SimReal Prothrombin Genotyping Kit

USER MANUAL

For in vitro Diagnostic Use

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1-Introduction

Prothrombin G20210A (also the **prothrombin 20210 mutation**, the **factor II mutation**, or the **prothrombin mutation**) is a genetic variant that approximately doubles or triples the risk of forming blood clots in the veins. The variant is commonly associated with the disease venous thromboembolism (VTE), which includes both deep vein thrombosis and pulmonary embolism. Most carriers, though, never develop VTE in their lifetime.

Prothrombin G20210A was identified in the 1990s, is almost exclusively present in Caucasians and is estimated to have originated in that population slightly over 20,000 years ago. About 2 to 3% of Caucasians carry the variant and it confers a 2- to 3-fold higher risk of VTE. Deficiencies in the anticoagulants Protein C and Protein S give a higher risk (5- to 10-fold). Behind non-O blood type and factor V Leiden, prothrombin G20210A is one of the most common genetic risk factors for VTE.

The polymorphism is located in a noncoding region of the prothrombin gene (3' untranslated region nucleotide 20210), replacing guanine with adenine. The position is at or near where the pre-mRNA will have the poly-A tail attached. The variant causes elevated plasma prothrombin levels (hyperprothrombinemia), possibly due to increased pre-mRNA stability. Prothrombin is the precursor to thrombin, which plays a key role in causing blood to clot (blood coagulation). G20210A can thus contribute to a state of hypercoagulability, but not particularly with arterial thrombosis. A 2006 meta-analysis showed only a 1.3-fold increased risk for coronary disease.

Heterozygous carriers who take oral contraceptives are at a 15-fold increased risk of VTE while carriers also heterozygous with factor V Leiden have an approximate 20-fold higher risk. In a recommendation statement on VTE, genetic testing for G20210A in adults that developed unprovoked VTE was misadvised, as was testing in asymptomatic family members related to G20210A carriers who developed VTE. In those who develop VTE, the results of thrombophilia tests (wherein the variant can be detected) rarely play a role in the length of treatment.

2- Product description

SimReal Prothrombin Genotyping Kit is an in vitro diagnostic kit designed to determine the genotype of prothrombin gene G21210A Mutation related to thrombophilia on the basis of in-vitro DNA amplification using Real-time PCR technology.

Mutation detection is based on amplification and detection of distinct alleles using corresponding labeled probes. The probes targeting normal (G21210) and mutant (A21210) alleles are labeled with FAM and HEX flourochrome, respectively.

3- Kit contents

Reagents	labels	volume
Master mix 2X	2X Real Time Mix	500 ul
Primer and probe Mix	Oligomix Factor II Prothrombin	100 ul
Heterozygote control	HET Factor II Prothrombin Positive control	20 ul
Mutant homozygote for G20210A	HOMO MUT Factor II Prothrombin Positive control	20 ul
Wildtype homozygote for G20210A	HOMO WT Factor II Prothrombin Positive control	20 ul
ddH2O		500 ul

4- Storage

All reagents of the SimReal Prothrombin Genotyping Kit should be stored at –20°C and are stable until their expiration date at recommended conditions.
Repeated freezing and thawing (> 3 x) should be avoided, as this may reduce the sensitivity. If the kit is to be used only intermittently, it is recommended to aliquot the reagents. Storage at +4°C should not exceed a period of five hours.

5- Test principle

SimReal Prothrombin genotyping Kit employs multiplex PCR. A fragment of the human Prothrombin gene, whether wild type or polymorphic, is amplified in a single reaction, using sequence-specific primers against mutant and wild-type alleles.

In Taq man real-time PCR the amplified product is detected via fluorescent dyes. Wild type allele is amplified and fluorescence detection is accomplished using the Cy-5 channel. Allele with G20210A polymorphism is amplified and fluorescence detection is accomplished using the FAM channel. Main advantages of the Real time PCR technique, compared to the conventional amplification techniques, are for example the possibility to execute a semi-automated analysis in which the time needed for the visualization of the amplicons is eliminated; and the absence of the post amplification sample manipulation that reduces the possible contamination phenomena.

6- Safety information

- Carefully read this instruction before starting the procedure
- Do not use the kit after its expiration date
- Keep the product away from heating sources and the direct light
- Avoid repeated freezing and thawing of the reagents, this may reduce the sensitivity of the test.
- Prepare quickly the Reaction mix on ice or in the cooling block.
- Mix the reagents of kit before used.
- Don’t move Equipment's and material from a working area to another room.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Wear disposable gloves to handle the reagents and the clinical samples and wash the hands at the end of work
- Wear separate gloves in each area.
- Wash the bench surfaces with 5% sodium hypochloride

7- Protocol

a) Genomic DNA Extraction

DNA-preparation from patients' blood according to standard procedures (e.g. Roche Diagnostics ‘High Pure PCR Template Preparation Kit).

