FMF NIRCA PROTOCOL

STEP 1.

After you have isolated patient's DNA and DNA from a healthy donor (wild type), you perform a nested PCR. The primers used to amplify exon 2 and exon 10 of the mefv gene are the following. For further information click HERE.

Exon 2

A' PCR

Upper primer

5' – AACTTTAATATCCAAGGGGATTC – 3'

Lower primer

5' – TTCTCTGCAGCCGATATAAAGTA – 3'

Nested PCR

Upper primer

5' – TAATACGACTCTATAGGGCTCTCCTCTGCCCTGAA – 3'

Lower primer

5' – ATTTAGGTGACACTATAGGAGGTGACCGAATGTTCTGG – 3'

Exon 10

A' PCR

Upper primer

5' – GATTGGCGCTCAGGCACAT – 3'

Lower primer

5' – GGCTCCGTGGGCACAGTAAC – 3'

Nested PCR

Upper primer

5' – TAATACGACTCACTATAGGGTGTGCTCTCCCCTACCA – 3'

Lower primer

5' – ATTTAGGTGACACTATAGGACACCTAGTCGGCATTC – 3'

* Nested upper primers have an intergrated T7 promoter and nested lower primers an SP6 promoter.

The PCR conditions for exon 2 are (MJ Research PTC-200 DNA Engine):

A' PCR	Nested PCR
94 °C for 8 min	94 °C for 8min
37 cycles of:	37 cycles of:
• 94 °C for 80 sec • 58 °C for 60 sec • 72 °C for 60 sec	• 94 °C for 60 sec • 59 °C for 60 sec • 72 °C for 60 sec

The PCR conditions for exon 10 are (MJ Research PTC- 200 DNA Engine):

A'PCR	Nested PCR
94 °C for 3 min	94 C for 3min
25 cycles of:	23 cycles of:
• 94 °C for 60 sec • 59 °C for 60 sec • 72 °C for 60 sec	• 94 °C for 60 sec • 59 °C for 60 sec • 72 °C for 60 sec

For **50 µl** PCR reaction we use:

- 5 µl **10x PCR buffer** (500mM KCl , 200mM Tris- HCl pH 8.4, Invitrogen Platinum Taq DNA polymerase kit, Cat.10966-034)
- 1.5 µl 50mM **$MgCl_2$** (included in Invitrogen Platinum Taq DNA polymerase kit)
- 0.4 µl 100mM **dNTPs** mixture (Invitrogen Cat. 10297-018)
- 50 pmol of each **primer**
- 0,4 µl 5U/µl Platinum Taq **DNA polymerase** (included in Invitrogen Platinum Taq DNA polymerase kit)
- 0.5 µl of template **DNA**
- Up to 50 µl **distilled H_2O**

It has to be noted that in order to amplify exon 2, 5µl DMSO is added in the 50 µl PCR reaction (both A' and nested PCR) because of the G-C rich content of the sequence.

For nested PCR, 1µl of A' PCR product is used as template.

