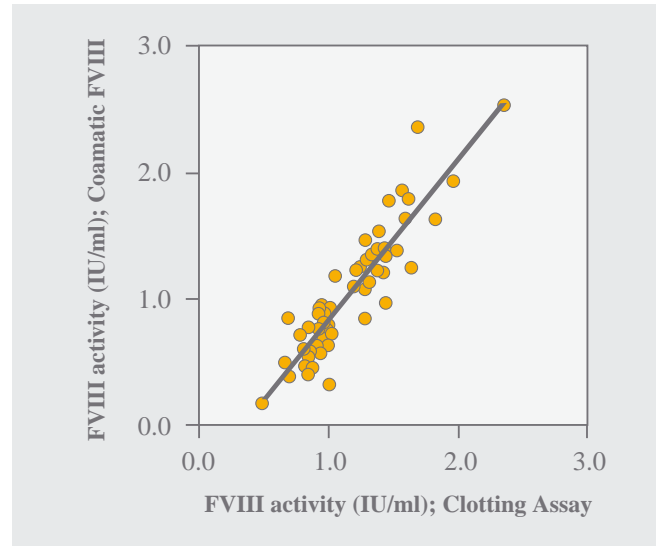


Fig. 4. Comparison of FVIII activities obtained by a clotting assay and Coamatic Factor VIII.

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 FVIII activity higher than 1.4 IU/ml, thus confirming earlier published data^{1,2}.

These results have been obtained by a simple modification of the existing applications and protocols, consisting in the pre-dilution 1:4 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 pre-dilution 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.

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HIRUDIN

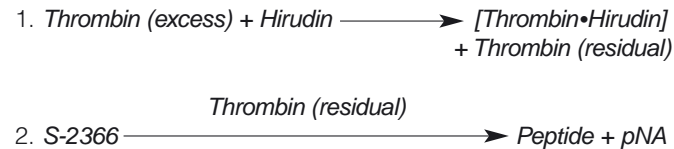
Determination of hirudin levels in plasma with the chromogenic substrate S-2366.

Background

Hirudin is a protein originating from the medical leech^{1,2}. It is the most potent thrombin inhibitor with a dissociation constant below 10^{-12} mol/l. Due to the production of hirudin through recombinant technology, this protein is now readily available in abundant amounts and hence it has become an interesting candidate as a new antithrombotic agent³⁻⁶. The therapeutic range of hirudin is roughly 0.5 – 2.5 µg/ml. Hirudin may be determined by ELISA methods, by clotting methods (APTT, thrombin time or Ecarin clotting time (ECT)) or by chromogenic methods^{7,8}. The latter is insensitive to oral anticoagulants⁹ and is suitable for automation. The ECT method is the most competitive of the clotting methods being essentially insensitive towards heparin. However it shows some sensitivity to changes in plasma concentrations of prothrombin and fibrinogen^{9, 10}. The chromogenic methods described here may be used for the monitoring of hirudin levels in plasma samples. The same principle may be used for the potency assessment of hirudin preparations. In this case the potency of hirudin is expressed in thrombin inhibitory units (TIU)¹¹ and the international standard for thrombin must be used.

Measurement principle

Thrombin is added in excess to the sample. Thrombin activity is neutralised in proportion to the amount of hirudin contained in the sample, and the remaining amount hydrolyses the chromogenic substrate S-2366. The pNA released upon hydrolysis of the substrate is then monitored photometrically at 405 nm.



Reagents

- S-2366, 25 mg** Art. No. 82 10 90
Reconstitute with 20 ml sterile water.
- Human Thrombin, 10 NIH-U (Sigma, T-9135)**
Reconstitute with 7.1 ml sterile water to obtain a solution 1.4 NIH-U/ml. The solution is stable for one week at 2-8°C
- Polybrene® (Sigma, H-9268)**
Dissolve the substance with water to obtain 1 mg/ml.
- Tris EDTA Buffer** Art. No. 82 33 66
10 ml stock solution
Buffer containing 0.5 mol/l Tris pH 8.4, 1.5 mol/l NaCl, 70 mmol/l Disodium-EDTA. An opened vial is stable for 2 months at 2-8°C. Before use, dilute 10 ml of the stock solution with 88.7 ml sterile water. Add 1.26 ml polybrene solution.
- Hirudin**
Prepare a stock solution of 500 µg/ml hirudin.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 20-25°C. Separate plasma carefully from the blood cells.

Standard and Sample Dilutions

Standards

Low range (0 – 2 µg/ml)

Hirudin µg/ml	Predilution		Final dilution	
	Hirudin Stock µl	Water µl	Diluted Hirudin µl	Plasma µl
2.0	200	300	10	1000
1.5	150	350	10	1000
1.0	100	400	10	1000
0.5	50	450	10	1000
0	-	-	-	1000

High range (0 – 10 µg/ml)

Hirudin µg/ml	Hirudin Stock µl	Plasma µl
10.0	20	1000
7.5	15	1000
5.0	10	1000
2.5	5	1000
0	-	1000

Samples and Standards

Dilution	Low dose range	High dose range
Sample/Standard	50 µl	25 µl
Buffer	800 µl	2000 µl

Microplate Assay Procedure

	Sample	Blank
Sample/Standard	50 µl	50 µl
Incubate at 37°C	3-4 min	-
Thrombin (20-25°C)	50 µl	-
Incubate at 37°C	2 min	-
S-2366 (37°C)	50 µl	-
Incubate at 37°C	2 min	-
Acetic acid, 20%	50 µl	50 µl
Water	-	100 µl

Read the absorbance at 405 nm, using a reference wavelength of 490 nm. The colour is stable for at least 4 hours. Subtract the absorbance for the blank from the absorbance of the corresponding standards and test plasma sample. Plot the corrected absorbances for the standards against hirudin concentrations in a lin-lin graph and draw the standard curve from linear regression. The concentration of hirudin in the test sample is calculated from the standard curve.

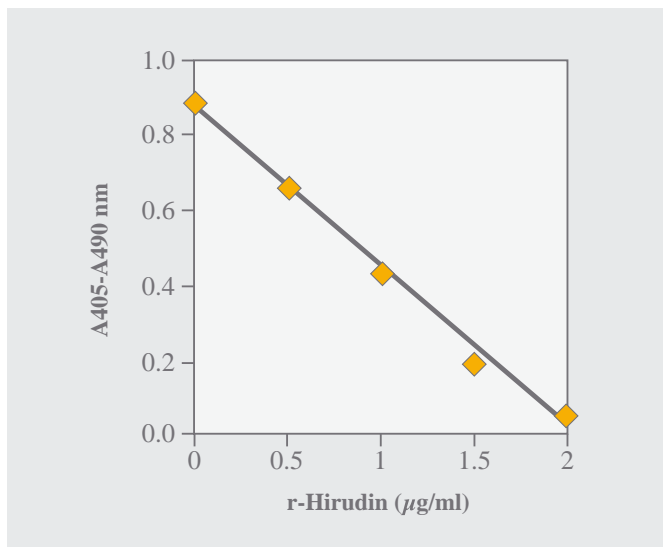


Fig. 1. Low range standard curve with the microplate method. Recombinant hirudin was used.

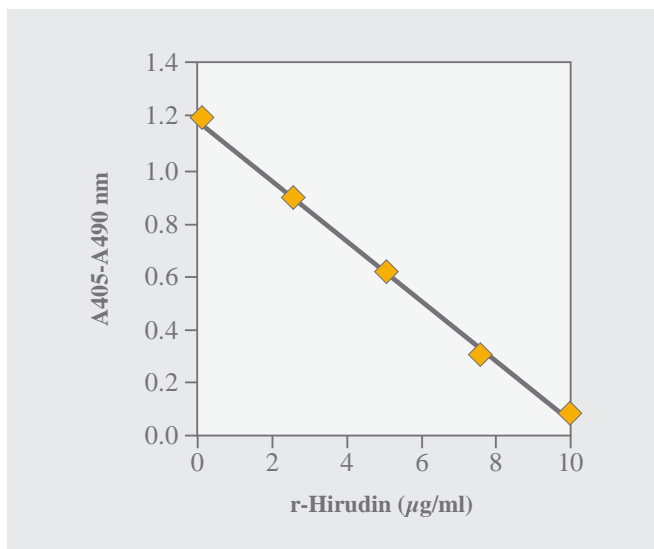


Fig. 2. High range standard curve with the microplate method. Recombinant hirudin was used.

