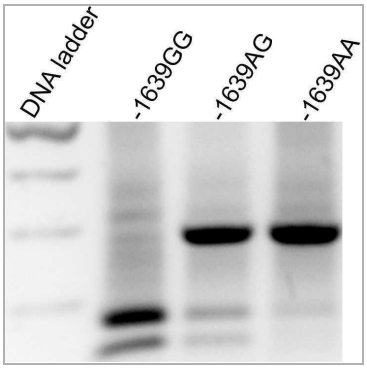


# Characterization of the VKORC1 and CYP2C9 genotypes V.4

Mirsada Causevic[1], Edin Begic[2,1]

[1]Sarajevo Medical School, Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina;
[2]General Hospital "Prim.Dr. Abdulah Nakas" Sarajevo, Bosnia and Herzegovina

mirsadacausevic

VERSION 4

MAR 14, 2023

OPEN ACCESS

**DOI:** dx.doi.org/10.17504/protocols.io.kxygx9edzg8j/v4

**Protocol Citation:** Mirsada Causevic, Edin Begic 2023. Characterization of the VKORC1 and CYP2C9 genotypes. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx9edzg8j/v4Version created by mirsadacausevic



**Protocol status:** Working
We use this protocol and it's working

**Created:** Mar 14, 2023

**Last Modified:** Mar 14, 2023

**PROTOCOL integer ID:** 78727

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**Keywords:** Vitamin K Epoxide Reductase, Vitamin KO Reductase, VKORC1, Cytochrome P-450, CYP2C9

ABSTRACT

Vitamin K antagonists (e.g. warfarin) are anticoagulants which represent widely prescribed drugs for prevention and treatment of thromboembolic disorders.

Warfarin's molecular target is vitamin K epoxide reductase enzyme and its metabolism is performed by the cytochrome P-450 2C9 enzyme, both of which are encoded by polymorphic genes - vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P-450 2C9 (*CYP2C9*), respectively.

Identification of the *VKORC1* and *CYP2C9* genotypes was recommended by the U.S. Food and Drug Administration in 2007, in order to guide the initial dosing of warfarin, the first oral vitamin K antagonist drug, to achieve the optimum anticoagulation and prevent hemorrhagic events.

In an effort to provide a lab protocol that will provide training in pharmacogenomics/pharmacogenetics (PGx) for undergraduate students of pharmacology or medicine, that can be performed in a laboratory that contains basic molecular biology equipment and by using reagents that are available worldwide at low cost, we revisited protocols published previously by other researchers.

We hope that it will provide the elementary understanding of the simplest molecular biology methods used to identify different *VKORC1* (*-1639G>A*) and *CYP2C9* genotypes, as well as understanding of a link between a specific genotype (or combination of genotypes) and phenotype relevant for a patient's drug response.

The protocol represents a step-by-step guide to using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method in determining *VKORC1 -1639G>A*, *CYP2C9*1/*1*, *CYP2C9*1/*2*, *CYP2C9*1/*3*, and *CYP2C9*2/*3* genotypes.

Based on the combination of the *VKORC1 -1639G>A* and *CYP2C9* genotypes, that is, the pattern of DNA fragments observed at the end of an experiment, which are visualized using agarose gel electrophoresis and DNA staining, a conclusion on an individual's sensitivity to warfarin and appropriate drug dose is reached.

A dataset containing the *VKORC1 -1639G>A* and *CYP2C9* genotypes characterized in 32 patients using this protocol was deposited at the Harvard Dataverse Repository that can be accessed at the following digital object identifier: https://doi.org/10.7910/DVN/SFPYCD. In the dataset, the patients were grouped into the following anticoagulant sensitivity/dosing categories, as recommended by the U.S. Food and Drug Administration's (FDA) label for warfarin: 1) standard anticoagulant sensitivity/dosing genotypes (N=20), 2) intermediate anticoagulant sensitivity/dosing genotypes (N=11), and 3) low anticoagulant sensitivity/dosing genotypes (N=1).