The DNA extracted can be stored for several months at ≤ -18°C.

b) Preparation of the PCR mix

For each experiment prepare a master mix of an appropriate volume for: 3 controls (*HET* G20210A, *HOMO WT, and HOMO MUT*), 1 reaction blank, n+1 samples. The reagents of the mix have to mix under this ratio:

component	Final concentration	Volume/reaction
Reaction Mix		
2x PCR Master Mix	*1x*	*10ul*
10 X oligomix	*1x*	*2ul*
ddH2O	-	*7ul*
Template DNA	≤100ng/reaction	*1ul*
Total reaction volume		***20ul***

After a mix preparation, aliquot 20µl of Master Mix in the tubes or in the micro plates for PCR than add in each tube 1µl (correspondent to ≤100ng) from the extracted DNA or control DNA and set in order the tubes in the instrument and start the program of amplification setting before.

c) *Real time PCR cycler programming*

Refer to the specific handbook of the equipment used but be sure to set the following thermal profile

Step	Time	Temperature	
PCR initial heat activation	2 min	95°c	1X
Denaturation	15 s	95°c	35X
Annealing/Extension*	60 s	60°c	

* Acquire florescent signal in green and yellow channels

Optional: Check the specifity of PCR product(s) by agarose gel electrophoresis.

8- Data analysis

The fluorescence in each channel indicates the hybridization of the probe

Channel 1 for FAM= Wild Type probe
Channel 2 for HEX= Mutated probe

If a sample shows fluorescence in channel 1, the sample is homozygous wild type.
If a sample shows fluorescence in channel 2, the sample is homozygous mutated.
If a sample shows fluorescence in all channels (1 and 2), the sample is heterozygous.

9- Troubleshooting

❖ *No fluorescent FAM and JOE signal in the samples*

1) Wrong channel has been chosen.

2) The instrument was not programmed correctly

3) Pipetting errors or omitted reagents

4) The kit was not conserved correctly or it was used beyond the expiry date

5) Inhibitory effect of the samples: genomic DNA with an insufficient purification and or insufficient extraction

6) The amplification reaction was inhibited

❖ *Variable Fluorescence intensity*

1) Air bubble is trapped in the PCR tubes

2) The reaction mix was not mixed well prior to aliquoting

3) Difference in concentration of the amplified DNA samples

❖ *Low fluorescence intensity*

1) Very low amount of DNA and/or low purity.

2) Decay of fluorophore or primers in the device due to unsuitable storage condition

3) Real Time Master Mix and Oligomix reagents were freeze and thawed for more than three times

10- Materials required, but not supplied with the kit

- ✓ Equipment and materials required for DNA extraction
- ✓ Micropipette (range: 0,5-10 µL; 2-20 µL; 10-100 µL; 20-200 µL; 100-1000 µL);
- ✓ Micro centrifuge max 12-14.000 rpm
- ✓ Real time amplification instrument
- ✓ Incubator
- ✓ Optical micro plate for real Time PCR
- ✓ Tubes of 0.2 ml with optical caps
- ✓ Talc-free disposable gloves
- ✓ Disposable sterile filter-tips (range: 0,5-10 µL; 2-20 µL; 10-100 µL; 20-200 µL; 100-1000 µL)
- ✓ Dnase- and Rnase-free sterile water.

11- Product use limitation:

- ✓ The histological sample is not suitable for this analysis
- ✓ This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.
- ✓ This product should be used in accordance with this user manual.

12-Quality control:

Predefined genotype control must be included correctly, otherwise the sample results is invalid.

13- Material Safety Data Sheet (MSDS)

SECTION 1–HAZARDOUS IDENTIFICATION

Emergency Overview: May cause irritation to skin, eyes, and respiratory tract, may affect kidneys.

HMIS and NFPA Ratings: 0 – Minimal or None, 1 – Slight, 2 – Moderate, 3 – Serious, and 4 – Severe

Health: 2
Flammability: 0
Reactivity: 1

SECTION 2–FIRST AID MEASURES

Eyes: Flush eyes with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating eyelids. Call a physician. Skin: Wash skin with soap and copious amount of water. Ingestion: If the person is conscious, wash out mouth with water. Call a physician. Inhalation: Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Call a physician.

SECTION 3–ACCIDENTAL RELEASE MEASURES

Personal Precautions: Avoid breathing or contact with vapors, mist of gas. Environmental Precautions: Do not let product enter drains. Methods for Cleaning Up: Cover with dry lime, sand, or soda ash. Sweep up and shovel. Place in covered container for disposal.

SECTION 4–EXPOSURE CONTROLS/PPE

Engineering Controls: Safety shower and eye wash. Mechanical exhaust. Personal Protective Equipment: Eye Protection: Safety goggles. Hand Protection: Compatible resistant gloves. Respiratory Protection: None required. Hygiene Measure: General practice, wash (hands and skin) thoroughly after handling. Remove and wash contaminated clothing.

SECTION5 –STABILITY AND REACTIVITY

Stability: Stable under recommended storage conditions. Materials to Avoid: Acid chlorides, Phosphorus halides, strong oxidizing agents, strong acids, strong reducing agents. Hazardous Decomposition Products: Carbon monoxide, Carbon dioxide, Sulfur dioxides. Hazardous Polymerization: Will not occur

SECTION 6–DISPOSAL CONSIDERATIONS

Dispose of container, unused contents and contaminated packaging in accordance with federal, state and local requirement. Contract with a licensed Chemical Waste Disposal Service. Dissolve or mix with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

SECTION 7 –OTHER INFORMATION DISCLAIMER

The information provided on the MSDS is furnished in good faith and based on our present knowledge. However, this MSDS shall not constitute a guarantee of any kind. Personnel

handling this material must make independent determinations of the suitability and completeness of information from all sources to assure proper use and disposal of this material and the safety and health of employees and customers. NEB assumes no additional liability or responsibility resulting from the use of, or reliance on this information. This product is for R&D use only. Not for drug, household or other uses.