

VKORC1 -1639G>A RealFast™ Assay



ViennaLab Diagnostics GmbH

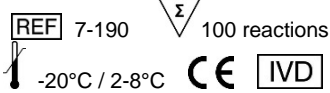
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1. Intended Use

The VKORC1 -1639G>A RealFast™ Assay is a fast and accurate real-time PCR test for the detection of the -1639G>A single nucleotide polymorphism (SNP) in the promoter region of the human *Vitamin K Epoxide Reductase Complex 1* (VKORC1) gene. This polymorphism is the most relevant tag-SNP associated with interpatient variability in dose requirements for oral anticoagulants, like warfarin, phenprocoumon or acenocoumarol. The qualitative assay discriminates the three possible VKORC1-1639G>A genotypes in a human DNA extract: GG (normal dose), GA (intermediate dose) or AA (low dose).

Reference sequence: HGVS: NG_011564.1 g.3588G>A; NCBI dbSNP: rs9923231.

2. Introduction

VKORC1 is a key protein in the vitamin K pathway and target of vitamin K antagonists, which are used as oral anticoagulants in the prevention and therapy of thromboembolic disorders. These drugs are widely prescribed, but their narrow therapeutic range and wide variation in drug response complicate treatment particularly at the beginning. Empiric dosing results in frequent dose changes as the international normalized ratio (INR) gets too high or too low, leaving patients at risk for bleeding (over-anticoagulation) or thromboembolism (under-anticoagulation). Apart from age, gender, body mass index and use of concomitant medication, the VKORC1 -1639G>A variant is the most important genetic factor affecting therapeutic dose requirements. Homozygous carriers of the A allele require a lower dose of oral anticoagulants than heterozygous or homozygous carriers of the G allele.

3. Kit Contents

RealFast™ 2x Genotyping Mix	1 vial	□ white cap	1000 µl
VKORC1 -1639G>A Assay Mix	1 vial	■ purple cap	550 µl
VKORC1 -1639G>A GG-Control	1 vial	■ green cap	75 µl
VKORC1 -1639G>A AA-Control	1 vial	■ red cap	75 µl

The RealFast™ 2x Genotyping Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system.

The VKORC1 -1639G>A Assay Mix consists of VKORC1 gene-specific primers and two allele-specific, dual-labeled hydrolysis probes. Controls representing VKORC1 -1639GG and -1639AA genotypes are supplied with the kit.

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

4. Storage and Stability

VKORC1 -1639G>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 120 bp fragment of the VKORC1 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 VKORC1 -1639GG samples the **HEX-labeled VKORC1 -1639G 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 VKORC1 -1639AA samples the **FAM-labeled VKORC1 -1639A 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 (VKORC1 -1639GA) both probes bind to the amplicons and generate intermediate signals in both channels.

5.2. Real-time PCR Instrument Compatibility

The VKORC1 -1639G>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 77 alleles testing positive for the VKORC1 -1639A polymorphism with a CE-marked reference kit. The VKORC1 -1639G>A RealFast™ Assay determined all 77 alleles as positive, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 95 alleles testing negative for the VKORC1 -1639A polymorphism with a CE-marked reference kit. The VKORC1 -1639G>A RealFast™ Assay determined all 95 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 VKORC1 -1639 **GG-Control** and VKORC1 -1639 **AA-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 (GA-Control), mix an aliquot of GG-Control and AA-Control in a ratio of 1:1.

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

7.3. Preparation of VKORC1 -1639G>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
VKORC1 -1639G>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 (-1639G)** and signals recorded in the **FAM channel (-1639A)**. 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 -1639GG and -1639AA genotypes, respectively. Data points clustered in the middle of the scatterplot represent heterozygous -1639GA genotypes. The NTC appears in the lower left corner.

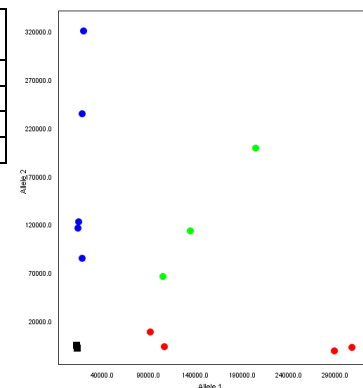
Controls	Amplification in FAM channel (520 nm)	Amplification in HEX channel (556 nm)	Genotype / Phenotype
GG-Control	NO	YES	GG / normal dose
GA-Control	YES	YES	GA / intermediate dose
AA-Control	YES	NO	AA / low dose
NTC	NO	NO	----

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 GG-Control (HEX-positive). Vice versa, set threshold value for the HEX channel just above the background fluorescent signal of the AA-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.