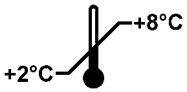
PRONTO® FMF Screen™

For the detection of the following mutations in the human pyrin gene:

M680I
M694V
M694I,
V726A
E148Q

Instructions for Use

REF 9904



Tests

PRONTO DIAGNOSTICS

CE IVD

INTENDED USE

The PRONTO® FMF Screen™ kit is a Single Nucleotide Primer Extension ELISA Assay intended for the qualitative *in vitro* detection of the M680I (c.2040 G>C and c.2040 G>A), M694V (c.2080 A>G), M694I (c.2082 G>A), V726A (c.2177 T>C) and E148Q (c.442G>C) mutations in the Pyrin gene, from amplified human DNA.

For *in vitro* diagnostic use

BACKGROUND

Familial Mediterranean Fever:

An autosomal recessive disease characterized by short periodic attacks of fever together with painful manifestations in the abdomen, chest, joints, or skin and recurring attacks of inflammation in the peritoneum, synovium or pleura. In most patients the first symptoms may appear by the age of 10 and in 90% of the patients by the age of 20. Fever is a constant feature but is rarely the sole symptom, usually preceded by pain. The most important complication of the disease is the development of amyloidosis. Amyloid nephropathy is independent of the severity of the other signs of the disease and may appear at early age. The manifestation are first protenuria, then nephrotic syndrome and renal failure. Treatment with colchicine reduces the symptoms and the frequency of the periodic attacks and may prevent the development of amyloidosis. The disease is found with a relatively high incidence in Mediterranean countries with a carrier rate of 1/5 in Turks, 1/6 in North African Jews and in 1/2 in Armenians. Four to six founder mutations account for about 80% of FMF chromosomes from typical cases (Armenians, Arabs, Jews and Turks). The MEFV gene is mapped to chromosome 16p13.3. Few mutations are responsible for the high frequency of the disease. There is a significant linkage desiquilibrium for all the major mutations in all the populations suggesting that they are ancient.

REFERENCES

1. Magal, N. et al: FASEB: www.faseb.org, (1999).
2. Ehrlich, G.: Annals of Internal Medicine. 129:581-582.
3. Touito, I.: Eur. J. Hum. Genet., 9(7):473-83. Review (2001).
4. Daniels, M. et al: Am. J. Med. Genet., 30;55(3):311-4 (1995).
5. Rogers, DB. et al: Am. J. Med. Genet., 34(2):168-72 (1989).

WARNINGS & PRECAUTIONS

- Reagents supplied in this kit may contain up to 0.1% sodium azide that is toxic if swallowed. Sodium azide has been reported to form explosive lead or copper azides in laboratory plumbing. To prevent the accumulation of these compounds, flush the sink and plumbing with large quantities of water.
- TMB Substrate solution is an irritant of the skin and mucous membranes. Avoid direct contact.
- The Stop Solution contains dilute sulfuric acid (1M), which is an irritant of the eyes and the skin. In case of contact with the eyes, immediately flush them with water. Do not add water to this product. In case of an accident or discomfort consult a physician (if possible, show the bottle label).
- In addition to reagents in this kit, the user may come in contact with other harmful chemicals that are not provided, such as ethidium bromide and EDTA. The appropriate manufacturers' Material Safety Data Sheets (MSDS) should be consulted prior to the use of these compounds.

ASSAY OVERVIEW

The PRONTO® procedure detects predefined polymorphisms in DNA sequences, using a single nucleotide primer extension ELISA assay.

1 **TARGET DNA AMPLIFICATION:** The DNA fragments that encompass the tested mutations are amplified. This amplified DNA is the substrate for the primer extension reaction.

2 **POST-AMPLIFICATION TREATMENT:** The amplified DNA is treated to inactivate free unincorporated nucleotides, so that they will not interfere with the primer extension reaction.

3 **PRIMER EXTENSION REACTION:** A single nucleotide primer extension assay is carried out in a 96-well thermoplate. Each well contains a 5'-labeled primer that hybridizes to the tested DNA next to the suspected mutation site, and a single biotinylated nucleotide species (corresponding to mutant or wild type), which complements the nucleotide base at the tested site. Each post-amplification treated sample is tested in two wells per mutation: the first well of each pair tests for the presence of the mutant allele (*mut*), while the second well tests for the presence of the normal allele (*wt*). The biotinylated nucleotide will be incorporated to the primer in the course of the reaction or not added, depending on the tested individual's genotype.

4 **DETECTION BY ELISA:** The detection of the biotinylated primers is carried out by an ELISA procedure: The biotin-labeled primers bind to a streptavidin-coated ELISA plate and are detected by a peroxidase-labeled antibody (HRP) directed to the 5' antigenic moiety of the primer.
A peroxidase reaction occurs in the presence of TMB-Substrate.

