



Intended Use and Application

Pefakit® PiCT® (Prothrombinase induced Clotting Time) is a plasma based functional assay for the quantitative determination of anticoagulant activities of heparin and heparinoids based on FXa and/or FIIa inhibition.

Introduction

Heparin and heparinoids like UFH, LMWH, pentasaccharide and danaparoid are anticoagulant drugs, which enhance the inhibitory action of the natural inhibitors antithrombin (AT) and partly also heparin cofactor II on FXa and/or FIIa [1]. They are therefore referred to as indirect inhibitors. In many studies the efficacy of these drugs for the prevention and treatment of thromboembolic events has been shown [2]. However these substances can also induce bleeding complications. In patients with renal insufficiency accumulation of the anticoagulant can occur following repeated application [3]. Before spinal anesthesia in anticoagulated patients a control of the anticoagulant levels is recommended [4]. In many clinics different anticoagulants are used at the same time.

A new generation of therapeutic anticoagulants are direct inhibitors for thrombin or Factor Xa (7). These are themselves directed against thrombin or Factor Xa and inhibit these enzymes without intervention via the antithrombin produced naturally in the body. For some of the direct inhibitors described so far no general monitoring is required, but in certain cases it is also preferable here to check their anticoagulatory effect (8, 9).

The availability of a universal test facilitates the simple monitoring of these substances.

Principle of the Method

The plasma sample is mixed with a reagent (R1) containing a combination of a defined amount of activated factor X (FXa), phospholipids and RVV-V, an enzyme from the venom of the snake *Daboia russelli* specifically activating factor V [5]. When measuring indirect FXa inhibitors and in the case of thrombin inhibitors (direct and indirect) the added FXa is inhibited during an incubation period of 180 s depending on the amount of AT-heparin complexes. The thrombin formed from prothrombin via the prothrombinase complex is also inhibited depending on the amount of indirect and/or direct thrombin inhibitors in the sample.

Following recalcification the prothrombinase complex is formed using the residual FXa, phospholipids, factor Va generated from factor V present in the sample and free calcium ions. Due to the factor V pre-activation by means of RVV-V, prothrombinase formation is not dependent on endogenous thrombin mediated factor V activation. Therefore the assay has been named "Prothrombinase-induced Clotting Time" (PiCT®). The time until the detection of clotting is recorded. Due to the incubation step, this procedure is referred to as the "2-step method".

Should **direct FXa inhibitors** be measured, the sample incubation step with the reagent (R1) is omitted. This procedure is therefore called the "1-step method".

Reagents

Reagent	Content
R1	Activator (FXa, RVV-V, Phospholipids, Hepes, Mannitol) 3 vials (lyophilisate, to be reconstituted in 2 ml of deionized water per vial). Incubate the reconstituted solution in closed vials for 30' at room temperature and swirl rigorously before use
R2	Start Reagent 3 vials containing 2 ml 25 mM CaCl ₂

Materials required but not provided

- Deionized water
- Calibrated pipettes (50–2000 µl)
- Automated or semi-automated coagulation instruments which employ mechanical or optical detection methods
- Calibrators and controls for quality control and for interpretation of results, as described in the respective section

Note: When using automated or semi-automated coagulation analyzers refer always to manufacturer's operator manual or ask for a detailed adaptation protocol.

Storage and Stability

The test kit may be used up to the expiry date given on the label when stored unopened at 2–8 °C.

Stability of the reagents after reconstitution in the original vial:

Reagent	Stability	
R1	-20 °C	4 weeks ^{a)}
	2–8 °C	3 days
	15 °C	48 hours (on-board)
	25 °C	12 hours (on-board)
R2	-20 °C	2 years
	2–8 °C	2 years
	15–25 °C	12 months

^{a)}Frozen reagents should be thawed at RT and mixed well before use. Freeze only once either in the original vial or as aliquots in Pefakit® Vials [REF 801140].

Quality Controls

Use Pefakit® Controls LMWH [REF 505-21], Pefakit® Controls UFH [REF 505-22], Pefakit® Controls Hirudin [REF 505-23] and Pefakit® Controls Danaparoid [REF 505-28] and Pefakit® Controls Argatroban [REF 505-29] as a control reference for validation of the assay. A control run should be made with each test series. For preparation and use of the controls refer to the instructions given in the corresponding control kit package inserts.

Ranges of expected values for LMWH, UFH, hirudin, danaparoid and argatroban controls are provided with each batch and given in a certificate attached to the box insert included in the respective kit. The precision of the measured values is guaranteed to a max. of 1.5 IU/ml for LMWH, 1.8 IU/ml for UFH, 3.0 µg/ml for hirudin, 1.0 U/ml for danaparoid and 1.2 µg/ml for argatroban. If values outside the specified range are obtained, a complete check of reagents should be made and the analysis should be repeated. If the problem persists a complete instrument check should be made and the analysis should be repeated.