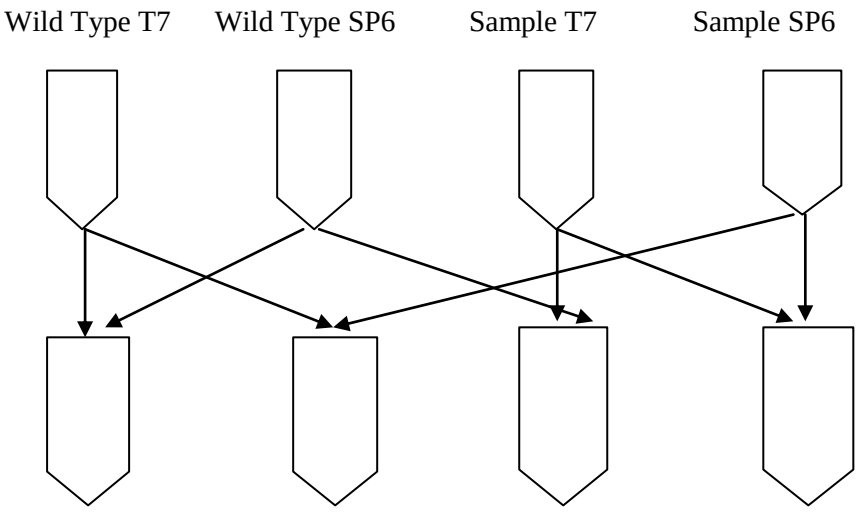
STEP 2. TRANSCRIPTION

The next step is the transcription of the nested PCR product into single strand specific RNA using T7 and SP6 polymerases. Each sample and one wild type PCR product (per sample), is transcripted separately into 2 different strand specific RNA molecules.

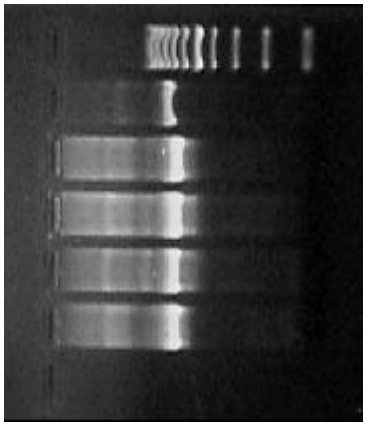
- For reaction volume of **10µl** we use :
  - 4µl distilled H_20
  - 2µl transcription buffer (200mM Tris.Cl, pH 8.0, 40mM $MgCl_2$, 10mM spermidine, 250mM NaCl, stored at -20 °C, Fermentas, included in Fermentas T7 and SP6 polymerase kits)
  - 1µl of 10mM rNTP mixture (Invitrogen Cat. 18109-017)
  - 0.25µl RNase inhibitor
  - 1µl of either T7 (Fermentas #EP 0111) or SP6 polymerase (Fermentas #EP0131)
  - 2µl of PCR product
- Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid
- Incubate at 37 °C for 70min
- Add 10µl of hybridization buffer (25mM EDTA : 25µl of 0.5M EDTA and 475µl DEPC water) (mix gently)
- Briefly centrifuge
- Incubate at 94°C for 3min
- Briefly centrifuge

STEP 3. HYBRIDIZATION

In this step we cross-hybridize the transcription products from the previous step in a fashion shown in this simplified diagram:



- 10µl of each T7 tube is cross-hybridized with 10µl of each SP6 tube (mix gently)
- Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid
- Incubate at 94°C for 3 min. Let hybrids reach room temperature slowly
- Briefly centrifuge
- Run hybridization product on 1.5% gel agaroze (2µl hybridization product + 1 µl loading dye).



