CYP2C9 mpx RealFast™ Assay

REF 7-225 / 7-228 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 CYP2C9 mpx RealFast™ Assay is a fast and accurate multiplex real-time PCR test for the simultaneous detection of the c.430C>T (CYP2C9*2) and c.1075A>C (CYP2C9*3) polymorphisms in the human *CYP2C9* gene. Both variants of the CYP2C9 enzyme, *2 and *3, exhibit decreased function leading to poor metabolism (PM) phenotypes for various drugs. Patients with low enzyme activity are at risk of adverse drug reactions or therapeutic failure, particularly for CYP2C9 substrates with a narrow therapeutic window, such as S-warfarin or phenytoin. The kit is designed to identify patients carrying one or two copies of the CYP2C9 *2 or *3 variants. In a human DNA extract the qualitative assay discriminates the possible CYP2C9 genotypes: normal *1*1, heterozygous *1*2, *1*3 or *2*3, homozygous *2*2 or *3*3.
Ref.Seq.: NM_000771.3: c.430C>T, dbSNP rs1799853 (CYP2C9*2) and c.1075A>C, dbSNP rs1057910 (CYP2C9*3).

2. Introduction

The CYP2C9 isoform is a drug-metabolizing enzyme of the cytochrome P450 superfamily that is highly expressed in the liver. It is estimated to be responsible for the clearance of up to 20% of all drugs undergoing phase I metabolism. CYP2C9*2 and CYP2C9*3, the two most common decreased function alleles among individuals of European ancestry, account for interindividual variability in drug response. For instance, individuals with PM phenotype (i.e. genotypes with homozygous or compound heterozygous *2 or *3 alleles) are at greater risk of severe bleeding during coumarin-based anticoagulation therapy, such as warfarin, acenocoumarol and - to a lesser extent - phenprocoumon. Furthermore, PM phenotypes experience more frequent symptoms of overdose when treated with the anti-epileptic drug phenytoin.

3. Kit Contents

		100 / 32 Rxn
RealFast™ 2x mpx **Probe Mix**	1 vial □ white cap	1000 / 320 µl
CYP2C9 mpx **Assay Mix**	1 vial ■ purple cap	550 / 550 µl
CYP2C9 ***1*1-Control**	1 vial ■ green cap	75 / 75 µl
CYP2C9 ***2*3-Control**	1 vial ■ red cap	75 / 75 µl

The RealFast™ 2x Probe Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The CYP2C9 mpx Assay Mix consists of *CYP2C9* gene-specific primers and four allele-specific, dual-labeled hydrolysis probes. Controls representing CYP2C9*1, *2 and *3 variants are supplied with the kit.

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

4. Storage and Stability

The CYP2C9 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 amplifiy a 184 bp (*2) and 140 bp (*3) fragment of the *CYP2C9* gene, respectively, 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.

CYP2C9 probe	Fluorophore	Channel
*2 (c.430T)	FAM	520 nm
*1 (c.430C)	HEX	556 nm
*3 (c.1075C)	ROX	605 nm
*1 (c.1075A)	Cy5	670 nm

In normal samples the ***1 (c.430C, c.1075A) 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 variant samples the hybridized ***2 (c.430T)** or ***3 (c.1075C) 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 *1*2 (c.430CT) or *1*3 (c.1075AC) samples both probes bind to the amplicons and generate intermediate signals in both channels.

5.2. Real-time PCR Instrument Compatibility

The CYP2C9 mpx 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®)
- ✓ 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 55 CYP2C9 *2 alleles and 35 CYP2C9 *3 alleles, both tested with a CE-marked reference kit. The CYP2C9 mpx RealFast™ Assay correctly determined all *2 and *3 alleles, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 307 alleles testing negative for CYP2C9 *2 and 327 alleles testing negative for CYP2C9 *3 with a CE-marked reference kit. The CYP2C9 mpx RealFast™ Assay correctly determined all negative *2 and *3 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 CYP2C9 ***1*1-Control** and CYP2C9 ***2*3-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 (*1/*2- and *1/*3-Control), mix an aliquot of *1*1-Control and *2*3-Control in a ratio of 1:1.

» ***Note:*** **1*1- and *2*3-Controls are potential sources of contamination. Make sure to handle them carefully.* «

7.3. Preparation of CYP2C9 mpx RealFast™ **Master Mixes:**

For each sample set up two reactions, one for CYP2C9*2 and one for CYP2C9*3.
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
CYP2C9 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 (mutiert)**. 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 variant 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 **CYP2C9 variants**
	FAM Green	HEX Yellow	ROX Orange	Cy5 Red	
*1*1-Control	NO	**YES**	NO	**YES**	*1*1 (normal)
*1*2 / *1*3-Control	**YES**	**YES**	**YES**	**YES**	*2*3 (heterozygous)
*2*3-Control	**YES**	NO	**YES**	NO	---- (positive control for *2 and *3)
NTC	NO	NO	NO	NO	----
Sample 1	**YES**	**YES**	NO	**YES**	*1*2 (heterozygous)
Sample 2	NO	**YES**	**YES**	**YES**	*1*3 (heterozygous)
Sample 3	**YES**	NO	NO	**YES**	*2*2 (homozygous)
Sample 4	NO	**YES**	**YES**	NO	*3*3 (homozygous)

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 *1*1-Control (HEX-/Cy5-positive). Vice versa, set threshold value for the HEX and Cy5 channels just above the background fluorescent signal of the *2*3-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* diagnostic 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.