Blood Collection and Sample Preparation

The patient should be at rest for 10 min prior sampling. Collect venous blood carefully in either 104 mM or 129 mM sodium citrate (volume ratio 9+1). Mix gently blood and anticoagulant directly after sampling, avoid foam formation. Centrifuge immediately at no less than 2000x g for at least 20 min at room temperature. Take care to avoid contaminations from the platelet layer into plasma when the plasma is separated from the cells. Never use a hemolytic plasma sample.

For storage freeze undiluted plasma rapidly at -70 °C in aliquots. Freeze only once. Avoid repeated freezing and thawing cycles. Thawing should be done rapidly (within 5 minutes) in a 37 °C water bath. For more information see NCCLS document H21-A2 [6].

Stability of undiluted samples (plasma):

-80 °C	2 years
-20 °C	2 years
15–25 °C	4 hours

Procedure

Reagents should be prepared as described above. Thaw frozen samples as described above ensuring negligible loss of activity of unstable coagulation factors and absence of cryoprecipitate. Invert thawed sample for homogenization.

The measurement of indirect FXa inhibitors and of indirect and direct thrombin inhibitors (e.g. UFH, LMWH, fondaparinux, danaparoid, argatroban, hirudin) is carried out using a **2-step method**:

Pipetting scheme 2-step method:

	Volume
Sample (or calibrator or control plasma)	50 µl
R1 Activator	50 µl
Incubation: 180 s, 37 °C	
R2 Start Reagent (37 °C)	50 µl

Record the clotting time (optically or mechanically).

Measurement of direct FXa-inhibitors like Rivaroxaban, is carried out using the **1-step method**.

Procedure:

A 1:1 mixture of reconstituted R1 activator and R2 start reagent is created.

The mixture must be created in one of the original bottles or in a vial from Pefakit® Vials [REF 801140].

Pipetting scheme 1-step method:

	volume
Sample (or calibrator or control plasma)	100 µl
Incubation: 60 s, 37 °C	
R1+R2 PiCT® Reagent Mix 1:1	60 µl

Record the clotting time (optically or mechanically).

Interpretation of the test results

The clotting time is prolonged with increasing concentration of anticoagulation in the blood. Calibration using Pefakit® PiCT® Calibrators LMWH [REF 505-11], Pefakit® PiCT® Calibrators UFH [REF 505-12], Pefakit® PiCT® Calibrators Hirudin [REF 505-13], Pefakit® PiCT® Calibrators Danaparoid [REF 505-18] and/or Pefakit® PiCT® Calibrators Argatroban [REF 505-19] enables determination of LMWH, UFH, hirudin, danaparoid and or argatroban anticoagulant activity. Both, LMWH and UFH calibrators are calibrated against the corresponding WHO standards. For preparation and use of the calibrators refer to the calibrators' instructions for use.

Specificity and Sensitivity

Pefakit® PiCT® is sensitive to both anti-Xa and anti-IIa activity. In the case of a therapy change from a heparin or heparinoid to an anti-FIIa anticoagulant like hirudin or argatroban a residual anti-FIIa activity of heparin or heparinoid will lead to additional prolongation of the measured clotting time.

The measurement range of quantification of anticoagulant activity for LMWH is 0–1.5 IU/ml, for UFH 0–1.8 IU/ml, for hirudin 0–3.0 µg/ml, for danaparoid 0–1.0 U/ml and for argatroban 0–1.2 µg/ml.

Precision and Reproducibility

Intra-assay and inter-assay precision and reproducibility of anticoagulant activity measurements were determined using Pefakit® Controls LMWH [REF 505-21] and Pefakit® Controls UFH [REF 505-22] on 5 different instruments. Over all instruments and measurements the coefficient of variation (CV) within the series (n = 5 to 7) and between test series on 9 different days was ≤7%. Trueness of the data was determined by comparing the means of the calculated anticoagulant activities for all series and all days with the certified values for the controls tested.

Deviation of the mean from the certified value was within a range of ±0.08 IU/ml. Thus the test has a very high precision and reproducibility for UFH and LMWH measurements on all instruments tested so far.

Within the frame of a multi-center study the precision and reproducibility was checked based on controls 1 and 2 for UFH and LMWH both within the series (20 measurements) and from day to day (10 days) under the conditions of a routine laboratory. The results of this study are given in the following table:

	Precision within the series (%CV)			
	UFH		LMWH	
	Control 1	Control 2	Control 1	Control 2
Center 1	2.9	2.9	4.4	2.3
Center 2	2.7	1.9	4.8	1.8
Center 3	2.9	2.7	3.3	5.0

	Precision day-to-day (%CV)			
	UFH		LMWH	
	Control 1	Control 2	Control 1	Control 2
Center 1	12.5	7.1	6.2	7.0
Center 2	10.2	5.5	8.6	6.3
Center 3	4.3	3.2	5.5	3.0

Limitations and Interferences

Most factor deficiencies have no influence on determination of anti-coagulant activity. In patient samples with antithrombin deficiency a reduced anticoagulant activity is detected. Clotting times of samples with known anticoagulant concentration are therefore lower than expected.

In samples highly deficient in factor V, prothrombin and/or fibrinogen (below 25% normal level) clotting times are clearly prolonged. Also lupus anticoagulants can lead to the detection of false-high anticoagulant activity.