→ 100 bp ladder

→ PCR product

→ Wild Type T7 – Wild Type SP6

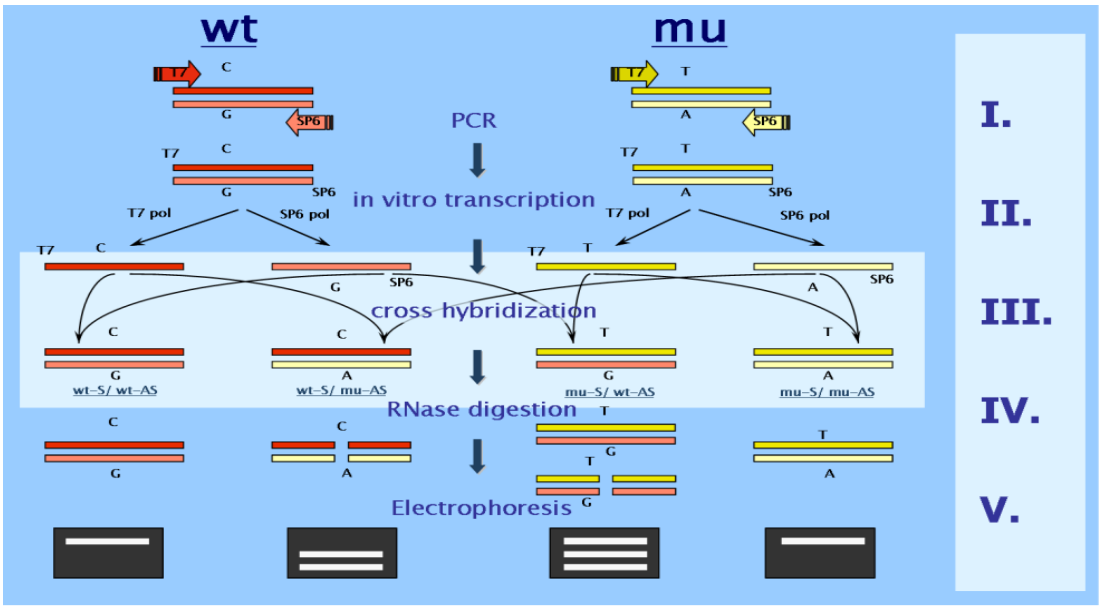
→ Wild Type T7 – Sample SP6

→ Sample T7 – Wild Type SP6

→ Sample T7 – Sample SP6

*It has to be noted that the cross-hybridizing procedure should not exceed 8mins at room temperature after finishing transcription and before the reaction tubes are returned to 94 °C.

A schematic overview of transcription – hybridization can be seen in the following diagram:



STEP 4. DIGESTION

In this assay, by using RNase 1 and RNase T1, we investigate possible mutation by cleaving mismatches in the RNA hybrids. Each hybridization product is subjected to three different digestion reactions that contain either RNase 1, RNase T1 or a mix of RNase 1 / RNase T1.

- Dilute RNases in digestion buffer (1 mM EDTA pH 8.0, 10mM Tris.HCl pH 7.5, 150mM NaCl, 3mM $CaCl_2$, 50 µg / ml ethidium bromide, stored at -20 °C). Dilutions for each RNase are :
  - RNAse 1 – 1:250 (Ambion Cat. #2294)
  - RNAse T1 – 1:3 (Fermentas #EN0542)
  - RNAse mix- 1/1 equal volumes mix from the previously diluted RNAses (1/T1)
- For each RNAse digestion (16µl reaction volume) we use:
  12 µl of RNAse diluted in digestion buffer
  4 µl of hybridization product
- Incubate at 37 °C for 45 min

STEP 5. ANALYSIS OF RNASE CLEAVAGE PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

Add 4 µl of gel loading solution (3M NaCl, 10mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 0.25% bromophenol blue, 10µg/ml ethidium bromide, 33% glycerol) that also serves as a stop solution for the digestion process.

Then the product is loaded on 1.5% agarose gel and photographed after approximately 45 min of electrophoresis at 120V so that each digestion fragment separates adequately from the other ones for the best visible result.

STEP 6. R202Q ANALYSIS

In order to control the incidence of R202Q alteration, nested PCR products of exon 2 are digested with the PvuII restriction enzyme (Invitrogen, Paisley, UK).

For reaction volume of **15µl** we use:

- 1.5 µl Reaction 6 buffer 10x
- 5U Pvu II
- 1 µg PCR product
- Up to 15 µl pH_2O

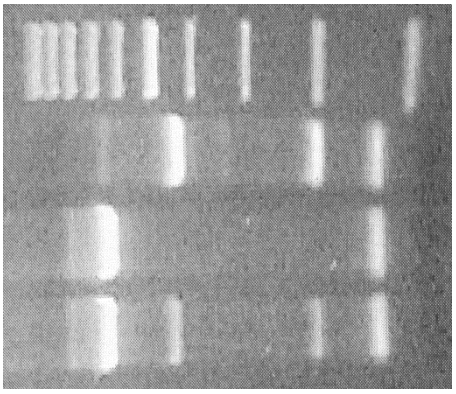
Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid.

The reaction conditions are (MJ Research PTC- 200 DNA Engine):

- 37 °C for 2 hours → incubation
- 65 °C for 20 min → inactivation

- Immediately on ice

Then the product is loaded on 1.5% agarose gel and photographed after approximately 45 min of electrophoresis at 120V so that each digestion fragment separates adequately from the other ones for the best visible result.



→ DNA ladder 100 bp

→ Homozygote

→ Normal

→ Heterozygote