PTH 20210G>A RealFast™ Assay

REF 7-120 Σ 100 reactions

-20°C / 2-8°C CE IVD

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1. Intended Use

The PTH 20210G>A RealFast™ Assay is a fast and accurate real-time PCR based test for the detection of the 20210G>A mutation in the human *Factor II* (*FII*) gene encoding the coagulation factor prothrombin (PTH). This point mutation is associated with hereditary thrombophilia. The kit is designed to identify patients at risk of venous thromboembolism. The qualitative assay discriminates the three possible PTH 20210G>A genotypes in a human DNA extract: GG (normal), GA (heterozygous) or AA (homozygous mutant).
Reference sequence: HGVS: NG_008953.1 g.25313G>A; NCBI dbSNP: rs1799963.

2. Introduction

PTH, also known as Factor II, is a proenzyme in the blood coagulation cascade. The 20210G>A mutation in the 3' untranslated region of the gene alters the polyadenylation site. The resulting increase in mRNA synthesis leads to higher prothrombin plasma levels, which in turn give rise to elevated thrombin generation and subsequently to excessive formation of fibrin clots.
The PTH 20210G>A mutation, showing a prevalence of 2 % in Caucasians, is the second most common genetic risk factor for venous thromboembolism. Heterozygous carriers are exposed to 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 genetic predispositions, obesity, hypertension, type 2 diabetes, smoking or intake of oral contraceptives, are even more predisposed to venous thrombotic events.

3. Kit Contents

RealFast™ 2x **Genotyping Mix**	1 vial	white cap	1000 µl
PTH 20210G>A **Assay Mix**	1 vial	purple cap	550 µl
PTH 20210G>A **WT-Control**	1 vial	green cap	75 µl
PTH 20210G>A **MUT-Control**	1 vial	red cap	75 µl

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

The RealFast™ 2x Genotyping Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The PTH 20210G>A Assay Mix consists of *FII* gene-specific primers and two 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

PTH 20210G>A 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 a gene-specific primer pair which amplifies a 110 bp fragment of the *FII* gene, and two dual-labeled, allele-specific hydrolysis probes which hybridize to the target sequence of the amplified fragment. 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.
In normal samples the **HEX-labeled** PTH 20210G>A **wild type probe** hybridizes to the complementary strand of the gene fragment. A strong fluorescence signal is detected in the HEX channel (556nm) and no or only a baseline signal in the FAM channel (520nm). Vice versa, in homozygous mutant samples the **FAM-labeled** PTH 20210G>A **mutant probe** binds to the gene fragment. A strong fluorescence signal is detected in the FAM channel and no or only a baseline signal in the HEX channel. In heterozygous samples both wild type and mutant probes bind to the amplicons and generate intermediate signals in both channels.

5.2. Real-time PCR Instrument Compatibility

The PTH 20210G>A RealFast™ Assay is validated for use with the AB 7500 Fast instrument.
The kit is compatible with various common real-time PCR instruments capable of recording FAM and HEX fluorescence:

- ✓ AB 7500 Fast (Applied Biosystems®)
- ✓ AB StepOne™ (Applied Biosystems®)
- ✓ CFX96™ (Bio-Rad)
- ✓ LightCycler® 480 (Roche)
- ✓ Mx3005P (Agilent Technologies)
- ✓ 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.*

When using AB StepOne™, set passive reference dye to "ROX"! «

The kit is supplied **without ROX**. For use with real-time PCR instruments requiring high ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT), add ROX at a final concentration of 1 µM to the 2x Genotyping Mix.

5.3. Assay Performance Specifications

Determination of **sensitivity** was performed on 49 alleles testing positive for the PTH 20210G>A mutation with a CE-marked reference kit. The PTH 20210G>A RealFast™ Assay determined all 49 alleles as positive, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 113 alleles testing negative for the PTH 20210G>A mutation with a CE-marked reference kit. The PTH 20210G>A RealFast™ Assay determined all 113 alleles as negative, 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) and HEX (556 nm) 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 **PTH 20210G>A WT-Control** and **PTH 20210G>A 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 PTH 20210G>A 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 Genotyping Mix	10 µl	250 µl
PTH 20210G>A 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:

AB 7500 Fast, StepOne™, CFX96™, LightCycler® 480, Mx3005P and **other** Peltier heating block-based **instruments**:

Cycles	Temp	Time	Steps
1	95°C	3 min	Initial denaturation
40	95°C	15 sec	Denaturation
	60°C	1 min	Annealing/Extension – **Data acquisition** on FAM and HEX channel

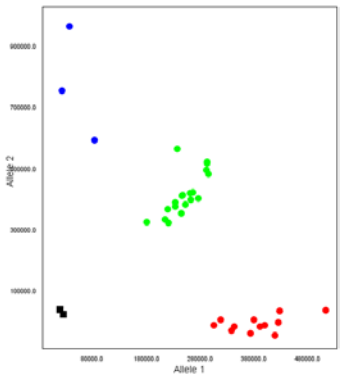
Rotor-Gene® 6000:

Cycles	Temp	Time	Steps
1	95 °C	3 min	Initial denaturation
40	95 °C	15 sec	Denaturation
	36-well rotor: **56 °C**	1 min	Annealing/Extension – **Data acquisition** on Green and Yellow channel
	72-well rotor: **60 °C**		

8. Data Analysis / Interpretation of Results

The genotype of each sample is determined by calculating the ratio between signals recorded in the **HEX channel (normal)** and signals recorded in the **FAM channel (mutant)**. Most real-time PCR software automatically resolves data of both 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	Amplification in **FAM** channel (520 nm)	Amplification in **HEX** channel (556 nm)	Genotype
WT-Control	NO	**YES**	normal
HET-Control	**YES**	**YES**	heterozygous
MUT-Control	**YES**	NO	homozygous mutant
NTC	NO	NO	----



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

Recommendations for Threshold Settings (C_q):
Set threshold value for the FAM channel just above the background fluorescent signal generated by the WT-Control (HEX-positive). Vice versa, set threshold value for the HEX channel just above the background fluorescent signal of the MUT-Control (FAM-positive).

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.