Aprotinin in the plasma up to a concentration of 800 CIU/ml has no influence on the test result. In contrast, protamin interferes with the test at a concentration of 1 IU/ml and higher. In order to make sure that the test is not disturbed by interference the plasma should be completely free of protamin.

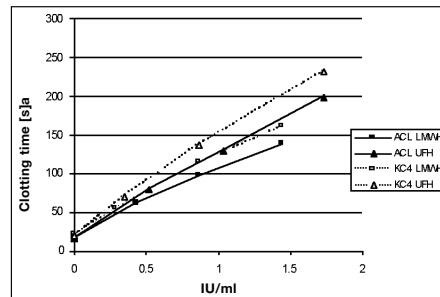
R1 (Activator) contains FXa which may lead to carry-over effects. FXa may contaminate other samples and interfere with them. Appropriate cleaning of instrument after this pipetting step is therefore mandatory and has to be part of the adaptation protocol.

Expected Values

Clotting times may vary when analyzed on different instruments. Minor variations can occur when different batches of the same reagent are used. Acceptable ranges of concentrations for low (C1) and high (C2) controls are given in the certificate attached to each control kit (Pefakit® Controls LMWH [REF 505-21], Pefakit® Controls UFH [REF 505-22], Pefakit® Controls Hirudin [REF 505-23], Pefakit® Controls Danaparoid [REF 505-28] or Pefakit® Controls Argatroban [REF 505-29]).

Please note that the dilution scheme may differ from instrument to instrument (refer to adaptation protocol). Typical calibration curves obtained with UFH Calibrator and LMWH Calibrator on different instruments are given in the following diagram:

Calibration on ACL-9000™ and KC4A™ Micro



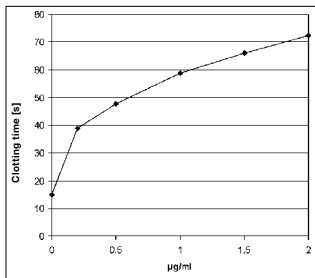
Ranges given in the certificates apply to all instruments (BCS®, KC-4/-10 A™ micro, CA-1500, ACL 9000™, STA®C und AMAX CS-190, CA-500 und ACL-Top).

In the following table expected values for the clotting time in seconds are given for plasmas of patients not under anticoagulation treatment (normal range) and for plasmas of patients treated with different types and, in the case of LMWH, different dosage (prophylactic/therapeutic) of anticoagulants. The clotting times were measured within the frame of a multi-center study using different analyzers (STA®-C, STA®-R, KC-10™). Ranges are combined for all centers and all instruments. Type and time of blood collection was chosen in such a way that peak levels after the last application could be detected. The ranges shown here can be used as an orientation help. These ranges have to be verified or modified based on the experience at the individual testing facility:

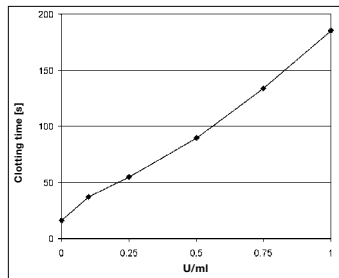
Patient Group	Clotting Time [s]		
	Median	Quartile 1-3	95% range
Normal Range (n=159)	25.3	21.8-27.1	19.2-30.6
UFH (n=93)	92.9	77.5-109.7	30.8-143.3
prophylactic LMWH (n=102)	62.8	45.8-73.2	29.4-110.7
therapeutic LMWH (n=124)	114.4	89.6-136.7	57.3-300.1
Fondaparinux (n=50)	61.9	53.8-68.2	50.2-86.4
Danaparoid (n=47)	90.1	65.1-116.8	46.8-144.6

The following diagrams show typical results obtained with dilution series in the clinical range of fondaparinux, danaparoid, argatroban hirudin and rivaroxaban:

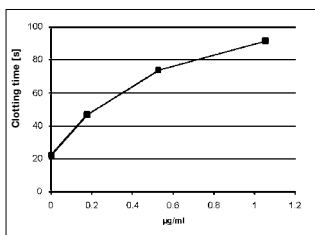
Fondaparinux (Arixtra®)



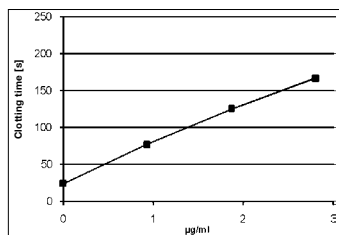
Danaparoid (Orgaran®)



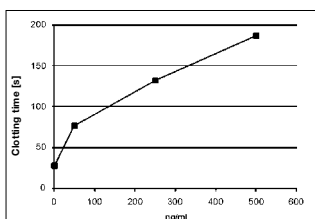
Argatroban (Argatra®)



Hirudin (Refludan®)



Rivaroxaban (Xarelto®)



Precautions

The reagent R1 contains products derived from animal blood and snake venom and has therefore to be handled as potentially infectious material.

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Trademarks

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