Pefakit® APC-R Factor V Leiden

REF 502-01 **IVD** **For in-vitro Diagnostic Use**

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 FVa-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*), in order to convert FV into FVa. Coagulation is triggered by the addition of a FVa 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 =** $\frac{\text{+APC clotting time}}{\text{–APC clotting time}}$	

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.

Semi-automatic ball coagulometer (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®XP		
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

CS-line		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	62	> 3.5
heterozygous	37	1.4–2.0
homozygous	2	1.0–1.1

ACL Top®line		
Genotype FV:Q^{506}	n	Ratio range (min/max)
negative	138	> 2.8
heterozygous	94	1.3–1.8
homozygous	1	1.0–1.1

STA®line		
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

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®XP(n=340), Semi-automatic ball coagulometer (micro) (n=290), CS-2100/CS-5100 (n=101), ACL Top®500 (n=233) and STA®C (n=244).
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

Accuracy was determined with one C1 FV-L Negative Control and one C2 FV-L Heterozygous Control each in a series of 20 measurements taken on the same day on three fully-automated analytical systems (BCS®XP, ACL Top®500, STA® C).
The coefficient of variation (CV%) for the clotting time and the ratio on all three analytical systems was ‹10% for the C1 FV-L Negative Control and ‹5% for the C2 FV-L Heterozygous Control.
Reproducibility was determined by taking two measurements/day on each of 20 different days on three fully-automated analytical systems (BCS®XP, ACL Top®500, STA® C) with one C1 FV-L Negative Control and one C2 FV-L Heterozygous Control each.
The following coefficients of variation (CV%) for the clotting time and the ratio were found with the two controls:

ACL Top® 500	Mean (ratio)	CV% (within each day)	CV% (day to day)	CV% total
C1 FV-L Negative Control	5.0	7.4	11.2	10.7
C2 FV-L Hetero-zygous Control	1.7	5.1	5.1	5.4

BCS® XP	Mean (ratio)	CV% (within each day)	CV% (day to day)	CV% total
C1 FV-L Negative Control	4.7	2.9	4.6	4.8
C2 FV-L Hetero-zygous Control	1.6	2.8	4.5	4.1

STA®-C	Mean (ratio)	CV% (within each day)	CV% (day to day)	CV% total
C1 FV-L Negative Control	5.5	8.7	7.6	9.3
C2 FV-L Hetero-zygous Control	1.5	1.6	3.6	3.7

Limitations and Interferences

Use of fresh or thawed frozen plasma samples makes no significant difference to the test result. Likewise, use of buffered or non-buffered citrated plasma does not interfere with the test result.

Experimental studies have shown that vitamin k antagonists (phenprocoumon, warfarin) and a deficiency of fibrinogen, prothrombin, Factor VIII, Factor X, ATIII, protein C and protein S up to 100%, and an excess of fibrinogen, Factor VIII, ATIII and TFPI up to 5 times normal values have no influence on the ratio or the sensitivity of the test. Hemolytic samples or contamination with platelets do not interfere with mechanical measurements. In contrast, optical methods can be influenced by hemolytic or lipemic plasma samples. Lupus anticoagulant antibodies have not been shown to interfere with the test. However, severe Factor V deficiency (‹50%) may lead to prolonged clotting times and thus reduce the discriminatory performance of the test. The presence of aprotinin (which inhibits the APC used in the test) and protamine in the patient's blood can considerably shorten clotting times, and this may also reduce discriminatory performance.

The addition of polybrene does not interfere with the measurement of plasma from heparinized patients up to concentrations of ‹2 IU/ml UFH, LMWH and ‹2 µg/ml pentasaccharide. Direct Factor Xa inhibitors (e.g. rivaroxaban) have no influence on the discriminatory performance of the test, whereas direct thrombin inhibitors (e.g. argatroban, dabigatran) interfere with discriminatory performance.
Following administration of aprotinin, protamine or direct thrombin inhibitors, it is therefore recommended either to delay blood sampling for the Pefakit® APC-R test for up to 24 hours or to determine the FVL mutation using PCR as an alternative method.

Warnings and Precautions

Reagent R1 (55025R1)

Warning – Reagent R1 contains bovine serum albumin (BSA).

Hazard statement:
H302 Harmful if swallowed.
Safety statement:
P264 Wash hands thoroughly after handling.

Reagent R1 contains material obtained from human plasma (‹0.1%) and should therefore be handled with caution, taking due note of the recommended precautions for biohazards.

Reagent R2 (55025R2)

Warning – Reagent R2 contains bovine serum albumin (BSA).

Hazard statement:
H302 Harmful if swallowed.
Safety statement:
P264 Wash hands thoroughly after handling.

Reagent R3 (55025R3)

Warning – Reagent R3 contains disodium dihydrogenethylenediaminetetraacetate, bovine serum albumin (BSA).

Hazard statements:
H302 Harmful if swallowed.
H332 Harmful if inhaled.
H373 May cause damage to the respiratory organs through prolonged or repeated exposure. Route of exposure: inhalation.
Safety statements:
P260 Do not breathe dust.
P264 Wash hands thoroughly after handling.
P310 Immediately call a POISON CENTER or doctor/ physician.

Reagent R4 (55025R4)

Reagent R4 contains human plasma (50–100%). Each donor unit used in the preparation of Dilution Plasma (R4) has been tested for antibodies against HIV1 and 2, Hepatitis C-Virus (HCV) antibodies, Treponema pallidum antibodies, as well as for Hepatitis B surface antigen and Hepatitis C und HIV 1 genome (PCR) and has been found to be negative. The tests used are CE-certified according to List A of the European Directive for IVDs (98/79/EC) and are subject to control by the responsible authority.
Nevertheless it should be assumed that no test can exclude the presence of blood-borne diseases with absolute certainty. Consequently, this reagent should be regarded as potentially infectious material and should be handled accordingly.

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