5 **INTERPRETATION OF THE RESULTS:** The results are determined either visually (substrate remains clear or turns blue) or colorimetrically (substrate remains clear or turns yellow) following the addition of the stop solution.

DISCLAIMER

- Results obtained using this kit should be confirmed by an alternative method.
- Confirmed results should be used and interpreted only in the context of the overall clinical picture. The manufacturer is not responsible for any clinical decisions that are taken.

The user of this kit should emphasize these points when reporting results to the diagnosing clinician or the genetic counselor.

CONTENTS OF THE KIT

FMF Screen Amplification Mix……..	1 vial (clear cap)	(0.95 mL)
PRONTO® Buffer 2	1 bottle	(3 mL)
Solution C	1 vial (yellow cap)	(130 µL)
Solution D	1 vial (red cap)	(100 µL)
ColoRed™ Oil	1 dropper bottle	(13 mL)
Assay Solution	1 bottle (green solution)	(100 mL)
Wash Solution (conc. 20x)	1 bottle	(100 mL)
HRP Conjugate	1 vial	(450 µL)
TMB Substrate	1 bottle	(40 mL)
Stop Solution (1M H_2SO_4)	1 bottle	(30 mL)
PRONTO® FMF Screen™ Plates	3 individually pouched plates	
Detection Plates	3 Streptavidin-coated ELISA plates	

STORAGE AND STABILITY

- Store at 2-8ºC. **Do not freeze**.
- Do not use the kit beyond its expiration date (marked on box label). Stability is maintained even when components are re-opened several times.
- Minimize the time reagents spend at room temperature.
- This kit has been calibrated and tested as a unit; **do not mix reagents from kits with different lot numbers.**

ADDITIONAL MATERIALS REQUIRED

- Taq DNA polymerase
- Deionized water (about two liters per kit)
- Thermowell plate or tubes (thin wall) for the post-amplification treatment
- Sterile pipette tips
- Troughs/reagent reservoirs - for use with the detection reagents
- Thermocycler for a 96-well microplate
- Multichannel pipettes (5-50 µL and 50-200 µL)
- Positive displacement pipettes (1-5 µL, 5-50 µL, 50-200 µL & 200-1,000 µL)
- Filtered tips

- ELISA reader with 450 nm filter (optional - 620 nm filter)
- Polaroid camera and color film to record results (optional)
- Automated microtiter plate washer or squirt bottle
- Vortex mixer
- Timer

ASSAY PROCEDURE

1 DNA AMPLIFICATION

1. **Dispense** 2 µL template DNA (from an initial concentration of about 150 ng/µL) to a thermoplate well or tube.
2. **Prepare** a Master Mix in a sterile vial, according to the volumes indicated in the table below, plus one spare reaction volume. Add the Taq DNA Polymerase to the amplification mix shortly before dispensing the mix. Mix gently by pipetting.

PCR Master Mix

Solution	Volume for one sample
Amplification mix FMF Screen	18.0 µL
Taq DNA Polymerase (5 u/µL)	0.5 µL

The following Taq DNA polymerases were validated for use with this procedure (lacking 3' → 5' exonuclease activity):

- PHARMACIA Cat. # 27-0799
- SIGMA Cat. # D -1806
- ROCHE Cat. # 1-146-165
- PROMEGA Cat. # M-1661
- BIOLINE Cat. # M95801B
- PERKIN ELMER Cat. # M801-0060

3. **Dispense** 18 µL Master Mix to each well or tube.

4. **Add** one drop of ColoRed™ oil to each well. Do not touch the wells with the tip of the oil bottle. Even when using a thermocycler with a hot lid, it is essential to use oil

5. **Place** the thermoplate well or tube in a thermocycler previously programmed with the following protocol:

Cycling Protocol			
1.	94ºC	2 minutes	
2.	94ºC	30 seconds	35 cycles
3.	59ºC	45 seconds	
4.	72ºC	30 seconds	
5.	72ºC	5 minutes	

6. To verify amplification, **subject** 5 µL of the amplified product to electrophoresis in a 2% agarose gel.

Sizes of amplified fragments:

Mutation	Position	Fragment size
M680I, M694V, M694I & V726A	Exon 10	357 bp
E148Q	Exon 2	201 bp

Limitation of the test:

Different Taq DNA polymerases and thermocyclers may influence the amplification yield dramatically. It is recommended to use a validated Taq DNA polymerase and a calibrated thermocycler.

