FV-PTH mpx RealFast™ Assay

REF 7-115 / 7-118 Σ 100 / 32 reactions

-20°C 2-8°C CE IVD

ViennaLab Diagnostics GmbH
Gaudenzdorfer Guertel 43-45
A-1120 Vienna, Austria
Phone: (+43-1) 8120156-0
info@viennalab.com
www.viennalab.com

1. Intended Use

The FV-PTH mpx RealFast™ Assay is a fast and accurate multiplex real-time PCR test for the simultaneous detection of the two most important thrombophilic mutations. The point mutation 1691G>A in the human coagulation *Factor V (F5)* gene, referred to as FV Leiden, and the prothrombin (PTH) 20210G>A mutation in the *Factor II (F2)* gene are associated with hereditary thrombophilia. The kit is intended to test patients suspected of having an increased risk for thrombotic disorders. The qualitative assay discriminates the three possible genotypes for each of the mutations in human genomic DNA: normal, heterozygous or homozygous mutant.
Reference sequence: FV: NG_011806.1 g.41721G>A; dbSNP: rs6025 / PTH: NG_008953.1 g.25313G>A; dbSNP: rs1799963.

2. Introduction

FV and PTH are major players in the coagulation cascade. The FV Leiden mutation causes a single amino acid exchange at position 506 (R506Q), which alters a cleavage site and thereby prevents efficient inactivation of FV. Persisting FV activity increases the risk of clot formation in veins. The PTH 20210G>A mutation in the 3' untranslated region results in increased mRNA synthesis and higher prothrombin plasma levels, which in turn lead to elevated thrombin generation and consequently to excessive formation of fibrin clots. Heterozygous FV Leiden carriers encounter a 5 to 10 times higher risk of having venous thrombosis, whereas homozygous carriers have a 50 to 100 times higher risk compared to non-carriers. Heterozygous PTH 20210G>A carriers encounter a 3-fold, homozygous carriers up to a 20-fold increased risk compared to non-carriers. Individuals with additional risk factors, like the presence of other thrombophilic mutations, obesity, hypertension, type 2 diabetes, smoking or intake of oral contraceptives, are even more predisposed to venous thrombotic events.

3. Kit Contents

			100 / 32 Rxn
RealFast™ 2x mpx **Probe Mix**	1 vial	white cap	1000 / 320 µl
FV-PTH mpx **Assay Mix**	1 vial	purple cap	550 / 550 µl
FV-PTH mpx **WT-Control**	1 vial	green cap	75 / 75 µl
FV-PTH mpx **MUT-Control**	1 vial	red cap	75 / 75 µl

The kit contains reagents for 100 / 32 reactions in a final volume of 20 µl each.

The RealFast™ 2x Probe Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The FV-PTH mpx Assay Mix consists of *F5* and *F2* gene-specific primers and four allele-specific, dual-labeled hydrolysis probes. Controls representing wild type (WT-Control) and homozygous mutant (MUT-Control) genotypes are supplied with the kit.

4. Storage and Stability

FV-PTH mpx RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -20°C. Alternatively, store at 2 to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid prolonged exposure to intense light. If stored correctly, the kit will retain full activity until the expiration date indicated on the label.

5. Product Description

5.1. Principle of the Test

The test is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains two gene-specific primer pairs which amplify a 142 bp fragment of the *F5* gene and a 110 bp fragment of the *F2* gene, as well as four dual-labeled, allele-specific hydrolysis probes which hybridize to the target sequences of the amplified fragments. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5' – 3' exonuclease activity of the Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product.

Hydrolysis probe	Fluorophore	Channel
FV mutant	FAM	520 nm
FV wild type	HEX	556 nm
PTH mutant	ROX	605 nm
PTH wild type	Cy5	670 nm

In normal samples the **wild type probes** generate a strong fluorescence signal in the HEX or Cy5 channel and no or only a baseline signal in the FAM or ROX channel. Vice versa, in homozygous mutant samples the hybridized **mutant probes** generate a strong fluorescence signal in the FAM or ROX channel and no or only a baseline signal in the HEX or Cy5 channel. In heterozygous samples, both wild type and mutant probes bind to the amplicons and generate intermediate signals in the respective channels.

5.2. Real-time PCR Instrument Compatibility

The FV-PTH mpx RealFast™ Assay is validated for use with various common real-time PCR instruments capable of recording FAM, HEX, Cy5 and ROX fluorescence:

- ✓ AB 7500 Fast (Applied Biosystems®)
- ✓ CFX96™ (Bio-Rad)
- ✓ LightCycler® 480 (Roche)
- ✓ MIC qPCR Cycler (bms)
- ✓ Rotor-Gene® 6000 (Qiagen)

» ***Note:*** *RealFast™ Genotyping QuickGuides for setting up and analyzing experiments on different types of instruments can be downloaded from www.viennalab.com.* «

The kit is **not suitable** for use with real-time PCR instruments requiring ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT) or for instruments without appropriate fluorescence detection channels.

5.3. Assay Performance Specifications

Determination of **sensitivity** was performed on 39 positive FV Leiden alleles and 37 positive PTH 20210G>A alleles, both tested with a CE-marked reference kit. The FV-PTH mpx RealFast™ Assay correctly determined all positive alleles, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 235 negative FV Leiden alleles and 247 negative PTH 20210G>A alleles, both tested with a CE-marked reference kit. The FV-PTH mpx RealFast™ Assay correctly determined all negative alleles, which equaled a true negative rate of 100%.