## ATTACHMENTS

Figure 1_VKORC1.jpg Figure 2_CYP2C9-1-1.jpg Figure 3_CYP2C9-1-2.jpg Figure 4_CYP2C9-1-3.jpg Figure 5_CYP2C9-2-3.jpg

## IMAGE ATTRIBUTION

Mirsada Causevic, Honours BSc, PhD, Sarajevo Medical School, Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina

## GUIDELINES

This protocol uses human whole blood as a starting material for extracting human, genomic DNA. Human whole blood could be a source of hepatitis B (HepB) virus infection (if taken from persons with HepB virus infection) to the individuals who work with human blood and, as such, represents health and safety or biological hazard.

## MATERIALS

Agarose - Sigma A9539-100G;
AvaII restriction endonuclease enzyme - New England BioLabs R0153S;
Bromophenol blue - Sigma B0126-25G;
Boric acid - Sigma B6768-500G;
DNA dye - Ethidium bromide - Sigma E1510-10ML;
DNA ladder (1) - BenchTop 100bp DNA Ladder - Promega G8291;
DNA ladder (2) - MiniSizer 50bp DNA Ladder - Norgen 11200;
dNTPs - dATP, dCTP, dGTP and dTTP - at a stock concentration of 100mM each - Promega U1330;
EDTA - Sigma EDS-500G;
Ethanol - Sigma 32205-1L;
Glycerol - Sigma G5516-500ml;
HCl - Sigma 30721-2.5L;
Isopropanol - 2-propanol - Sigma 59304-500ml;
KpnI restriction endonuclease enzyme - Promega R6341;
NaCl - Honeywell-Fluka 31434-1KG;
NsiI restriction endonuclease enzyme - New England BioLabs R0127S;
MspI restriction endonuclease enzyme - New England BioLabs R0106S;
Proteinase K - Sigma P2308;
SDS - Sigma L3771-100G;
GoTaq G2 DNA Polymerase - Promega M7845;
Trizma base - Sigma RDD008-1KG.

## SAFETY WARNINGS

Ethidium bromide, which is used during the preparation of agarose gels, is a DNA intercalating dye and it can, therefore, act as a mutagen. It should be handled with care and only when wearing gloves and safety glasses.

## ETHICS STATEMENT

The patients with a diagnosis of atrial fibrillation (N=32), who donated one blood sample each for this study, were recruited at the Maglaj Health Centre, Maglaj, Bosnia and Herzegovina, according to the Declaration of Helsinki from 2013, and following bioethical approval by the Institutional Review Board of the Maglaj Health Centre, from the 11th of November 2019.

## BEFORE START INSTRUCTIONS

In order to protect the individuals from the potential HepB virus infection while working with human blood, the U.S.A. Centers for Disease Control and Prevention (CDC) issued a recommendation for HepB vaccination to be carried out in individuals before they start working with human blood. Vaccine-induced antibody titer to the HepB virus of ≥10 mIU/mL should be present in individuals before commencing work with human blood.

# Genomic DNA extraction

1 Patients' whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at -20°C until use. Genomic DNA extraction from the human whole blood, that is, leukocytes, was carried out according to the protocol described by Subbarayan PR and colleagues (doi: 10.2144/02336bm10), with modifications.

Upon thawing of the blood, for every patient's blood sample, five (5) sterile tubes were labeled and 300 µl of the whole blood was added. This was the amount of the starting material that yielded ample amount of human, genomic DNA at the end of the DNA extraction protocol.

2 Concentrations of human, genomic DNA was determined by using Qubit 4 Fluorometer (Q33226, Invitrogen and Thermo Fisher Scientific).

Subsequently, for polymerase chain reactions (PCRs) 100 ng of genomic DNA was used in every PCR reaction.

# Identification of the *VKORC1 -1639G>A* genoytpes

3 Identification of the *VKORC1 -1639G>A* genotypes was performed according to the polymerase

chain reaction-restriction fragment length polymorphism (PCR-RFLP) method-based protocol published by Sconce EA and colleagues (doi: 10.1182/blood-2005-03-1108).

