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THROMBOPHILIA MULTIPLEX REAL-TIME PCR KIT (3 MUTATIONS)
Cat. No: 10R-20-03

INTRODUCTION

In Thrombophilia, blood has an increased tendency to form potentially dangerous clots. Hereditary defects in one or more of the clotting factors can cause to excessive blood clot formation called thrombosis. Thrombophilia Multiplex Real-Time PCR Kit (3 Mutations) includes; FII Prothrombin, FV Leiden and MTHFR 677 mutations/polymorphisms [1-2].

INTENDED USE

Thrombophilia Multiplex Real-Time PCR Kit (3 Mutations) can detect FII Prothrombin, FV Leiden and MTHFR 677 mutations/polymorphisms in whole blood samples by using qualitative Real-Time PCR method.

TARGETED USER

For professional use only. Testing should be performed by professionals trained in molecular techniques.

PRINCIPLE OF THE SYSTEM

During the PCR reaction, the DNA polymerase cleaves the probe at the 5′ end and separates the reporter dye from the quencer dye only when the probe hybridizes perfectly to the target DNA. This cleavage results in the fluorescent signal which is monitored by Real-Time PCR detection system. An increase in the fluorescent signal (C_T) is proportional to the amount of the specific PCR product [3,4].

PRODUCT SPECIFICATION

Each isolated DNA should be tested with all master mixes separately. The kit provides reagents in a **"ready-to-use"** master mix format which has been specifically adapted to 5′ nuclease PCR for SNP analysis. The test system is designed by SNP Biotechnology for use with sequence specific primers and probes.
The fluorescence of mutation analysis is FAM, HEX/JOE and Texas Red. Also each master mix contains an internal control labelled with CY5 dye (See Table 2). Internal Control is Zinc Finger Protein Gene, X Linked – ZFX (OMIM: 314980).
The limit of detection (LOD) for the Thrombophilia Multiplex Real-Time PCR Kit (3 Mutations) was determined as 1 ng/µl.

SYSTEM CONTENTS

Reagents	10 rxns	20 rxns	50 rxns
TRP-3 Mix 1	200 µl	400 µl	1000 µl
TRP-3 Mix 2	200 µl	400 µl	1000 µl
Control DNA*	30 µl	30 µl	60 µl

Table 1: Kit content

* Control DNA is a synthetic plasmid containing some of the mutation regions. Expected results for synthetic control DNA should be FII and 677 Heterozygote. There should be no amplification in other mixes and dyes except CY5. Since to Control DNA is a synthetic plasmid, amplification plots of synthetic control DNA may appear slightly different from the sample DNA. Please gently vortex and then spin centrifuge for 1-2 seconds before using the Control DNA.

STORAGE

- All reagents should be stored at – 20 °C and dark.
- All reagents can be used until the expiration date on the box label.
- Repeated thawing and freezing (>4X) should be avoided, as this may reduce the sensitivity of the assay.

SAMPLE COLLECTION

Thrombophilia Multiplex Real-Time PCR Kit (3 Mutations) is approved for use with whole blood samples.
- Standard precautionary instructions must be followed by all healthcare professionals during the collection and transportation of whole blood samples.
- Whole blood samples should be collected in appropriate containers before delivery to the laboratory.
- Freezing and thawing of samples should be avoided.

DNA EXTRACTION

Blood samples should be collected in appropriate sterile EDTA tubes and can be stored at +4°C up to one month. For more than one month specimen should be stored at -20°C. It is advised to gently mix the tube (with EDTA) after collection of blood to avoid coagulation.
Our system optimized according to GeneAll® Exgene™ Blood SV. It is advised to elute DNA with 150 µl elution buffer for better results.