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TFPI (Tissue Factor Pathway Inhibitor, type - I)

Determination of TFPI activity in plasma with S-2222 (microplate method) .

Background

Human TFPI is a modular protein synthesized primarily by the vascular endothelium under normal physiologic conditions; small amounts are also expressed by monocytes and macrophages.

The regulatory activity of TFPI is directed towards the extrinsic initiation pathway of the coagulation cascade involving binding and direct inhibition of factor Xa. It also inhibits the Tissue Factor/ factor VIIa complex in a factor Xa dependent fashion. TFPI expression can be modulated in several cell types in response to various inflammatory stimuli.

In vivo TFPI is distributed in three pools: 80-85% is associated with endothelial cell-surface, and it is released into plasma after injection of heparin; 10% circulates in plasma primarily in association with the lipoproteins and a small amount in free form, and 3% is found in platelets.

TFPI levels are elevated in pregnancy and lower in the newborn than those in the adult, while recently a significant reduction was found in woman taking combined oral contraceptives, suggesting a potential underlying cause for an increased risk for thrombosis. The role of TFPI in several thrombotic, inflammatory and malignant conditions is already established.

Measurement Principle

TFPI activity assay is based on the ability of TFPI in the sample to inhibit TF/FVIIa catalytic activity, in presence of FXa. Plasma is incubated for a prolonged time, allowing the formation of inactive TF/FVIIa/TFPI/FXa complexes. Fibrin polymerization inhibitor, I-2882, is added to prevent formation of cross-linked fibrin.

After first incubation, residual TF/FVIIa catalytic activity is determined by the addition of FX and a selective chromogenic substrate, S-2222.

Reagents

1. TBS/BSA/Polybrene® Buffer
(0.05 M Tris-HCL, 0.15 M NaCl, pH 7.5, supplemented with 2.0 mg/ml BSA and 2.0 µg/ml Polybrene®)
Dissolve 6.057 g Tris base, 8.766 g NaCl, 2.0 g BSA, and 2.0 mg Polybrene® in 900 ml H₂O, adjust the pH to 7.5 with 2 M HCl and top up to 1000 ml with H₂O

2. Acetic acid (50 % (V/V))
Add 125 ml 100 % Acetic acid to 125 ml H₂O

3. CaCl₂ (50 mM)
Dissolve 0.7351 g CaCl₂ - 2H₂O in 100.0 ml H₂O

4. I-2882 (10mg/ml) Art.No. 82 38 15 10
Dissolve the vial content (40 mg) with 4.0 ml H₂O

5. Factor Xa, bovine (7,1 nkat/ml) Art.No. 82 09 85
Dissolve the vial content (71 nkat) with 10.0 ml H₂O

6. Recombinant Human Tissue Factor
(RecombiPlasTin, Instrumentation Laboratory
Art.No. 49 73 27 50)
Dissolve the vial content with 5.0 ml diluent

7. Recombinant Factor VIIa (30 kIE/ml)
(NovoSeven, Novo Nordisk)
Dissolve the vial content (240 kIE = 4.8 mg) with 8.5 ml diluent

8. Factor X, bovine (2 U/ml) Art.No. 82 22 39
Dissolve the vial content (2 U) in 1.0 ml H₂O

9. S-2222 (2.7 mM) Art.No. 82 03 16
Dissolve the vial content (25 mg) in 12.49 ml H₂O

10. HCl 2M

11. Normal plasma

Additional Material

Fridge or ice-bath
Thermostat at 37°C
Water bath at 37°C
Microplates, flat-bottom
Lids for microplates or Parafilm®
Spectrophotometer 405 nm

Storage Conditions

Store the Reagents 1 and 3-7 at 2-8°C or on ice until use it. Reagents 8 and 9 can be prepared during the first incubation step (30 min) and should have room temperature. All the reconstituted reagents can be used over a period of at least three weeks at 2-8°C. Avoid contamination by microorganism.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000xg for 20 minutes at 20-25°C. Separate plasma carefully from the blood cells. Plasma can be stored in aliquots at -70°C.

Standard Curve

- Dilute Normal Plasma 1:10 with ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)
- Dilute this 1:10 dilution (= 10 %) further to 7.5 %, 5.0 %, 3.75 %, 2.5 %, 1.25 %, 0.625 % and 0.313 % with ice cold TBS/BSA Buffer/Polybrene® (Reagent 1).

Note: an aliquot of 100 µl of each dilution (10 % - 0 %) is sufficient for the standard curve.

Sample Dilution

Normal Samples

10 µl Plasma + 390 µl ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)

Heparinized Samples

10µl Plasma + 790µl ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)

Preparation of the Combined Reagent

(sufficient for 1 plate)

1. Factor VIIa 10µl
2. Tissue Factor 125µl
3. Factor Xa 125µl
4. I-2882 125µl
5. CaCl₂ 2500µl
6. TBS/BSA Buffer 7115µl

Preparation of the Substrate Reagent

(sufficient for 1 plate)

1. Factor X 0.65 ml
2. TBS/BSA Buffer 1.95 ml
3. S-2222 2.60 ml

Microplate Assay Procedure

Generally, the determination of double values is recommended

Standard or Sample Dilution	25µl
Combined Reagent	100µl
Incubate the microplate covered with a lid for 30 min. at 37 °C	
Substrate Reagent	50µl
Incubate the microplate covered with a lid for 30 min. at 37 °C	
Acetic acid	50µl

Blank: For the Blank determined with the 10%-standard, 50 µl acetic acid are added to the wells before the Substrate Reagent.

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An overview of the EUROPEAN PHARMACOPOEIA and of the UNITED STATES PHARMACOPEIA

With the advent of the chromogenic technology, and the validation of several methods and applications over the last two decades, the use of chromogenic assays has been extended from the research and/or clinical laboratories, to the quality control laboratories in pharmaceutical companies.

Chromogenix, leader in chromogenic technology, has always been involved in collaborating at international level for the development of such procedures. The aim of the following paragraphs is to provide a summary of the principal plasma-derived products, of the blood-coagulation related drugs and of the chromogenic assays as described in the European and the United States Pharmacopoeia.

You will find some of the recommendations reported in both the Pharmacopoeias, with particular reference to the application of chromogenic assays. However most of the information, concerning the production procedures, or assays with no relevance for the purpose of this text, have been deliberately not described. Thus, for a more comprehensive information on the Pharmacopoeia protocols, the consultation of the original text is strongly recommended.

In Europe and in United States, the regulation about licensing, marketing, testing medicines differs for several aspects. Here below there is a summary of the different authorities.

The European authorisation system EMEA, the European Agency for the Evaluation of Medicinal Products, is operating since 1995 with the aim to regulate licensing and surveillance of human and veterinary medicinal products within Europe. The European system operates following both a centralised procedure of authorisation, for product derived from biotechnology and high technology and a decentralised procedure, for most of the conventional medicines (please visit www.eudra.org).

From the cooperation between the Council of Europe and the Commission of the European Communities, in 1964 started the European Pharmacopoeia (www.phEur.org). Respectively in 1975 and 1981 for human and veterinary medicines, two EEC Directives made the monographs of the European Pharmacopoeia obligatory for marketing authorisation dossiers. In 1994 the European Directorate for the Quality of Medicines, EDQM, previously in charge of the European Pharmacopoeia Secretariat, also became responsible for the European network of laboratories for the quality control of medicines.

The European Pharmacopoeia is the official compendium published by EDQM. It is now at the fourth edition (2002); it is completed by 3 supplements a year.