2 POST-AMPLIFICATION TREATMENT

1 **Prepare** a post-amplification treatment mix shortly before use. Combine in a single test tube the volumes appearing in the following table, multiplied by the number of tested samples, plus one spare volume.

Post-Amplification Mix

Solution	Volume for one sample
PRONTO® Buffer 2	90.0 µL
Solution C	4.0 µL
Solution D	3.0 µL

2 **Mix** gently by pipetting this solution in and out five times. Do not vortex.

3 **Add** 97 µL of the post-amplification mix into each well or tube containing 15 µL of each amplified DNA sample.
Ensure that the solution you add becomes well mixed with the DNA sample by pipetting.

4 **Add** one drop of ColoRed™ oil to each tube. Do not touch the tube with the tip of the oil bottle. Even when using a thermocycler with a hot lid, it is essential to use oil.

5 **Incubate** for 30 minutes at 37ºC, then for 10 minutes at 95ºC in a thermocycler.

If not used immediately, the treated sample can be kept at 2-8ºC for a maximum of four hours.

3 PRIMER EXTENSION REACTION

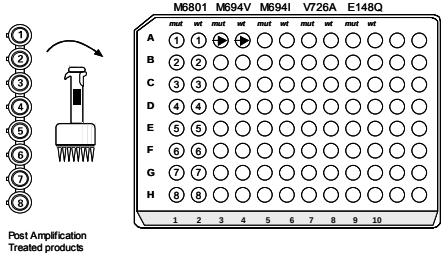
1 **Program** the thermocycler as follows:

Cycle	Temperature	Time
Start:	94º C	15 sec
20 cycles:	94º C	30 sec
	60º C	10 sec
End:	18-25º C - Cool down to room temperature	

2 **Take** a PRONTO® Plate out of its pouch. Notice the color at the bottom of the wells. For each mutation tested, use a pink well (*mut*) and a blue well (*wt*). Mark the plate with the ID numbers of your test.
If you intend to use less than a full plate, you can cut the plate and return the unused portion to the pouch. If you do this, seal the pouch immediately with its desiccant card inside.

3 **Dispense** 8 µL of post-amplification treated DNA into the first ten wells in row A (see Fig. 1). Continue with the remaining samples. It is possible to transfer up to eight samples simultaneously using a multichannel pipette. Ensure that the solution is at the bottom of each well by inspecting the plate from below. Be sure that the well does not contain air bubbles.

Figure 1: Scheme for dispensing Post Amplification treated DNA samples into the PRONTO® FMF Screen™ Plate



Recommendation:
Use a new set of tips for each column. Alternatively use the same set of tips, but do not touch the bottom of the wells.

4 **Tilt** the plate and add one drop of ColoRed™ oil to each well. Do not touch the well with the tip of the oil bottle. Even when using a thermocycler with a hot lid, it is essential to use oil.

5 **Turn on** the thermocycler and start the cycling protocol.

6 When the thermal cycling is complete, you can proceed to the ELISA assay, or store the reaction products in the refrigerator and carry out the visualization steps within 24 hours.

4 ELISA ASSAY - COLOR DEVELOPMENT

The ELISA assay consists of the following steps:

- **Binding** the biotinylated primer to the Streptavidin-coated plate.
- **Washing** away the unbound primer.
- **Incubating** with the HRP conjugate.
- **Washing** away the unbound conjugate.
- **Incubating** with the TMB Substrate (color development).

The results of this assay can be determined in one of two ways:

a **Visually**: by monitoring the development of the blue color.

or

b **Colorimetrically:** by adding Stop Solution and measuring the absorbance using an ELISA reader at a wavelength of 450 nm (yellow color).

! Before proceeding with the ELISA assay make your choice of visual or colorimetric determination of results.

PREPARATION

- All components used in the detection step should reach room temperature before starting the assay.
- Dilute the 20x Wash Solution to 1x with deionized water
  Dilute solution may be kept at 18-25º C for up to one month.

- Peel off the plastic cover of the Detection Plate. Mark the side of the plate with the kit name and test number.
- Place the PRONTO® plate and the Detection plate side by side, oriented in the same direction (see Fig. 2).

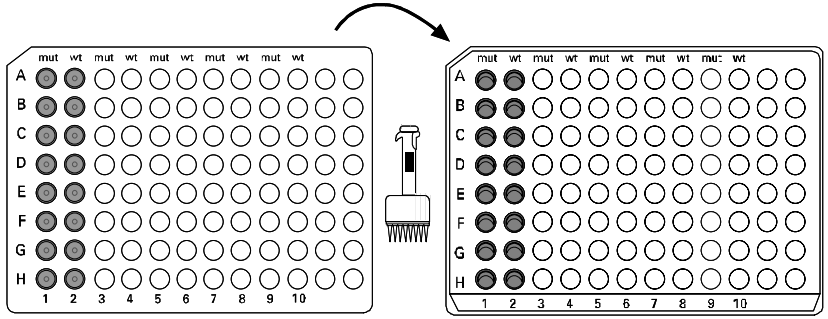
TRANSFER TO THE DETECTION PLATE

1 **Fill** a reagent reservoir / trough with the green colored Assay Solution. About 11 mL will be required for a 96-well plate.