MUTATION / POLYMORPHISMS - DYE TABLE

Tubes	Mutations/Polymorphisms	Dyes
Mix 1	FII Wild Type	FAM
	FV Leiden Wild Type	HEX/JOE
	MTHFR 677 Wild Type	Texas Red
	Internal Control	CY5
Mix 2	FII Mutant	FAM
	FV Leiden Mutant	HEX/JOE
	MTHFR 677 Mutant	Texas Red
	Internal Control	CY5

Table 2: Tubes- mutations / polymorphisms - dyes.

PROCEDURE

- Different test tubes should be prepared for each master mix.
- Leave the master mixes* and controls at RT to melt.
- Before starting work, mix the master mixes gently by pipetting
- For each sample, pipet **20 µl master mix** with micropipets of sterile filter tips to each optical white strips or tubes.
- Add **5 µl DNA** into each tube. Please do not pipette DNA before and after addition into well.
- Optical caps are closed, it is recommended to spin the plates/strips at low speed for a short time.
- Run with the programme shown below.

*Master mixes include HotStart Taq DNA Polymerase.

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PCR PROGRAMME

95 °C	3 Min.	Holding
95 °C	15 Sec.	30 Cycles
60 °C	1 Min.	

Table 3: PCR Programme

Fluorescent dyes are FAM, HEX/JOE, Texas Red and CY5.

This system can be used with the following devices;
- Bio-Rad CFX96, Opus 96
- ABI Prism ® 7500/7500 Fast
- Mic qPCR Cycler

For other four or more channel Real-Time PCR devices (which can read FAM, HEX/JOE, Texas Red and CY5 dyes), a trial run is recommended.

If you use;
ABI Prism® system, please choose **"none"** as passive reference and quencher.
Mic qPCR Cycler, please adjust gain settings, **"Green Auto Gain"** to **20** and "**Yellow Auto Gain**" to **10**

Supplied Materials
- White PCR plates/strips with optical covers*

*The PCR Plate/strip tube and caps seriously affect the amplification curve quality. Therefore, white PCR plates/strips and optical caps provided by the manufacturer should be used with the kit.

Required Materials (Not Provided)
- PCR Cabinet
- Vortex Mixer
- Desktop Microcentrifuge (For 2.0ml tubes and PCR strip tubes), plate spin for studies using PCR plates.
- Automated or spin column based DNA isolation Kit
- Disposible powder-free laboratory gloves
- Micropipettes (0.5ml-1000ml)
- Micropipette tips
- Standard laboratory equipments.

DATA ANALYSIS

After the run is completed data are analysed using the software with FAM, HEX/JOE, Texas Red and CY5 dyes. The below results were studied with Bio-Rad CFX96. The threshold values for all dyes were set to 500, based on experiments conducted using the Bio-Rad CFX96 Real-Time PCR system, the GeneAll® Exgene™ Blood SV Isolation Kit, and white PCR strips supplied by SNP Biotechnology. Threshold values may vary depending on the PCR device, DNA isolation kit, and the type or brand of PCR strips/tubes used.

Internal control amplification plots must be seen in all wells except NTC and has been labelled with CY5 dye. The C_T value of internal controls should be **$21 \leq C_T \leq 27$**. These values are optimised according to the GeneAll® Exgene™ Blood SV Isolation Kit and Bio-Rad CFX96 Real-Time PCR Device. C_T values may vary ±2/3 cycle according to the other DNA isolation systems and Real-Time PCR devices.

Amplification plots of mutations can be analysed by FAM, HEX/JOE and Texas Red dyes. The C_T value should be between **$21 \leq C_T \leq 27$**. These values are optimised according to the GeneAll® Exgene™ Blood SV Isolation Kit and Bio-Rad CFX96 Real-Time PCR Device. C_T values may vary ±2/3 cycle according to the other DNA isolation systems and Real-Time PCR devices.
- Homozygous wild type sample gives amplification signal only with wild type master mix.
- Heterozygous sample gives amplification signal both with wild type and mutant master mixes.
- Homozygous mutant sample gives amplification signal only with mutant master mix.
- The difference of the C_T value with wild type and mutant amplification plots should be **≤3** for heterozygote sample. If it is **$4 \leq C_T \leq 6$**, test should be repeated, if **>6**, the late plot should be considered as non-spesific.