The United States authorisation system

Food and Drug Administration (FDA; www.fda.gov) is the governmental body in United States, who is responsible to enforce laws and regulations on foods, drugs, medical devices and cosmetics. FDA is organised in five centres: Center of Biologics Evaluation and Research (CBER); Center for Drug Evaluation and Research (CDER); Center for Devices and Radiological Health (CDRH); Center for Food Safety and Applied Nutrition (CFSAN); Center for Veterinary medicine (CVM).

The official methods for laboratory procedures are, according to FDA, those described in the United States Pharmacopoeia (USP; www.usp.org) and in the Code of Federal Regulations. In absence of a particular method, FDA may also utilise the Official Methods of Analysis of the Association of Analytical Chemists (AOAC; www.aoc.org), or may recommend a procedure or a published method. The current issue of the United States Pharmacopoeia is the USP25-NF20.

Chromogenix believes that the information set forth in this section is in accord with current recommendations and/or requirement of European and US Pharmacopoeia at the time of publication, they accept no legal responsibility for any errors or omissions and make no warranty, expressed or implied, with respect to material contained herein. In view of the ongoing research, changes in government regulations and the constant flow of information relating to these areas the reader is urged to review and evaluate the information provided by the European and US Pharmacopoeia as the primary reference.

EUROPEAN PHARMACOPOEIA 2002 FOURTH EDITION

Plasma derived products and haemostasis related drugs: The Monographs

Monograph	Tests recommended (E.P. reference methods)	Test Principle*	Assays Recommended (E.P. reference methods)	Assay Principle*
Human Plasma for Fractionation 2002:0853	<ul style="list-style-type: none"> • Anti-HIV-1 • Anti-HIV-2 • HBsAg • Anti-HCV • Total protein (2.5.9) • Factor VIII (2.7.4) 	- - - - - - Chromogenic	N/A	-
Albumin Solution, Human 2002:0255	<ul style="list-style-type: none"> • pH (2.2.3) • Total protein (2.5.9) • Protein composition (2.2.31) • Molecular size distribution (2.2.29) • Haem (2.2.25) • Prekallikrein activator (2.6.15) • Aluminium (Method I, 2.2.23) • Potassium (Method I, 2.2.22) • Sodium (Method I, 2.2.22) • Sterility (2.6.1) • Pyrogens (2.6.8) 	- - - - - - Chromogenic - - - - - -	N/A	-
Human Normal Immunoglobulin for Intravenous Administration 2002:0918	<ul style="list-style-type: none"> • Solubility (monograph) • pH (2.2.3) • Osmolality (2.2.35) • Total protein (2.5.9) • Protein composition (2.2.31) • Distribution of molecular size (2.2.29) • Anticomplementary activity (2.6.17) • Prekallikrein activator (2.6.15) • Anti-A and anti-B Haemagglutinins (2.6.20) • Water (2.5.12) • Sterility (2.6.1) • Pyrogens (2.6.8) • Antibody to hepatitis B surface antigen (2.7.1) 	- - - - - - - Chromogenic - - - - - -	N/A	-

* Only chromogenic, clotting or fibrin clot lysis methods are indicated



Monograph	Tests recommended (E.P. reference methods)	Test Principle*	Assays Recommended (E.P. reference methods)	Assay Principle*
Human Coagulation Factor VII 2002:1224	<ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Osmolality (2.2.35) • Total protein (2.5.9) • Water (2.5.12) • Activated coagulation factors (2.6.22) • Heparin (2.7.12) • Thrombin (monograph) • Sterility (2.6.1) • Pyrogens (2.6.8) <p>Consistency of the method of production:</p> <ul style="list-style-type: none"> • Factor II (2.7.18) • Factor IX (2.7.11) • Factor X (2.7.19) 	<p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>Clotting</p> <p>Chromogenic</p> <p>Clotting</p> <p>–</p> <p>–</p> <p>Chromogenic</p> <p>Clotting</p> <p>Chromogenic</p>	<ul style="list-style-type: none"> • Factor VII activity (2.7.10) • Factor II activity (2.7.18) • Factor IX activity (2.7.11) • Factor X activity (2.7.19) 	<p>Chromogenic</p> <p>Chromogenic</p> <p>Clotting</p> <p>Chromogenic</p>
Human Coagulation Factor VIII 2002:0275	<ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Osmolality (2.2.35) • Total protein (2.5.9) • Haemagglutinins anti-A and anti-B (2.6.20) • Hepatitis B surface antigen (2.7.1) • Water (2.5.12) • Sterility (2.6.1) • Pyrogens (2.6.8) 	<p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p>	<ul style="list-style-type: none"> • Factor VIII activity (2.7.4) • von Willebrand factor activity (for products intended for treatment of von Willebrand disease) 	<p>Chromogenic</p> <p>Ristocetin based</p>
Human Coagulation Factor IX 2002:1223	<ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Osmolality (2.2.35) • Total protein (2.5.9) • Activated coagulation factors (2.6.22) • Heparin (2.7.12) • Water (2.5.12) • Sterility (2.6.1) • Pyrogens (2.6.8) • Factors II, VII and X for evaluation of consistency of the method of production 	<p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>Clotting</p> <p>Chromogenic</p> <p>–</p> <p>–</p> <p>–</p> <p>Not specified</p>	<ul style="list-style-type: none"> • Factor IX activity (2.7.11) 	<p>Clotting</p>

* Only chromogenic, clotting or fibrin clot lysis methods are indicated



Monograph	Tests recommended (E.P. reference methods)	Test Principle*	Assays Recommended (E.P. reference methods)	Assay Principle*
Human Prothrombin Complex 2002:0554	<ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Osmolality (2.2.35) • Total protein (2.5.9) • Activated coagulation factors (2.6.22) • Heparin (2.7.12) • Thrombin (monograph) • Water (2.5.12) • Sterility (2.6.1) • Pyrogens (2.6.8) 	<p>–</p> <p>–</p> <p>–</p> <p>–</p> <p>Clotting</p> <p>Chromogenic</p> <p>Clotting</p> <p>–</p> <p>–</p> <p>–</p>	<ul style="list-style-type: none"> • Factor IX (2.7.11) • Factor VII (2.7.10) • Factor II (2.7.18) • Factor X (2.7.19) 	<p>Clotting</p> <p>Chromogenic</p> <p>Chromogenic</p>
Heparin Calcium 2002:0332	<ul style="list-style-type: none"> • Appearance of solution (2.2.1) • pH (2.2.3) • Protein and nucleotidic impurities (2.2.25) • Nitrogen (2.5.9) • Calcium (2.5.11) or Sodium (Method I, 2.2.23) • Heavy metals (2.4.8) • Loss on drying (2.2.32) • Sulphated ash (2.4.14) • Sterility (2.6.1) • Bacterial endotoxin (2.6.14) 	–	<ul style="list-style-type: none"> • Heparin activity (2.7.5) 	Clotting
Heparin Sodium 2002:0332		–		
Heparins, Low-Molecular-Mass 2002:0828	<ul style="list-style-type: none"> • pH (2.2.3) • Nitrogen (2.5.9) • Heavy Metals (2.4.8) • Calcium (2.5.11) or Sodium (Method I, 2.2.23) • Loss on drying (2.2.32) • Molar ratio of sulphate ions to carboxylate ions (2.2.38) • Sterility (2.6.1) • Bacterial Endotoxin (2.6.14) 	–	<ul style="list-style-type: none"> • Anti-Factor Xa Activity (monograph) • Anti-Factor IIa Activity (monograph) 	<p>Chromogenic</p> <p>Chromogenic</p>
Urokinase 2002:0695	<ul style="list-style-type: none"> • Appearance of solution (2.2.1) • Hepatitis B surface antigen (monograph) • Thromboplastic contaminants (monograph) • Molecular fractions (2.2.30) • Total protein (2.5.9) • Sterility (2.6.1) • Pyrogens (2.6.8) 	<p>–</p> <p>–</p> <p>Clotting</p> <p>–</p> <p>–</p> <p>–</p> <p>–</p>	<ul style="list-style-type: none"> • Urokinase activity (monograph) 	Fibrin clot lysis time

