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PENTAPHARM LTD

Pefakit® APC-R Factor V Leiden

REF **502-01**

IVD - For In-Vitro Diagnostic Use

CE

Intended Use and Application

Pefakit® APC-R Factor V Leiden is a plasma based functional assay for the determination of resistance to activated protein C caused by the factor V Leiden mutation (FV:Q^{506}).

Introduction

Activated protein C (APC) resistance is the most frequent hereditary defect associated with deep vein thrombosis. Over 95% of the APC resistance phenotype can be explained by the Factor V Leiden mutation [1,2,3,4,5,6]. This defect is caused by point mutation in the factor V gene resulting in a replacement of the amino acid Arg 506 by a Gln residue [2,3,7]. The heterozygous (het) defect is associated with a 5 to 10 fold, the homozygous (hom) defect with a 50 to 100 fold increased thrombosis risk [5,8,9].

There are two possibilities of detecting factor V (FV) Leiden. Plasma based functional assays identifying the phenotype expression of the defect [1] or genotype determination which can be done by PCR technology [10].

Principle of the Method

Pefakit® APC-R Factor V Leiden is a plasma-based functional clotting assay and differs from other functional APC resistance tests by acting specifically on the prothrombinase complex level. It is based on a FV-dependent prothrombin activator isolated from snake venom. Robustness and specificity of the assay is enhanced by elimination of possible disturbing influences by factors upstream the coagulation cascade and independency from calcium. Interference from UFH, LMWH and Pentasaccharide in the blood sample is precluded by a heparin inhibitor added to reagents 1 and 2.

Sample plasma is pre-diluted with reagent 4 (dilution plasma) and incubated at 37°C with FV activator from snake venom (RVV-V from *Daboia russelli*). Coagulation is triggered by the addition of a FV dependent prothrombin activator from snake venom from *Notechis scutatus* in the absence of calcium. The clotting times are recorded and the ratios (clotting time in the presence of APC / clotting time in the absence of APC) are calculated.

Reagents

Reagent	Content
R1	**APC / RVV-V (+APC) Reagent** (APC, RVV-V, Polybrene, Hepes, BSA) 3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)
R2	**RVV-V (-APC) Reagent** (RVV-V, Polybrene, Hepes, BSA) 3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)
R3	**PTA Reagent** (Prothrombin Activator, EDTA, Hepes, BSA) 3 vials (lyophilisate, to be reconstituted in 4.0 ml of deionized water per vial)
R4	**Dilution Plasma** (Human Plasma, processed) 3 vials (lyophilisate, to be reconstituted in 2.0 ml of deionized water per vial)

Incubate reconstituted solutions R1-R4 in closed vials for 30' at room temperature and swirl gently before use. **Attention:** Extended incubation of reagent R4 may - due to its high protein content - cause a phase separation characterized by a clear solution with a fine, whitish layer on its surface. This may be erroneously interpreted as coagulation. Therefore, the reagent must absolutely be brought in its initial homogeneous and slightly cloudy form just before use.

Materials required but not provided

- Deionized water
- Calibrated pipettes (1000 - 5000 µl)
- Automated or semi-automated coagulation instruments using mechanical or optical detection methods

  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:

Reagent	Stability	
R1	-20°C	6 months
	2-8°C	14 days
	15-25°C	24 hours (on-board)
R2	-20°C	6 months
	2-8°C	14 days
	15-25°C	24 hours (on-board)
R3	-20°C	6 months
	2-8°C	14 days
	15-25°C	24 hours (on-board)
R4	-20°C	6 months
	2-8°C	14 days
	15-25°C	24 hours (on-board)

Frozen reagents should be thawed at 37°C and gently mixed before use. Freeze only once.

Quality Controls

Use Pefakit® APC-R Factor V Leiden Controls (REF 502-21) as a control reference for the validation of the assay. Negative control or wild-type (neg) shows normal response to APC whereas heterozygous control (het) shows response to the presence of the heterozygous type of FV:Q^{506} mutation. A control run should be made with each test series.

For preparation, use and interpretation of the controls, refer to the instructions and certified ranges mentioned in the package insert of the corresponding control kit.

Different clotting times will be obtained with different types of instruments depending on the clot detection principle. If values outside the certified range (ratio) 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. As a general rule hemolytic plasma samples should not be used.

For storage freeze undiluted plasma rapidly at -70°C in aliquots. Freeze only once. Avoid repeated freezing and thawing cycles. To ensure negligible loss of activity of labile coagulation factors and absence of cryoprecipitate, thawing should be done rapidly (within 5 min) in a water bath at 37°C. For more information see NCCLS document H21-A2 [11].

Stability of undiluted samples (plasma):

-80°C	at least 1 year
-20°C	2 months
2-8°C	24 hours
15-25°C	4 hours

Procedure

Prepare reagents and samples as described above. Mix gently thawed sample for homogenization, avoid foam formation. Determine +APC clotting time (clotting time in the presence of Activated Protein C), -APC clotting time (clotting time in the absence of Activated Protein C) and calculate the ratio according to the following scheme:

		+APC	-APC
	Sample or control plasma	30 µl	30 µl
R4	**Dilution Plasma**	20 µl	20 µl
		mix prior to use	mix prior to use
R1	**APC/RVV-V (+APC) Reagent**	50 µl	-
R2	**RVV-V (-APC) Reagent**	-	50 µl
	Incubation	**3 min, 37°C**	**3 min, 37°C**
R3	**PTA Reagent**	50 µl	50 µl
		determine clotting time	determine clotting time
	Ratio calculation	Ratio = +APC clotting time / -APC clotting time	

Interpretation of the test results

Differentiation of homozygous, heterozygous and negative samples is based on the typical ratio ranges measured with genotyped patient plasma samples (see tables below). These ratios may vary depending on laboratory, instrument and lot. Therefore, it is recommended to establish individual ranges and cut-offs for each laboratory and each instrument (if necessary also for each lot) by testing series of known genotyped patient plasmas.