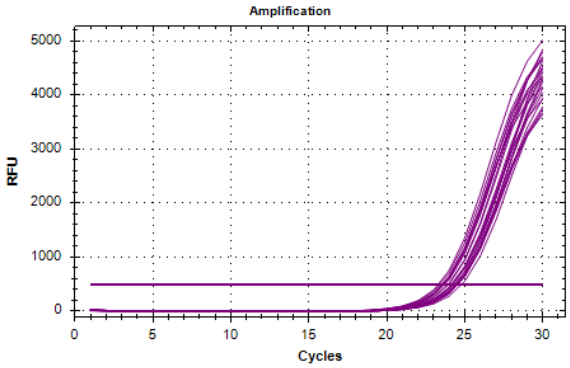

Figure 1: Internal Control plots – CY5 Dye

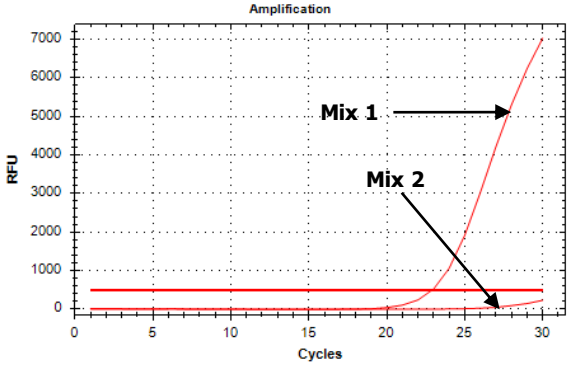

Figure 2: 677 Homozygous Wild Type Sample (Texas Red Dye)

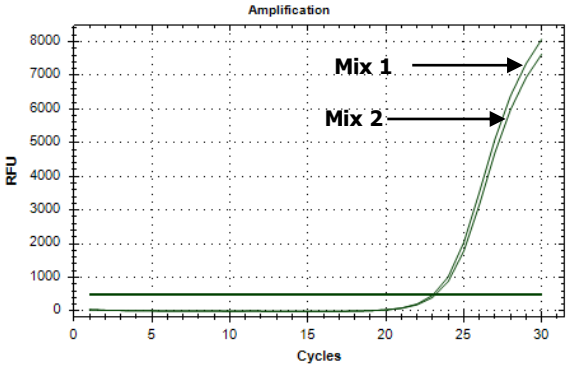

Figure 3: FV Leiden Heterozygous Sample (HEX/JOE Dye)

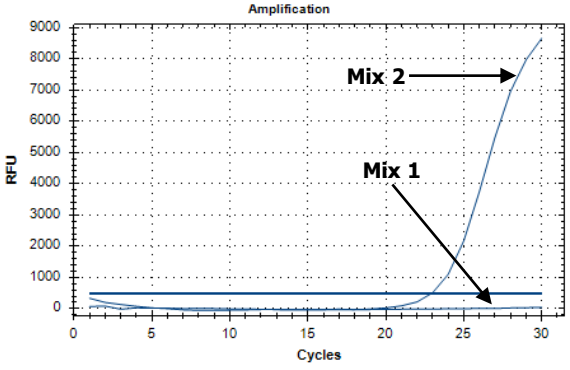

Figure 4: FII Prothrombin Homozygous Mutant Sample (FAM Dye)

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CAUTIONS

- All reagents should be stored at suitable conditions.
- Do not use the PCR master mixes forgotten at room temperature.
- Thaw PCR master mix at room temperature and slowly mix by inverting before use.
- Shelf-life of PCR master mix is 12 months. Please check the manufacturing data before use.
- Only use in vitro diagnostics.