* Only chromogenic, clotting or fibrin clot lysis methods are indicated





Monograph	Tests recommended (E.P. reference methods)	Test Principle*	Assays Recommended (E.P. reference methods)	Assay Principle*
Streptokinase 2002:0356	<ul style="list-style-type: none"> • pH (2.2.3) • Streptodornase (monograph) • Streptolysin (monograph) • Loss on drying (2.2.32) • Sterility (2.6.1) • Pyrogens (2.6.8) • Abnormal toxicity (2.6.9) 	<ul style="list-style-type: none"> – – – – – – – 	<ul style="list-style-type: none"> • Streptokinase activity (monograph) 	Fibrin clot lysis time
Alteplase for Injection 2002:1170	<ul style="list-style-type: none"> • Appearance of solution (2.2.1) • pH (2.2.3) • Solubility (monograph) • Protein content (2.2.25) • Single-chain content (2.2.29) • Monomer content (2.2.29) • Water (2.5.12) • Bacterial endotoxin (2.6.14) • Sterility (2.6.1) 	<ul style="list-style-type: none"> – – – – – – – – – 	<ul style="list-style-type: none"> • Alteplase activity (monograph) 	Fibrin clot lysis time
Fibrin Sealant Kit 2002:0903	<p>Component 1: Fibrinogen concentrate</p> <ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Stability of solution (monograph) • Water (2.5.12) • Sterility (2.6.1) <p>Component 2: Thrombin preparation</p> <ul style="list-style-type: none"> • pH (2.2.3) • Solubility (monograph) • Water (2.5.12) • Sterility (2.6.1) 	<ul style="list-style-type: none"> – – – – – – – – – – – – – – 	<p>Component 1: Fibrinogen concentrate</p> <ul style="list-style-type: none"> • Clottable protein (monograph) <p>Or</p> <ul style="list-style-type: none"> • Clotting assay (monograph) <p>• Factor XIII (monograph)</p> <p>Component 2: Thrombin preparation</p> <ul style="list-style-type: none"> • Thrombin activity (monograph) 	<p>–</p> <p>Clotting</p> <p>–</p> <p>Clotting</p>

* Only chromogenic, clotting or fibrin clot lysis methods are indicated



THE EUROPEAN PHARMACOPOEIA: CHROMOGENIC ASSAYS

The recommended reagents and methods of the European Pharmacopoeia were compared with the Chromogenix products and assays, with the intention to provide a guideline for the use of Chromogenix reagents in pharmaceutical companies, or in laboratories where the European Pharmacopoeia recommendations are followed. Please refer to the Pharmacopoeia compendium for a detailed description of the tests and assay procedures.

Determination of Anti- Factor IIa activity of LMW Heparins (2002:0828)

The thrombin assay is based on inhibition of an excess of thrombin by antithrombin in the presence of heparin. The residual thrombin will hydrolyse a chromogenic substrate. Antithrombin is added in excess, which means that the heparin concentration will be rate determining for the reaction. The amount of released chromophore is inversely proportional to the heparin concentration in the sample. The Antithrombin and the substrate mentioned in this method are in accordance with the antithrombin and substrate available from Chromogenix as shown in the table below.

Reagents

	E.P.	Chromogenix
Reference and Test solutions	0.015 – 0.075 IU anti-FIIa activity/ml. In Buffer A	–
Factor IIa	Human thrombin. Working solution: 5 IU/ml in Buffer A	–
Antithrombin	Purified from human plasma by heparin agarose chromatography. The specific activity should be at least 6 IU per mg. Working solution: 0.5 IU/ml in Buffer A.	Antithrombin, 10 IU, Art. No. 82 07 20 Antithrombin, 25 IU, Art. No. 81 07 96 Antithrombin is purified from human plasma by heparin sepharose chromatography. The specific activity is above 6 IU/mg.
Chromogenic substrate	D-Phe-Pip-Arg-pNA Working solution: 0.5 mM in Buffer B.	S-2238, 25 mg Art. No. 82 03 24 D-Phe-Pip-Arg-pNA
Buffer A	50 mM Tris, pH 7.4 150 mM NaCl 1% BSA	–
Buffer B	50 mM Tris, pH 8.4 175 mM NaCl 7.5 mM NaEDTA	–

Determination of Anti-FXa activity of LMW Heparins (2002:0828)

The factor Xa assay is based on inhibition of an excess of factor Xa by antithrombin in the presence of heparin. The residual factor Xa will hydrolyse a chromogenic substrate. Antithrombin is added in excess, which means that the heparin concentration will be rate determining for the reaction. The amount of released chromophore is inversely proportional to the heparin concentration in the sample. The factor Xa, antithrombin and the substrate mentioned in this method are in accordance with, factor Xa, antithrombin and substrate available from Chromogenix as shown in the table below.

Reagents

	E.P.	Chromogenix
Reference and Test solutions	0.025 – 0.2 IU anti-FXa activity/ml In Buffer A	–
Factor Xa	Bovine FXa. Proenzyme, obtained from bovine plasma and activated by a suitable activator as Russel's Viper Venom. The working solution (in Buffer A) should give in the assay a $\Delta A/\text{min} = 0.15-0.20$	FXa, 71 nkat _{S-2222} Art. No. 82 09 85 Factor Xa prepared from bovine plasma and activated by Russel's Viper Venom. Use in the range of 1 – 3 nkat/ml to obtained the required $\Delta A/\text{min}$
Antithrombin	Purified from human plasma by heparin agarose chromatography. The specific activity should be at least 6 IU per mg. Working solution: 1 IU/ml in Buffer A.	Antithrombin, 10 IU, Art. No. 82 07 20 Antithrombin, 25 IU, Art. No. 81 07 96 Antithrombin is purified from human plasma by heparin sepharose chromatography. The specific activity is above 6 IU/mg.
Chromogenic substrate	N-α-Z-D-Arg-Gly-Arg-pNA Working solution: 0.5 mM in Buffer B.	S-2765, 25 mg Art. No. 82 14 13 N-α-Z-D-Arg-Gly-Arg-pNA
Buffer A and B	Refer to the anti-FIIa method	Not available



Determination of Antithrombin activity of Human Antithrombin concentrates (2.7.17)

The antithrombin activity of the preparation is determined by its ability to inactivate thrombin in the presence of an excess of heparin. The residual thrombin hydrolyses a thrombin-specific chromogenic substrate releasing pNA. The amount of released pNA, is monitored photometrically at 405 nm and is inversely proportional to the amount of antithrombin.

The reagents reported in the table are used to determine antithrombin. The substrate and thrombin from Chromogenix can be used for this purpose since their characteristics fulfil the E.P. requirement.

Reagents

	E.P.	Chromogenix
Reference and Test solutions	0.005 – 0.0133 IU/ml in Buffer B	–
Thrombin	Bovine thrombin. Working solution: 2 IU/ml in Buffer C	Bovine Thrombin Art.No. 82 07 12 1 vial: 53±5 nkat that corresponds approximately to 21 NIH-U or 25 IU ^(S-2238)
Chromogenic substrate	D-Phe-Pip-Arg-pNA In Buffer A	S-2238, 25 mg Art. No. 82 03 24 D-Phe-Pip-Arg-pNA
Buffer A	50 mM Tris, pH 8.4 7.5 mM Na-EDTA 175 mM NaCl	–
Buffer B	Buffer A containing: 1% BSA 15 IU/ml heparin	–
Buffer C	Buffer A containing: 1% BSA	–

Determination of Prekallikrein activator activity in Albumin and Immunoglobulin preparations (2.6.15)

The E.P. method for the determination of prekallikrein activator (PKA) in human plasma derived products, is based on the activation of a prekallikrein fraction (from human blood: the preparation method is described in the monograph), with the sample. The PKA present in the sample activates prekallikrein to kallikrein, which hydrolyses a chromogenic substrate. The amount of released pNA, is monitored spectrophotometrically at 405 nm and is directly proportional to the amount of PKA present in the sample. The E.P. recommends the use of a chromogenic substrate specific for kallikrein. Two substrate sequences are indicated, as shown in the table below. One of these sequences corresponds to the substrate S-2302.