Limit of detection: 0.2 ng genomic DNA (per reaction). Recommended DNA concentration: 2 to 20 ng/µl genomic DNA.

6. Materials Required but not Supplied

Real-time PCR instrument with FAM (520 nm), HEX (556 nm), ROX (605 nm) and Cy5 (670nm) filters, instrument-compatible reaction vessels, disposable powder-free gloves, vortexer, mini-centrifuge for 2.0 ml tubes, tube racks, set of calibrated micropipettes (0.5 – 1000 µl), sterile tips with aerosol-barrier filter, molecular grade water, DNA extraction system, freezer, biohazard waste container.

7. Experimental Protocol

7.1. DNA Extraction

DNA extraction reagents are **not supplied** with the kit.
DNA isolated from various specimens (e.g. whole peripheral blood, dried blood spots, buccal swabs or saliva) can be used. Ensure extracted DNA is suitable for amplification in terms of concentration, purity and integrity.
For accurate genotype calling, the DNA amount per reaction should be within the range of 10 to 100 ng for all samples.

7.2. PCR Controls

Always include a **No Template Control** (NTC) in each experiment to confirm absence of potential contamination. It is advisable to run the NTC (use PCR-grade water instead of DNA) in duplicate.

Always include the FV-PTH mpx **WT-Control** and FV-PTH mpx **MUT-Control** as positive reference signals for your unknown samples. Some real-time PCR software, e.g. AB 7500 Fast, requires signals for all three possible genotypes for correct allelic discrimination. In order to obtain a heterozygous control (HET-Control), mix an aliquot of WT-Control and MUT-Control in a ratio of 1:1.

» ***Note:*** *WT- and MUT-Controls are potential sources of contamination. Make sure to handle them carefully.* «

7.3. Preparation of FV-PTH mpx RealFast™ **Master Mix**:

Gently vortex and briefly centrifuge all solutions after thawing. Set up PCR at room temperature. Prepare sufficient **Master Mix** for all your reactions (N samples + positive controls + negative controls) plus at least one additional reaction to compensate for pipetting inaccuracies:

Component	per reaction	e.g. 24+1 reactions
RealFast™ 2x Probe Mix	10 µl	250 µl
FV-PTH mpx Assay Mix	5 µl	125 µl
Master Mix	**15 µl**	375 µl

Dispense **15 µl Master Mix** into each well. Add **5 µl** purified **DNA** or **Control** template to reach a final reaction volume of 20 µl.
To minimize risk of contamination, always pipette templates in the following order: first NTC, then samples, last positive controls. Immediately close reaction vessels.

» ***Note:*** *Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed.* «

7.4. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for allelic discrimination / genotyping experiments. Place the samples into the thermal cycler and run the following program:

Program			**AB 7500 Fast, CFX96™, LightCycler® 480,** and **other** Peltier heating-block based **instruments**	**MIC, Rotor-Gene® 6000** (36-well & 72-well rotor)
Cycles	Temp	Time	Steps	Steps
1	95°C	3 min	Initial denaturation	Initial denaturation
40	95°C	15 sec	Denaturation	Denaturation
	60°C	1 min	Annealing/Extension – **Data acquisition** on FAM, HEX, ROX and Cy5 channels	Annealing/Extension – **Data acquisition** on Green, Yellow, Orange and Red channels

8. Data Analysis / Interpretation of Results

The genotype of each sample is determined by calculating the ratio between signals recorded in the **HEX** or **Cy5 channel (normal)** and signals recorded in the **FAM** or **ROX channel (mutant)**. Most real-time PCR software automatically resolves data of two channels into clusters in a scatterplot. Data points plotted along the x- and y-axes correspond to normal and homozygous mutant genotypes, respectively. Data points clustered in the middle of the scatterplot represent heterozygous genotypes. The NTC appears in the lower left corner.

Controls / Samples	Amplification in channel				Genotype **FV** / **PTH**
	FAM Green	HEX Yellow	ROX Orange	Cy5 Red	
mpx WT-Control	NO	**YES**	NO	**YES**	normal FV / normal PTH
mpx HET-Control	**YES**	**YES**	**YES**	**YES**	heterozygous FV / heterozygous PTH
mpx MUT-Control	**YES**	NO	**YES**	NO	homozygous mutant FV / homozygous mutant PTH
NTC	NO	NO	NO	NO	**----**
Sample 1	**YES**	**YES**	NO	**YES**	heterozygous FV / normal PTH
Sample 2	**YES**	NO	NO	**YES**	homozygous mutant FV / normal PTH
Sample 3	NO	**YES**	**YES**	**YES**	normal FV / heterozygous PTH
Sample 4	NO	**YES**	**YES**	NO	normal FV / homozygous mutant PTH

Some instrument software needs manual threshold settings for accurate genotype calling.

Recommendations for Threshold Settings (C_q):
Set threshold value for the FAM and ROX channels just above the background fluorescent signal generated by the WT-Control (HEX-/Cy5-positive). Vice versa, set threshold value for the HEX and Cy5 channels just above the background fluorescent signal of the MUT-Control (FAM-/ROX-positive).
Samples crossing the threshold line beyond C_q 37 give invalid results and must be repeated.

To analyze acquired data, please follow your instrument software instructions.

9. Warnings and Precautions

- For *in vitro* diagnostics use only.
- Always use disposable powder-free gloves and wear suitable lab coat when handling specimens and reagents.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use pipettes dedicated for PCR setup only, use aerosol-guarded pipette tips.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.
- Do not use expired kits or kit components.