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FMF MULTIPLEX REAL-TIME PCR KIT (26 MUTATIONS)
Cat. No: 11R-20-26

INTRODUCTION

Familial Mediterranean Fever (FMF) is an autosomal recessive disorder characterized by recurrent attacks of fever and polyserositis. It affects primarly people of Mediterranean, mostly non-Ashkenazi Jews, Araps and Turks. FMF Multiplex Real-Time PCR Kit (26 mutations) analysis twentysix mutations/polymorphisms of Mediterranean Fever gene (MEFV), which has been identified in exon 1; R42W, E84K, in exon 2; L110P, E148Q, E148V, E167D, E230K/Q, T267I, P283L, G304R, in exon 3; R354W, R408Q, P369S, in exon 5; F479L, in exon 9; I591T in exon 10; R653H, M680I (G/C-A), I692DEL, M694I, M694V, K695R, V726A, A744S, R761H. Kit is covering 99.8% mutations/polymorphisms rate of FMF in the Anatolian, Middle East countries and many other countries [1-10].

INTENDED USE

FMF Multiplex Real-Time PCR Kit (26 Mutations) can detect in exon 1; R42W, E84K, in exon 2; L110P, E148Q, E148V, E167D, E230K/Q, T267I, P283L, G304R, in exon 3; R354W, R408Q, P369S, in exon 5; F479L, in exon 9; I591T in exon 10; R653H, M680I (G/C-A), I692DEL, M694I, M694V, K695R, V726A, A744S, R761H mutations/ polymorphisms of MEFV gene 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 [11,12].

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, Texas RED and Quasar 705. Also each master mix contains an internal control labelled with CY5 dye (See Table 2). Internal Control is Prothrombin gene – FII (OMIM: 176930).

SYSTEM CONTENTS

Reagents	10 rxns	20 rxns	50 rxns
FMF-26 Mix 1	200 µl	400 µl	1000 µl
FMF-26 Mix 2	200 µl	400 µl	1000 µl
FMF-26 Mix 3	200 µl	400 µl	1000 µl
FMF-26 Mix 4	200 µl	400 µl	1000 µl
FMF-26 Mix 5	200 µl	400 µl	1000 µl
FMF-26 Mix 6	200 µl	400 µl	1000 µl
FMF-26 Mix 7	200 µl	400 µl	1000 µl
FMF-26 Mix 8	200 µl	400 µl	1000 µl
FMF-26 Mix 9	200 µl	400 µl	1000 µl
FMF-26 Mix 10	200 µl	400 µl	1000 µl
FMF-26 Mix 11	200 µl	400 µl	1000 µl
FMF-26 Mix 12	200 µl	400 µl	1000 µl
Control DNA*	90 µl	90 µl	150 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 I692del Wild Type, M694I Wild Type, M680I Homozygote Mutant, K695R Wild Type, A744S Wild Type, M694V Homozygote Mutant, V726A Homozygote Mutant and R761H Homozygote Mutant. . Amplification plots of synthetic control DNA may appear slightly different from the sample DNA.Since to Control DNA is a synthetic plasmid, it does not amplify CY5 dye and amplification plots of 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.

MUTATION /POLYMORPHISMS DYE TABLE

Tubes	Mutations/Polymorphisms	Dyes
Mix 1	P369S Wild Type	FAM
	E84K Wild Type	HEX / JOE
	A744S Wild Type	Texas Red
	I692DEL Wild Type	Quasar 705
	Internal Control	CY5
Mix 2	P369S Mutant	FAM
	E84K Mutant	HEX / JOE
	A744S Mutant	Texas Red
	I692DEL Mutant	Quasar 705
	Internal Control	CY5
Mix 3	G304R Wild Type	FAM
	E148V Wild Type	HEX / JOE
	M694V Wild Type	Texas Red
	R42W Wild Type	Quasar 705
	Internal Control	CY5
Mix 4	G304R Mutant	FAM
	E148V Mutant	HEX / JOE
	M694V Mutant	Texas Red
	R42W Mutant	Quasar 705
	Internal Control	CY5
Mix 5	E148Q Wild Type	FAM
	F479L Wild Type	HEX / JOE
	V726A Wild Type	Texas Red
	R653H Wild Type	Quasar 705
	Internal Control	CY5
Mix 6	E148Q Mutant	FAM
	F479L Mutant	HEX / JOE
	V726A Mutant	Texas Red
	R653H Mutant	Quasar 705
	Internal Control	CY5
Mix 7	M694I Wild Type	FAM
	T267I Wild Type	HEX / JOE
	E167D Wild Type	Texas Red
	R408Q Wild Type	Quasar 705
	Internal Control	CY5
Mix 8	M694I Mutant	FAM
	T267I Mutant	HEX / JOE
	E167D Mutant	Texas Red
	R408Q Mutant	Quasar 705
	Internal Control	CY5
Mix 9	M680I Wild Type	FAM
	P283L Wild Type	HEX / JOE
	L110P Wild Type	Texas Red
	I591T Wild Type	Quasar 705
	Internal Control	CY5
Mix 10	M680I Mutant	FAM
	P283L Mutant	HEX / JOE
	L110P Mutant	Texas Red
	I591T Mutant	Quasar 705
	Internal Control	CY5
Mix 11	K695R Wild Type	FAM
	E230K/Q Wild Type	HEX / JOE
	R761H Wild Type	Texas Red
	R354W Wild Type	Quasar 705
	Internal Control	CY5
Mix 12	K695R Mutant	FAM
	E230K/Q Mutant	HEX / JOE
	R761H Mutant	Texas Red
	R354W Mutant	Quasar 705
	Internal Control	CY5

Table 2: Tubes- mutations/polymorphisms- dyes.