REFERENCES

1. Andreas Gardemann, Tanja Arsic, Norbert Katz, Harald Tillmanns, Friedrich Wilhelm Hehrlein and Werner Haberbosch. "The Factor II G20210A and Factor V G1691A Gene Transitions and Coronary Heart Disease". Thromb Haemost 1999; 81: 208–13.
2. Eulo Lupi-Herrera, María Elena Soto-López, Antonio de Jesús Lugo-Dimas, Marcela Elizabeth Núñez-Martínez, Ricardo Gamboa, Claudia Huesca-Gómez, Lilia Mercedes Sierra-Galán, Verónica Guarner-Lans. "Polymorphisms C677T and A1298C of MTHFR Gene: Homocysteine Levels and Prothrombotic Biomarkers in Coronary and Pulmonary Thromboembolic Disease".Clinical and Applied Thrombosis/Hemostasis, Volume 25: 1-8.
3. Yolanda S Lie and Christos J Petropoulos. "Advances in quantitative PCR technology: 5' nuclease assays". Current Opinion in Biotechnology Volume 9, Issue 1, February 1998, Pages 43-48.
4. Luis Ugozzoli and R. Bruce Wallace. "Allele-Specific Polymerase Chain Reaction". A Companion to Methods in Enzymology Vol. 2, No. 1, February, pp. 42-48, 1991.

SYMBOLS AND DESCRIPTIONS

REF	Catalog Number	CE	CE Mark
LOT	Lot Number	UDI	Unique Device Identifier (01)Device Identifier (17)Expiry Date (10)Lot Number
	Manufacturer	Σ	Test Quantity
	Fragile	-20 °C	Storage Temperature
	Protect from directly sunlight	IVD	In Vitro Diagnostics
	Expiry Date		

Table 4: Symbols and descriptions

DISPOSAL OF KIT

Dispose of it according to the legal regulations of your region

TROUBLESHOOTING PROBLEMS AND SOLUTIONS

Problem	Reason	Solution
Internal control does not work/ low amplification	Absence of DNA / DNA extraction problems	Repeat test
	Absence of DNA / DNA extraction problems	• DNA extraction should be repeated. • DNA extraction should be replaced with one of the recommended methods.
	Sample is containing PCR inhibitor(s)	
No target gene amplification curves in some samples for both wild type and mutant mixes.	Absence of DNA / not added into well	Repeat test
	Absence of DNA / DNA extraction problems	• DNA extraction should be repeated. • DNA extraction should be replaced with one of the recommended methods.
	Sample is containing PCR inhibitor(s)	
No target DNA/internal control amplification curves in all wells	Error in temperature/time settings in PCR program	Correct any errors in the temperature/time settings in the PCR Program and repeat the test.
	Sample is containing PCR inhibitor(s)	• DNA extraction should be repeated. • DNA extraction should be replaced with one of the recommended methods.
Positive control result and/or C_T values are lower or higher than the value mentioned in User Manual.	Error in temperature/time settings in PCR program	Correct any errors in the temperature/time settings in the PCR Program and repeat the test.
C_T values are not valid (higher or lower) according to User Manual	Excessive or insufficient DNA sample	• Repeat the test. • DNA extraction should be repeated.
Low and/or invalid amplification curves	Stability problems arising from repeated thawing and freezing (>4X)	Repeated thawing and freezing (>4X) should be avoided, as this may reduce the sensitivity of the assay.
	Sample is containing PCR inhibitor(s)	• DNA extraction should be repeated. • DNA extraction should be replaced with one of the recommended methods.
	Stability problems arising from unavailable storage conditions.	All reagents should be stored at – 20 °C and dark.
	Bubble formation or pipetting error during pipetting	After adding the master mix and sample, it is recommended to spin the plates/strips at low speed for a short time.
For further questions, please contact us **tech@snp.com.tr**		

Table 5: Troubleshooting problems and solutions