Expected Values

Typical ratio ranges for PCR-genotyped patient plasmas on different devices are shown in the table below.

KC-4/-10 A™ micro		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	99	≥ 3.0
heterozygous	166	1.3 - 1.9
homozygous	25	0.9 - 1.1

BCS® (Behring Coagulation System)		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	143	≥ 3.0
heterozygous	170	1.4 - 2.2
homozygous	27	0.9 - 1.1

CA-1500 / CA-7000		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	235	≥ 3.0
heterozygous	56	1.5 - 1.8
homozygous	2	1.0 - 1.1

ACL 9000™		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	127	≥ 3.0
heterozygous	119	1.4 - 2.1
homozygous	24	0.9 - 1.2

STA® C		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	134	≥ 2.9
heterozygous	83	1.3 - 1.8
homozygous	27	0.9 - 1.1

AMAX CS-190		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	74	≥ 3.0
heterozygous	62	1.2 - 2.3
homozygous	10	0.9 - 1.1

When using these tables, following restrictions should be considered:

1. These are examples and **no** reference ranges or cut-offs guaranteed by the manufacturer.
2. Certain interference factors (refer to „Limitations and Interferences“) may cause ratio values which cannot clearly be attributed to a particular genotype, or may lead to clotting times that exceed the maximum admitted detection time of the instrument. In these cases, further investigation by PCR and the determination of individual factors are absolutely essential.

Sensitivity and Specificity

With the samples tested so far Pefakit® APC-R Factor V Leiden provided 100% sensitivity and 100% specificity for carriers of heterozygous and homozygous FV:Q^{506} mutation as determined by BCS® (n=340), KC-4/-10 A™ micro (n=290), CA-1500/CA-7000 (n=293), ACL 9000™ (n=270), STA® C (n=244) and AMAX CS-190 (n=146).

Due to the functional detection technique the assay is supposed to detect other FV mutations leading to APC-R phenotype as well. However their prevalence is very low compared to the FV Leiden mutation.

Accuracy and Reproducibility

With 2 genotyped plasma samples (neg/het) a series of 25 measurements were taken on the same day on 2 different fully automated analytical systems (BCS®, CA-500). Correlation of variance (CV) was determined based on the ratio. For both instruments and plasma genotypes the CV was below 5%.

In a further study on the STA® C analyzer, a series of 10 tests on each of 5 consecutive days were done using a heterozygous and a negative (wild-type) control. The CV of the clotting times and the ratios within each day and over all 5 days was ≤5.0 for the negative control and <3.0 for the heterozygous control.

The values obtained during these 5 days were within the following ranges:

	C1 FV-L Negative Control			C2 FV-L Heterozygous Control		
	+APC(s)	-APC(s)	Ratio	+APC(s)	-APC(s)	Ratio
min	132.9	24.2	5.3	39.2	25.4	1.5
max	166.3	25.7	6.7	42.6	27.7	1.6

Limitations and Interferences

No significant differences are observed when fresh or frozen plasma samples are used. It does neither matter whether buffered or un-buffered citrate plasma is used.

There is no significant influence on ratio or test sensitivity in case of Fibrinogen, Prothrombin, FVIII, FX, ATIII, Protein C, or Protein S deficiency (up to 100%) or excess of Fibrinogen, FVIII, ATIII, or TFPI (up to 5 times normal value). Mechanical measurements are neither influenced by the hemolytic blood samples nor by samples containing platelet residues. In contrast, optical methods can be influenced by the use of hemolytic or lipemic plasmas. Lupus anticoagulant antibodies did not influence the test. But a high Factor V deficiency (<50%) may lead to strongly elevated clotting times and thus may lead to loss of discrimination performance. The presence of Aprotinin (which inhibits the APC used in this test) and Protamine in the patient's blood can considerably shorten the clotting times, which may also lead to loss of discrimination power.

Due to the addition of Polybrene the prescribed assay procedure allows for the analysis of plasma from anticoagulated patients at heparin levels ≤2 IU/ml (UFH and LMWH) or pentasaccharide levels ≤2 µg/ml. The effect of direct thrombin inhibitors such as Hirudin or Argatroban is not inhibited by Polybrene. Hirudin in the patient plasma has a strong effect on clotting times and thus precludes proper discrimination of the different genotypes.

Therefore, after treatment of patients with Aprotinin, Protamine or direct thrombin inhibitors, it is recommended to either wait at least 24 hours before blood sampling for the test, or determine the FVL by a PCR method.

Precautions

Each donor unit used in the preparation of Dilution Plasma (R4) has been tested for antibodies against HIV Type 1 and 2, Hepatitis C-Virus antibodies, Treponema pallidum antibodies as well as Hepatitis B surface-antigen and Hepatitis C genome by PCR. The tests used are all CE certified tests according to list A of the European Directive for IVDs (98/79/EC) and are under supervision by the responsible European governmental authority. The plasmas were found to be negative on the tested parameters. However, since no test can completely rule out the presence of blood borne diseases these control plasmas have to be handled as potentially infectious material.

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