Reagents

	E.P.	Chromogenix
Measuring range	Up to 35 IU/ml	–
Prekallikrein	Prepared from human blood; refer to the monograph for procedure	Purification described in the Chromogenix Research Method for PKA.
Chromogenic substrate	N-benzoyl-Pro-Phe-Arg-pNA or D-Pro-Phe-Arg-pNA	S-2302, 25 mg Art. No. 82 03 40 D-Pro-Phe-Arg-pNA
Buffer	50 mM Tris pH 8.0 150 mM NaCl	Not available

Comments

As reported by Kerry P.J. et al. (Br J Haematol 60, 345-352, 1985), in 1984 the 1st International Standard for PKA (82/530) was assigned against the FDA PKA reference preparation No. 2 and the 1st British reference preparation for PKA. Over thirteen laboratories, participating in this study, eleven used a chromogenic method with the substrate S-2302.

Heparin measurements in coagulation factor concentrates (2.7.12)

The assay recommended in the E.P. for measuring the heparin activity in coagulation factor concentrates, is a thrombin based chromogenic assay. Thrombin, added in excess, is inhibited by antithrombin in the presence of heparin. The residual thrombin hydrolyses the chromogenic substrate. Antithrombin is added in excess, which means that the heparin concentration will be rate determining for the reaction. The amount of released chromophore is inversely proportional to the heparin concentration in the sample. The thrombin, antithrombin, Tris-EDTA-buffer and the substrate mentioned in this method are all in accordance with the reagents available from Chromogenix.

Reagents

	E.P.	Chromogenix
Test/Reference solutions	Obtain a solution with 0.25 IU/ml in Buffer A	–
Reference solution	Obtain a solution with 0.25 IU/ml in Buffer B	–
Thrombin	Bovine thrombin. Working solution: 20 IU/ml	Bovine Thrombin Art.No. 82 07 12 1 vial: 53±5 nkat that corresponds approximately to 21 NIH-U or 25 IU ^(S-2238)
Antithrombin	Purified from human plasma by heparin agarose chromatography. The specific activity should be at least 6 IU per mg. Working solution: 3 IU/ml	Antithrombin, 10 IU, Art. No. 82 07 20 Antithrombin, 25 IU, Art. No. 81 07 96 Antithrombin is purified from human plasma by heparin sepharose chromatography. The specific activity is above 6 IU/mg.
Chromogenic substrate	A thrombin specific chromogenic substrate.	S-2238, 25 mg Art. No. 82 03 24 D-Phe-Pip-Arg-pNA
Buffer A	7 g/l sodium chloride 6 g/l sodium citrate pH 7.3	
Buffer B	Tris-EDTA-buffer 50 mM Tris, pH 8.4 6 mM EDTA 190 mM NaCl	Tris-EDTA-buffer Art. No. 82 36 66 50 mM Tris 7.5 mM EDTA 150 mM NaCl pH 8.4



Determination of human Factor II (2.7.18)

Human coagulation factor II is assayed following specific activation to form factor IIa which is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate. The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

The substrate Chromogenix can be used for this purpose since its characteristics fulfil the E.P. requirement.

Reagents

	E.P.	Chromogenix
Reference and Test solutions	Dilute the preparation with Dilution Buffer to obtain a solution of 0.015 IU/ml of FII	-
Viper venom specific factor II activator	Ecarin (Echis carinatus)	-
Chromogenic substrate	H-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride Or specific chromogenic substrate for Factor IIa Reconstitute according to the manufacturer's instructions	S-2238, 25 mg Art. No. 82 03 24
Dilution Buffer	6.06 g/l tris (hydroxymethyl) aminomethane 17.53 g/l sodium chloride 2.79 g/l (ethylenedinitrilo) tertra-acetic acid 1 g/l bovine albumine or human albumine pH 8.4	-

Determination of Factor VII (2.7.10)

The E.P. method for the determination of factor VII is a chromogenic assay in which factor VII is assayed by its activity as a factor VIIa-tissue factor complex in the activation of factor X, in the presence of calcium and phospholipids.

The assay consists of two steps: the factor VII dependent activation of factor X, and the cleavage of the factor Xa specific chromogenic substrate. There is a linear relation between the rate of factor Xa formation and the factor VII concentration.

The Coaset Factor VII kit is in compliance with the E.P. recommendations, except for the albumin concentration in the working buffer, which can be easily adjusted by adding extra albumin.

The strategy for measuring factor VII concentrates using Coaset Factor VII and according to E.P. is:

1. Prepare a buffer containing 1%BSA
2. Dilute sample and standard to about 1 IU/ml;
3. Dilute further 1:1000, 1:2000 and 1:5000 in buffer;
4. Run standards and samples in duplicates from duplicate independent dilution;
5. Calculate the potency of the test preparation by the statistical method.

Reagents

	E.P.	Coaset Factor VII
Factor X	Human or bovine; 10 -350 nmol/l (1st step)	Bovine Factor X; Concentration within the range (1st step)
Thromboplastin	Natural or synthetic; suitable for use in prothrombin time determination	Human placenta thromboplastin; suitable for use in prothrombin time determination
Calcium ions	15 - 25 mmol/l in the thromboplastin reagent	13 mmol/l in the thromboplastin +FX reagent mixture
Albumin; Buffer	1% HSA or BSA; pH 7.3 - 8.0	1% BSA** pH 7.4
Chromogenic substrate	FXa specific; 3-5 aa+chromophore; 0.2 - 2 mmol/l (final)	S-2765 (FXa specific); 4 residues; 0.6 mmol/l (final)
Assay	Two-step	Two-step
FVII activity in the assay	< 0.005 IU/ml 1st step	0.0001 - 0.001 IU/ml standard curve 1st step
FX activation time	Suitable to obtain a linear dose-response	7 min (microplate procedure)

** The concentration of BSA of the working buffer of Coaset FVII as described in the package insert protocol is 0.2%. However, since albumin and Tris buffer are two different components in the kit, the concentration of BSA can be adjusted to 1% by adding additional albumin.

Determination of Factor VIII (2.7.4)

The E.P. method for the determination of factor VIII is a chromogenic assay in which factor VIII is assayed by its activity as a cofactor in the activation of factor X, by factor IXa in the presence of calcium and phospholipids.

The assay consists of two steps: the factor VIII dependent activation of factor X, and the cleavage of the factor Xa specific chromogenic substrate. There is a linear relation between the rate of factor Xa formation and the factor VIII activity.

The Coamatic Factor VIII kit is completely in compliance with the E.P. recommendations as shown in the table below. The Coatest FVIII and Coatest FVIII:C/4 kits are also in compliance with the E.P. recommendations. In fact by adjusting the BSA concentration of the working buffer to 1% BSA, all the requirements are fulfilled.

The strategy for measuring factor VIII concentrates using Coamatic Factor VIII according to E.P. is:

1. Dilute sample and standard to about 1 IU/ml in FVIII deficient plasma;
2. Dilute further 1:81, 1:162 and 1:324 in buffer;
3. Run standards and samples in duplicates from duplicate independent dilution
4. Calculate the potency of the test preparation by the statistical method for a slope/ratio assay (E.P. 2002, method 5.3)

For information on the use of Coatest FVIII and Coatest FVIII:C/4 for measuring Factor VIII concentrates please contact your local Distributor.

Reagents

	E.P.	Coamatic Factor VIII
Factor X	Human or bovine; 10 –350 nmol/l (1 st step)	Bovine Factor X; Concentration within the range (1 st step)
Factor IXa	Human or bovine; 1 – 100 nmol/l (1 st step)	Bovine Factor IXa; Concentration within the range (1 st step)
Factor VIII activator	Human or bovine thrombin or prothrombin	Bovine thrombin;
Phospholipids	Natural sources or synthetic; 1 – 50 µmol/l (1 st step); 15-35% is: phosphatidylserine	Synthetic phospholipids; Concentration within the range (1 st step); Phosphatidylserine within the requirements
Calcium ions	5 – 15 mmol/l (1 st step)	6.7 mmol/l (1 st step)
Albumin; Buffer	1% HSA or BSA; Tris buffer pH 7.3 – 8.0	1% BSA; Tris buffer pH 7.9
Chromogenic substrate	FXa specific; 3-5 aa+chromoph.; 0.2 – 2 mmol/l (final)	S-2765 (FXa specific); 4 residues; 0.6 mmol/l (final)
Inhibitor	To stop factor Xa generation and inhibit thrombin	I-2581 co-lyophilised with the substrate (thrombin inhibitor)
Assay	Two-step	Two-step
FVIII activity in the assay	< 0.03 IU/ml 1 st step	0.0015 - 0.0088 IU/ml standard curve 1 st step (normal assay range)
FX activation time	2 – 5 min	2 min (microplate method)

Determination of human Factor X (2.7.19)

Human coagulation factor X is assayed following specific activation to form factor Xa.

Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate. The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

The substrate S-2222 corresponds to one of the sequences indicated by E.P.

Reagents

	E.P.	Chromogenix
Reference and Test solutions	Dilute the preparation with Buffer to obtain a solution of 0.18 IU/ml of FX	–
Russell's viper venom specific factor X activator (RVV)	Vipera Russellii	–
Chromogenic substrate	N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide-dihydrochloride Or specific chromogenic substrate for Factor Xa Reconstitute according to the manufacturer's instructions Diluted 1 in 6 in Buffer	S-2222, 25 mg Art. No. 82 03 16
Buffer	3.7 g/l tris (hydroxymethyl) aminomethane 2.1 g/l imidazole 0.02 g/l hexadimethrine bromide 1 g/l bovine albumine or human albumine pH 8.4	–



UNITED STATES PHARMACOPEIA

About USP

USP (United States Pharmacopeia), established in 1820, is a private, voluntary, not-for-profit organization. More than 1,000 volunteer health care professionals, scientists, academicians, and government officials compose USP's "family", which is organized into three bodies:

(1) Convention Membership; (2) Board of Trustees; and (3) Council of Experts. A staff of more than 300 supports the work of USP's family.

Mission

USP promotes the public health by establishing and disseminating officially recognized standards of quality and authoritative information for the use of medicines and other health care technologies by health professionals, patients, and consumers.

Vision

The vision of the USP is to be a leader in advancing the health of the public by creating a unique knowledge base on medicines and other health care technologies. This knowledge base will contain state-of-the-art standards to assure the quality of these technologies and authoritative information to promote their appropriate use. We shall make this knowledge base accessible to people throughout the world.

Drug Standards

USP works closely with the U.S. Food and Drug Administration (FDA), the pharmaceutical industry, and the health professions, to establish authoritative drug standards.

These standards are enforceable by the FDA and by other countries, and recognized worldwide as the hallmark of quality. More than 3,700 standards monographs are published in the United States Pharmacopeia and the National Formulary (USP-NF), the official drug standards compendia. USP also provides more than 1,300 premier chemical Reference Standards to carry out tests specified in USP-NF.

USP's chemical Reference Standards are a key element in developing high-quality pharmaceuticals worldwide. They are used to carry out the test methods specified in the USP-NF.

USP 25-NF 20

The current compendia, USP 25-NF 20 are effective January 1, 2002 and provide recognized standards for drugs, excipients, medical devices, diagnostics, dietary supplements (botanicals), and nutritional supplements (vitamins and minerals). The USP-NF include standards for strength, quality, purity, identity, packaging, labeling and storage, general requirements and processes for tests and assays, and more.

Pharmacopeial Forum

The Pharmacopeial Forum (PF), the essential companion to the USP-NF, provides an opportunity for participation in the standards development and revision process. PF provides for public comment the immediately binding revisions in addition to the proposed changes to official USP-NF drug standards.

For further information on USP services and products, please contact USP at +1 301 8168227 or www.usp.org.



THE U.S. PHARMACOPEIA: CHROMOGENIC ASSAYS

The recommended reagents and methods of USP have been compared with the Chromogenix products and assays, with the intention to provide a guideline for the use of Chromogenix reagents in pharmaceutical companies, or in laboratories where the USP recommendations are followed. Please refer to the Pharmacopoeia compendium for a detailed description of the tests and assays procedures.

Anti-FXa activity of Heparin

The activity of Heparin Sodium and Heparin Calcium, should be determined by both a clotting assay and a chromogenic assay. The chromogenic assay consists essentially in the measurement of the anti-FXa activity of the test preparation against the USP Heparin Sodium Reference Standard. Antithrombin, FXa and the chromogenic substrate from Chromogenix are suitable for this test as reported in the table below.

Reagents

	USP	Chromogenix
USP Heparin Standard	0.25, 0.188, 0.125, 0.0625 and 0.312 USP Heparin unit per ml	-
Antithrombin	From human plasma with a specific activity not less than 4 U/mg. Working solution: 1 U/ml in Buffer pH 8.4.	Antithrombin, 10 IU, Art. No. 82 07 20 Antithrombin, 25 IU, Art. No. 81 07 96 The protein is purified from human plasma and the specific activity is above 6 IU/mg.
Factor Xa	From bovine plasma. FXa obtained by activation of FX with Russell's viper venom. The working solution is 20 U/ml in water. When tested in the blank procedure the absorbance change per minute is 0.65-0.70.	FXa 71 nkat (S-2222) Art. No. 82 09 85 Factor X prepared from bovine plasma and activated by Russell's viper venom. Reconstitute with 6 ml water to obtain a solution 11.8 nkat/ml which should give an absorbance change per minute in the range required if tested with S-2222.
Chromogenic substrate	Suitable for anti-FXa activity and reactive at a concentration ≤ 2.5 mM	S-2222, 25 mg Art. No. 82 03 16 Km: 1.1 mM
Buffer pH 8.4	50 mM Tris, pH 8.4 7.5 mM EDTA 175 mM NaCl 0.1% PEG 6000	-

Alteplase

The biological potency of alteplase is determined by an assay based on the fibrin clot lysis time. For the identification of the product however, a qualitative method based on the hydrolysis of a chromogenic substrate is recommended. The sequence of the chromogenic substrate is corresponding to the sequence of the substrate S-2288, which is H-D-Ile-Pro-Arg-pNA.





ABOUT THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS (www.isth.org)

HISTORY

The International Society on Thrombosis and Haemostasis grew out of the International Committee for the Standardization of the Nomenclature of the Blood Clotting Factors, which was formed in 1954 at a meeting in Basel, Switzerland. The Committee's agenda, which was expressed in its original name, gradually expanded, while the name was shortened to "International Committee on Thrombosis and Haemostasis (ICTH)"

In 1969, the Society was formed to provide a wider forum for interested scientists. The Committee now functions as the Society's working arm. To avoid confusion between the two entities, in 1987 the ICTH was renamed the "Scientific and Standardization Committee (SSC) of the ISTH."

PURPOSE

The International Society on Thrombosis and Haemostasis, Inc. (ISTH), a non-profit medical research organization, seeks to foster and advance science relating to thrombosis, haemostasis, and vascular biology; to provide a forum for discussion of these problems; to encourage research in these areas; to focus the attention of scientists of the several relevant disciplines on these matters; to foster the diffusion and exchange of ideas through publications and meetings; and to create internationally accepted standards for nomenclature and research methods.

ORGANIZATION

The ISTH, Inc. is a non-profit corporation with 2,000 members from over 50 countries. The Society is governed by a 15-member board of directors, the Council. Officers are President, Vice-President, Council Chairman, and Secretary/Chairman-elect. Ex officio members include the Executive Director, the Editor of Thrombosis and Haemostasis, and the Chairman of the SSC. Members of the SSC are nominated by the Society at large, elected by Council, and represent at least 12 different countries. The SSC currently supports 19 Scientific Subcommittees and Registries. The International Headquarters of the ISTH was organized in 1989 and may be contacted at:

*CB# 7035
UNC Medical School
Chapel Hill, NC 27599-7035 USA
tel. 919 929 3807
fax: 919 929 3935*

MEMBERSHIP

Membership in the Society may be obtained by application to the Executive Director and payment of annual dues. Benefits of membership include voting privileges in Society business and constitutional matters; nomination and election of Council members; nomination of candidates for election to the SSC; nomination of Congress Presidents; discount registration fees for biennial Congresses; and an annual subscription to the Society's journal, Thrombosis and Haemostasis. For more information, contact the Executive Director at ISTH headquarters. To get an application, print out the ISTH application page.