**4** PCR reactions were assembled as follows: reactions of 25 µl contained 1) sterile, milliQ-$H_2O$; 2) 1xPCR buffer (for GoTaq G2 DNA polymerase, Promega), from a stock of 5xPCR buffer; 3) 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (U1330, Promega), from a stock of 2.5 mM; 4) 1 µM forward primer, from a stock of 25 µM; 5) 1 µM reverse primer, from a stock of 25 µM; 6) 100 ng of genomic DNA and 7) 2.5 U GoTaq DNA polymerase (M7845, Promega).

Sequences of the PCR primers were as follows: 1) Forward primer: 5'-GCCAGCAGGAGAGGGAAATA-3'; 2) Reverse primer: 5'-AGTTTGGACTACAGGTGCCT-3'.

PCR cycles used for the amplification of the *VKORC1* gene promoter, which were based on the touchdown PCR protocol published by Korbie and Mattick in 2008, were as follows:
1) 95°C for 3 min (1 cycle);
2) 95°C for 2 s, 60°C for 2 s, 72°C for 30 s (2 cycles);
3) 95°C for 2 s, 59°C for 2 s, 72°C for 30 s (2 cycles);
4) 95°C for 2 s, 58°C for 2 s, 72°C for 30 s (2 cycles);
5) 95°C for 2s, 57°C for 2s, 72°C for 30 s (2 cycles);
6) 95°C for 2 s, 56°C for 2 s, 72°C for 30 s (2 cycles);
7) 95°C for 2 s, 55°C for 2 s, 72°C for 30 s (2 cycles);
8) 72°C for 7 min (1 cycle);
9) 4°C for 2 h (1 cycle).

**Expected result**

The expected size of the PCR product is 290 bp.

**5** In order to characterise the *VKORC1 -1639G>A* genotypes by using the PCR-RFLP method, the following reactions were set up with MspI restriction endonuclease enzyme:
1) 25 µl of the PCR product;
2) 3 µl of the 10xCutSmart Buffer (B7204S, New England Biolabs);
3) 1 µl of sterile, milliQ-$H_2O$;
4) 1 µl of MspI enzyme (R0106S, New England Biolabs). Total reaction volume: 30 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTris, boric acid, EDTA (TBE) buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images

were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

**Expected result**

The following pattern of DNA fragments can be expected after the MspI restriction endonuclease enzyme reaction (Figure 1):

1) GG or *VKORC1 -1639GG* genotype is characterized by two (2) DNA fragments of 168 and 122 base pairs (bp);

2) AG or *VKORC1 -1639AG* genotype is characterized by three (3) DNA fragments of 290, 168 and 122 bp;

3) AA or V*KORC1 -1639AA* genotype is characterized by one (1) DNA fragment of 290 bp.

## Identification of the *CYP2C9* genoytpes

6 Identification of the *CYP2C9* genotypes (**1/*1*, **1/*2*, **1/*3*, and **2/*3*) was performed according to the PCR-RFLP method-based protocol published by Sullivan-Klose TH and colleagues (doi: 10.1097/00008571-199608000-00007) and Yasar U and colleagues (doi: 10.1006/bbrc.1998.9992).

7 PCR reactions were assembled as follows: reactions of 25 µl contained 1) sterile, milliQ-$H_2O$; 2) 1xPCR buffer (for GoTaq G2 DNA polymerase, Promega), from a stock of 5xPCR buffer; 3) 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (U1330, Promega), from a stock of 2.5 mM; 4) 1 µM forward primer, from a stock of 25 µM; 5) 1 µM reverse primer, from a stock of 25 µM; 6) 100 ng of genomic DNA and 7) 2.5 U GoTaq DNA polymerase (M7845, Promega).

7.1 Sequences of the PCR primers for AvaII restriction endonuclease enzyme reactions were as follows: 1) AvaII-Forward primer: 5'-TACAAATACAATGAAAATATCATG-3'; 2) AvaII-Reverse primer: 5'-CTAACAACCAGACTCATAATG-3'.