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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

FMF Multiplex Real-Time PCR Kit (26 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.

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.

PCR PROGRAMME

95 °C	3 Min.	Holding
95 °C	15 Sec.	30 Cycles
62 °C	1 Min.	

Table 3: PCR Programme

Fluorescent dyes are FAM, HEX/JOE, Texas Red, Quasar 705 and CY5.

This system can be used with the following devices;
- Bio-Rad CFX96, Opus 96

For other five or more channel Real-Time PCR devices (which can read FAM, HEX/JOE, Texas Red, Quasar 705 and CY5 dyes), a trial run is recommended.

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, Quasar 705 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, Texas Red and Quasar 705 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.

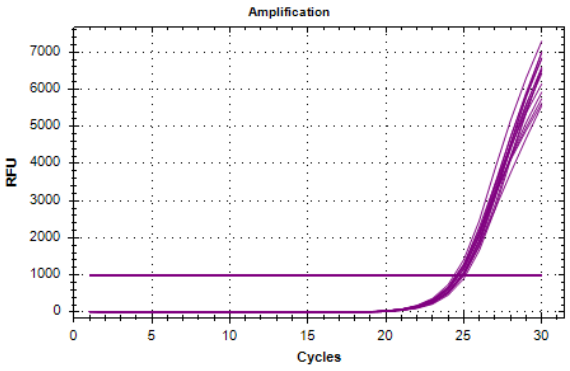

Figure 1: Internal Control plots – CY5 Dye

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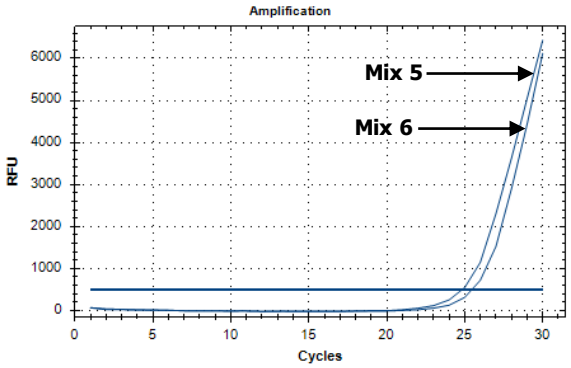

Figure 2: E148Q Heterozygous Sample (FAM Dye)

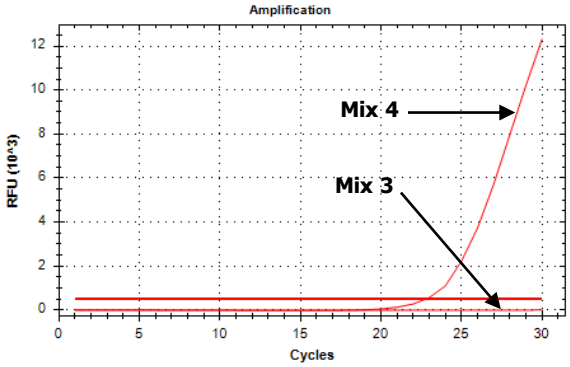

Figure 3: M694V Homozygous Mutant Sample (Texas Red Dye)

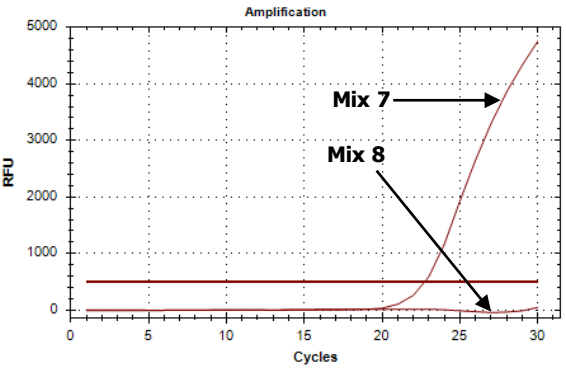

Figure 4: R408Q Homozygous Wild Type Sample (Quasar 705 Dye)

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.

DISPOSAL OF KIT

Dispose of it according to the legal regulations of your region

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

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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

Technical Note on Real-Time PCR Limitations
In Familial Mediterranean Fever (FMF) testing, the presence of the **K695R mutation in a homozygous mutant state** may compromise the reliable amplification or detection of the adjacent **M694I mutation.**
This state can result in **reduced signal intensity or a false-negative result for M694I.** This limitation stems from the inherent nature of Real-Time PCR and its reduced ability to differentiate between closely spaced nucleotide changes.
To ensure accurate interpretation:
- Be aware of this potential interaction when analyzing results involving codons 694 and 695.
- In cases of clinical suspicion or discordant results, confirmatory testing using high-sensitivity methods like sequencing is strongly recommended.