JOURNAL

Since 1972, the journal Thrombosis and Haemostasis (formerly Thrombosis et Diathesis Haemorrhagica) has been the official journal of the ISTH. The journal is published monthly in two annual volumes of six issues each. Thrombosis and Haemostasis is the official vehicle for publications of the Society and the SSC. Subscription information is available from:

*F.K. Schattauer Verlag, P.O.B. 10 45 45, D-70040 Stuttgart 10, Germany. FAX: 49 (0)711 229 8750
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ABOUT THE SECONDARY STANDARD FOR COAGULATION WORKING GROUP

THE SSC WORKING GROUP ON COAGULATION STANDARDS

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United Kingdom
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e-mail: thubbard@nibsc.ac.uk

HISTORY

The SSC Working Group on Coagulation Standards was formed as a result of a meeting between SSC members and Diagnostic Manufacturers to discuss the need to offer a uniform plasma standard for use by the manufacturers in labeling their coagulation calibrators.

To achieve this goal, the Working Group was established and is presently composed of an Executive Board, the participating Diagnostic Manufacturers, and a group of Expert Laboratories to label the analytes.

A request for bids to produce a calibrator (secondary standard) to assign potency for test kit calibrators of coagulation factors was subsequently sent to interested manufacturers. The Executive Board reviewed the bids and selected a supplier for this secondary standard.

Immuno AG produced lot #1 of the SSC Secondary Standard. A large number of SSC members and their laboratories have devoted time and effort to calibration of this secondary standard. Between 8-15 laboratories were involved in the standardization of each analyte.

THE SSC/ISTH SECONDARY COAGULATION STANDARDS (LOT#2)

Announcement of the change in procedure for ordering the SSC/ISTH Secondary Coagulation Standard which will go into effect with the shipping of Lot#2

The SSC/ISTH secondary plasma standard for coagulation factors is available to manufacturers of coagulation diagnostic reagents. The secondary standard was calibrated against WHO International Standards by multiple laboratories (see Table for assigned values and estimates of inter-laboratory variability).

This reagent is the result of a collaboration between the manufacturers of diagnostic reagents and the SSC.

Producers of coagulation reagents may order from:

Dr. Anthony R. Hubbard PhD
Division of Haematology
NIBSC
Blanche Lane
South Mimms
Potters Bar
Hertfordshire EN6 3QG
United Kingdom
fax: +44 1707 646730
e-mail: thubbard@nibsc.ac.uk

- Order size: minimum of 100 vials and multiples of 50 vials thereafter
- Delivery: 3 weeks after receipt of order; all orders to be despatched by courier at ambient temperature at the cost of the recipient
- Price: US \$ 2.50 per vial handling charge plus cost of courier despatch (a single invoice will be raised to cover above costs)
- Payment: 30 days after invoice
- Labelling: ISTH/SSC Plasma, Secondary Coagulation Standard Lot#2 Expiration End June 2006 Reconstitute with 1 ml purified water Store at -20°C or below
- Caution: Human Blood Product

Analyte	Value	CV%
Factor II	0.89 IU	4.4
Factor VII:C	1.05 IU	4.9
Factor VIII:C	0.66 IU	6.9
Factor IX	0.94 IU	2.9
Factor X	0.94 IU	1.5
von Willebrand Factor		
Ristocetin CoFactor	0.71 IU	13.8
Antigen	0.95 IU	7.9
Protein C		
Activity	0.96 IU	3.2
Antigen	0.97 IU	3.3
Protein S		
Total Antigen	0.88 IU	4.4
Free Antigen	0.92 IU	7.7
Function	0.73 IU	6.8
Antithrombin		
Activity	0.92 IU	3.4
Antigen	0.93 IU	9.2
Fibrinogen	2.4 mg	3.2





ABOUT THE INTERNATIONAL SOCIETY FOR FIBRINOLYSIS AND PROTEOLYSIS

HISTORY

The ISFP (formerly: International Society for Fibrinolysis and Thrombolysis) was established in 1992 with the goals to have a legal entity to represent the fibrinolysis community, to organise its international congresses and to unite the investigators in the field of fibrinolysis and proteolysis in one professional society. According to the bylaws (published in FIBRINOLYSIS, 7, i-iii, 1993) "The objectives of the Society, which has no profit motive, are exclusively scientific and educational in nature. Specifically, the objectives include the furtherance of scientific research relating to fundamental and medical aspects of Fibrinolysis, Proteolysis and Thrombolysis, the furtherance of research in related disciplines, the interchange of ideas through the organisation of scientific conferences and the stimulation of scientific activities for the developments in Fibrinolysis, Proteolysis and Thrombolysis".

MEMBERSHIP

The ISFP has at present about 250 members from 35 different countries. The annual membership fee (50 USD for 2002) entitles to a reduction on the registration fee for the International Congress of the Society (Münich, September 8-13, 2002). Junior membership, free of charge, is possible for colleagues under the age of 30.

OFFICERS

E.K.O. Kruithof (Switzerland), Chairman
N. Booth (UK), Chairperson-Elect
O. Matsuo (Japan), Secretary / Treasurer
H.R. Lijnen (Belgium), Executive Director

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T. Ny, Sweden
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D. Rifkin, USA
D. Rijken, The Netherlands
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P.J. Declerck, Belgium
R.L. Medcalf, Australia

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Chairperson of the SSC/ISTH

Sponsoring Members

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Dade Behring Marburg
Diagnostica Stagò
Genentech, Inc.
Schering AG
Serbio
Technoclone

Web site

[http:// www.fibrinolysis.org](http://www.fibrinolysis.org)

For more information or membership application, please contact:

H.R. Lijnen

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ABOUT THE NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL (NIBSC) - A WHO INTERNATIONAL LABORATORY FOR BIOLOGICAL STANDARDS

ACTIVITIES

NIBSC is a multi-disciplinary scientific establishment which has a national and international role in the standardisation and control of biological substances used in medicine. Such substances include bacterial and viral vaccines and products derived from human blood. Batches of these medicines must be independently assessed before they are released on to the market and NIBSC performs this regulatory function for the UK as a part of the European batch release system.

The quality and validity of the control work undertaken at NIBSC is accredited by the United Kingdom Accreditation Service (UKAS) to comply with the International Standard ISO 17025.

The Institute is also a World Health Organisation (WHO) International Laboratory for Biological Standards and as such prepares and evaluates biological reference substances including the definitive WHO International Biological Standards which serve the pharmaceutical, regulatory and research communities.

A full programme of fundamental and developmental research is undertaken at NIBSC to underpin its control and standardisation activities.

STAFF

NIBSC has about 300 staff of whom two-thirds are post-doctoral or graduate scientists. Staff are encouraged to publish in peer reviewed journals and a publications list is available. Many staff are members of UK, European and International committees and working parties which define future requirements for ensuring the quality and safety of biological medicines.

HISTORY

The origins of the scientific work of this Institute date back to 1928 and the activities of Medical Research Council's National Institute for Medical Research situated at Mill Hill in London. In the 1970's the responsibility for the control and standardisation part of the work was transferred to the newly established National Institute for Biological Standards and Control (NIBSC). Since 1976 the Institute has been directly funded by the UK Health Departments. Originally situated at Hampstead in London, in 1987 NIBSC moved some miles north to purpose-built accommodation in South Mimms in Hertfordshire.

The Biological Standards Act of 1975 established the National Biological Standards Board, which manages NIBSC. The Act also established the Institute as the UK's Official Medicines Control Laboratory for biological medicines and laid down its statutory functions.

For further information, to request our comprehensive catalogue of biological reference materials or to order standards:

Telephone +(44) 1707 654753

Fax +(44) 1707 646730

E-mail: standards@nibsc.ac.uk.