PCR cycles used with the above primers were as follows:

1) 94°C for 5 min (1 cycle);
2) 94°C for 60 s; 55°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

**Expected result**

The expected size of the PCR product is 691 bp.

**7.2** In order to characterise the *CYP2C9 *1/*1* or **1/*2* genotypes by using the PCR-RFLP method, the following reactions were set up with AvaII restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xCutSmart Buffer (B7204S, New England Biolabs);
3) 7 µl of sterile, milliQ-$H_2O$;
4) 1 µl of AvaII enzyme (R0153S, New England BioLabs). Total reaction volume: 20 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

Expected result

The following pattern of DNA fragments can be expected after the AvaII restriction endonuclease enzyme reaction:

1) **1/*1* or *CYP2C9 *1/*1* genotype is characterized by two (2) DNA fragments of 527 and 164 base pairs (bp) (Figure 2).
In addition, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4) (Figure 2).
Furthermore, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6) (Figure 2).

2) **1/*2* or *CYP2C9 *1/*2* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp (Figure 3).
In addition, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4) (Figure 3).
Furthermore, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6) (Figure 3).

**7.3** Sequences of the PCR primers for NsiI restriction endonuclease enzyme reactions were as follows: 1) NsiI-Forward primer: 5'-AATAATAATATGCACGAGGTCCAGAGATGC-3'; 2) NsiI-Reverse primer: 5'-GATACTATGAATTTGGGACTTC-3'.

PCR cycles used with the above primers were as follows:
1) 94°C for 5 min (1 cycle);
2) 94°C for 60 s; 60°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

Expected result

The expected PCR product is running between 100 and 200 bp markers when compared to a DNA ladder.

**7.4** In order to characterise the *CYP2C9 *1/*3* or **2/*3* genotypes by using the PCR-RFLP method, the following reactions were set up with NsiI restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xNEBuffer 3.1 (B7203S, New England Biolabs);
3) 7 µl of sterile, milliQ-$H_2O$;
4) 1 µl of NsiI enzyme (R0127S, New England BioLabs). Total reaction volume: 20 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

**Expected result**

The following pattern of DNA fragments can be expected after the NsiI restriction endonuclease enzyme reaction:

1) **1/*3* or *CYP2C9 *1/*3* genotype is characterized by two (2) DNA fragments of 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme (Figure 4).
In addition, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (Figure 4).
Furthermore, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6) (Figure 4).

2) **2/*3* or *CYP2C9 *2/*3* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme (Figure 5).
In addition, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (Figure 5).
Furthermore, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6) (Figure 5).

**7.5** Sequences of the PCR primers for KpnI restriction endonuclease enzyme reactions were as follows: 1) KpnI-Forward primer: 5'-AATAATAATATGCACGAGGTCCAGAGGTAC-3'; 2) KpnI-Reverse primer: 5'-GATACTATGAATTTGGGACTTC-3'.

PCR cycles used with the above primers were as follows:

1) 94°C for 5 min (1 cycle);
2) 94°C for 60 s; 60°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

**Expected result**

The expected PCR product is running between 100 and 200 bp markers when compared to a DNA ladder.

**7.6** In order to characterise the *CYP2C9 *1/*3* or **2/*3* genotypes by using the PCR-RFLP method, the following reactions were set up with KpnI restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xBuffer J (R009A, Promega);
3) 0.5 µl Bovine Serum Albumin Acetylated, 10mg/ml (R3960, Promega);
4) 6.5 µl of sterile, milliQ-$H_2O$;
5) 1 µl of KpnI enzyme (R6341, Promega). Total reaction volume: 20 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

Expected result

The following pattern of DNA fragments can be expected after the KpnI restriction endonuclease enzyme reaction:

1) **1/*3* or *CYP2C9 *1/*3* genotype is characterized by two (2) DNA fragments of 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme (Figure 4).
In addition, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4) (Figure 4).
Furthermore, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (Figure 4).

2) **2/*3* or *CYP2C9 *2/*3* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme (Figure 5).
In addition, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4) (Figure 5).
Furthermore, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (Figure 5).