Visit our home page: <http://www.nibsc.ac.uk>

The following categories of reference materials in the Haemostasis, Thrombosis, Transfusion Science & Immunohaematology fields are available from NIBSC.

- 1) Coagulation Factors and Inhibitors
 - a) Coagulation Factor Concentrates (eg FVII, FVIII, FIX, Antithrombin)
 - b) Diagnostic Plasma Standards (eg FVIII, FIX, Protein C, Antithrombin)
- 2) Antithrombotic Drugs
 - a) Heparin
 - b) LMW Heparin
- 3) Fibrinolysis
 - a) Streptokinase
 - b) Urokinase
 - c) t-PA
 - d) Plasmin
- 4) Immunohaematology
 - a) Platelet Immunology
 - b) Red Cell Immunology
 - c) Anti-D
- 5) General Haematology
 - a) Ferritin
 - b) Haemoglobin
 - c) Vitamin B12
 - d) Folate



ABOUT THE INTERNATIONAL THROMBOPHILIA EXTERNAL QUALITY ASSESSMENT SCHEME (EQAS) AND THE ECAT FOUNDATION

HISTORY

The ECAT made its debut in 1981 as a European concerted Action on Thrombosis. Since 1989 the ECAT began with the quality control of assays relevant in thrombophilia. Since 1993 the ECAT Quality Assessment Scheme is an independent foundation. At present (2002) 350 laboratories participate in the ECAT Foundation EQAS.

PURPOSE

The purpose of the scheme is to check and improve the performance of laboratory assays relevant in thrombophilia.

ORGANISATION

Board:

Prof. C.Kluft, Leiden, NL (chairman)
 Dr F. Haverkate, Leiden, NL (vice-chairman)
 Mr. D.J. Cuilenburg, Leiden, NL (treasurer)
 Dr J.J.M.L. Hoffmann, Eindhoven, NL
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Advisors:

Dr. J. Arnout, Leuven, B
 Prof. R.M.Bertina, Leiden, NL
 Prof. J.Jespersen, Esbjerg, DK
 Dr. Ph. De Moerloose, Geneva, CH

Director:

Mr. P. Meijer, Leiden, NL

MEMBERSHIP

Laboratories all over the world interested in participation are invited to participate providing they have adequate facilities, have remitted the required fee and quick mailing of samples is possible.

PROCEDURE

Laboratories receive 4 times per year freeze-dried plasma's. They should perform the assays using methods in routine in their own laboratory. Results show each laboratory its deviation from the mean value, and where possible, according to the different methodology used for the assay of one and the same component. Results are presented in graphs and tables.

The following assays are included:

Plasma Tests

Thrombophilia module	Protein C Pathway Test
• Antithrombin	Lupus Anticoagulant
• Protein C (activity and antigen)	D-Dimer
• Protein S (activity)	Factor VIII (elevated levels)
• Protein S (total and free antigen)	Homocysteine
• APC Resistance	

Molecular Biology

Set A

- Factor V
- Prothrombin mutation 20210
- MTHFR mutation

Set B

- HFE (H63 and C282)
- ApoE
- ApoB100

Set C

- Alpha-1-Proteinase Inhibitor
- UGT-1A
- ACE I/D

The modules for Molecular Biology are provided in co-operation with the German Society for Clinical Chemistry (DGKC). The samples for the molecular biology modules are distributed twice a year.

For detailed information, prices and subscription please contact:

P. Meijer
 PO Box 2215
 2301 CE LEIDEN, the Netherlands
 Phone + 31.71.5181496
 Fax + 31.71.5181330
 E-mail: P.Meijer@pg.tno.nl

Or visit our web site:

www.ecat.nl

MEETINGS

The ECAT Foundation organizes participant's meetings. The next meeting will hold on 8 November 2002.

Description	510 (K) clearance*	Art. No.	Page
Anti-B2 GPI			
Coaliza® Anti-B2 GPI IgG			17
Coaliza® Anti-B2 GPI IgM			17
Anti-Cardioliipin			
Coaliza® Anti-Cardioliipin		82 3377 63	17
Antithrombin			
Coamatic® Antithrombin	Yes	82 1991 63	7
Coamatic® AT 400		82 2320 63	7
Coamatic® LR AT		82 2957 63	7
Coacute® Antithrombin R	Yes	82 2122 63	19
APC™ Resistance			
Coatest® APC™ Resistance	Yes	82 2643 63	8
Coatest® APC™ Resistance-S	Yes	82 2916 63	8
Coatest® APC™ Resistance V	Yes	82 3120 63	9
Coatest® APC™ Resistance V-S	Yes	82 3138 63	9
V-DEF Plasma	Yes	82 3146 63	9
Factor VII			
Coaset® Factor VII		82 1900 63	11
D-Dimer			
Coamatic® D-Dimer		82 4011 63	13
Factor VIII			
Coamatic® Factor VIII	Yes	82 2585 63	12
Coatest® Factor VIII	Yes	82 1033 63	12
Coatest® Factor VIII:C/4	Yes	82 1918 63	12
Heparin			
Coamatic® Heparin	Yes	82 3393 63	14
Coatest® LMW Heparin/Heparin	Yes	82 1363 63	14
Coatest® Heparin	Yes	25 5539 63	14
Coacute® Heparin	Yes	82 1660 63	19
PAI			
Coaliza® PAI		82 1975 63	15
Coatest® PAI		82 1686 63	15
Plasmin Inhibitor			
Coamatic® Plasmin Inhibitor	Yes	82 3187 63	18
Plasminogen			
Coamatic® Plasminogen	Yes	82 2452 63	18
Protein C			
Coamatic® Protein C	Yes	82 2098 63	10
Protein S			
Coaliza® Protein S-Free	Yes	82 3567 63	10
Coamatic® Protein S-Free		82 4003 63	10
Soluble Fibrin			
Coatest® Soluble Fibrin		82 2965 63	13
VWF			
Coamatic® VWF		82 4029 63	11
TAFI			
Coaliza® TAFI		82 4045 63	16
t-PA			
Coaliza® t-PA		82 1538 63	16
Coaset® t-PA		82 1447 63	16

* This is the 510 (K) clearance status at time of printing. Please contact your local distributor for updated information.

Description	510 (K) clearance*	Art. No.	Page
Calibrators and Controls			
Calibration Plasma		82 3534 63	20
Control Plasma-Normal		82 3542 63	20
Control Plasma-Abnormal Level 1 & 2		82 3559 63	20
Calibration Plasma LMW-Heparin		82 3500 63	21
Control Plasma LMW-Heparin		82 3492 63	21
Control Plasma APC Resistance Level 1	Yes	82 2650 63	21
Control Plasma APC Resistance Level 2	Yes	82 2668 63	21
D-Dimer Controls		82 4037 63	21
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Bioreagents			
Antithrombin 10 IU		82 0720 39	22
FX		82 2239 63	22
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Antithrombin 25 IU		81 0796 39	22
Plasmin		81 0655 39	22
Prothrombin Activator Diluent		82 3526 63	22
Plasminogen		81 0663 39	23
Thrombin		82 0712 63	23
t-PA		82 1157 39	23
t-PA Stimulator		82 2130 63	23
Tris BSA Buffer		82 3518 63	24
Tris EDTA Buffer		82 3666 63	24
I-2882		82 3815 10	24
Chromogenic Substrates			
S-2222™		82 0316 39	25
S-2238™		82 0324 39	25
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S-2266™		82 0480 39	25
S-2288™		82 0852 39	26
S-2302™		82 0340 39	26
S-2314™		•	26
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S-2732™		•	28
S-2765™		82 1413 39	29
S-2767™		•	29
S-2772™		82 3013 63	29
S-2782™		•	29
S-2787™		•	30
S-2846™		•	30

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AUTOMATE METHOD LIBRARY

Chromogenix maintains a library of more than 250 fully validated automate methods. This means that we can provide you with a method for running most of our assays on the latest haemostasis analysers.

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CHROMOGENIX

An Instrumentation Laboratory Company

Chromogenix -
Instrumentation Laboratory SpA
V.le Monza, 338 - 20128 Milano (Italy)
www.chromogenix.com