2 Using a multichannel pipette **add** 100 µL of Assay Solution to the bottom of each well in column 1 of the PRONTO® Plate. Gently mix by pipetting in and out 3-4 times.

3 **Without changing tips, transfer** 100 µL from each well in this column to the first column in the Detection Plate (see Fig. 2).
Ensure that the solution at the bottom of all wells of the PRONTO® *Plate has turned green by inspecting them from below.*

Figure 2: Transferring the primer extension products from the PRONTO® Plate to Detection Plate.



4 **Repeat** this procedure, using a new set of tips for each column. It is essential to maintain the order of the samples.
10 µL of oil carried over or 10 µL of the sample left behind will not significantly affect the detection process.

5 **Incubate** for 10 minutes at room temperature (18-25ºC).

DETECTION BY ELISA

Procedure	Visual Detection (Blue color)	Colorimetric Detection (yellow color)
6 While the incubation of assay solution is taking place, dilute the Conjugated HRP in Assay Solution. For every detection plate used (96-well), about 11 mL of diluted conjugate is required. **This solution should be freshly prepared each time the test is run.**	Dilution: 1:100 (110 µL of conjugated HRP into 11 mL Assay Solution)	Dilution: 1:300 (36 µL of conjugated HRP into 11 mL Assay Solution)
7 Empty the plate and wash four times with 350 µL 1x Wash Solution. Ensure that the plate is relatively dry after the last wash step.	✓	✓
8 Add 100 µL of freshly diluted conjugated HRP to all the wells, with a multichannel pipette.	✓	✓
9 Incubate at room temperature.	10 minutes	10 minutes
10 Wash the plate as in step 7.	✓	✓
11 Add 100 µL TMB-Substrate to each well with a multichannel pipette and incubate at room temperature (18-25ºC) until blue color appears.	15 minutes	15 minutes
12 Add 100 µL of Stop Solution to each well with a multichannel pipette. The solution will turn yellow immediately.	–	✓
13 The results can be documented using a Polaroid camera with color film (for example - Fuji FP-100C), or by reading the absorbance using an ELISA reader (signal wavelength setting).	O.D. 620 nm	–
14 Within two hours read the absorbance using an ELISA reader (single wavelength setting).	–	O.D. 450 nm

VALIDATION OF THE RESULTS

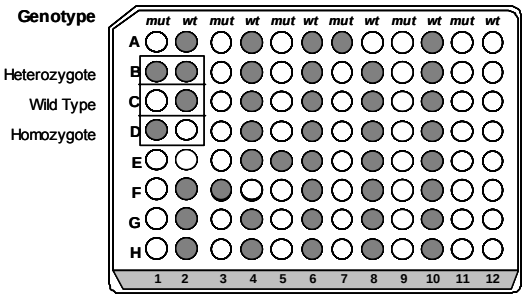
For Visual Detection:

For every mutation site tested, at least one of the wells should develop a deep blue color. Otherwise, the results are invalid for the relevant mutation (Fig. 3).

For Colorimetric Detection:

For every mutation site tested, at least one of the two wells should yield an reading O.D. ≥ 0.50.

Figure 3: Visual Interpretation of Genotypes



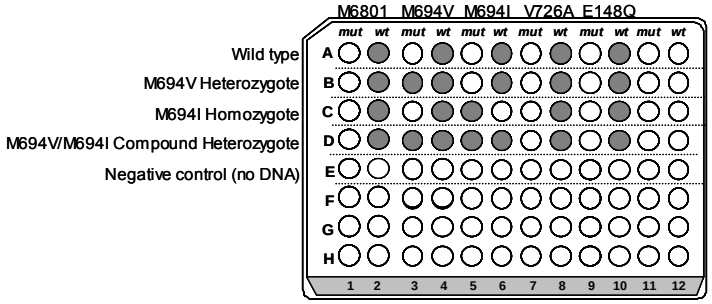
INTERPRETATION OF RESULTS

Important: Heterozygous or homozygous mutant results should be confirmed by retesting. It is recommended to repeat the test with newly extracted DNA.

Criteria for Visual Interpretation

A deep blue color indicates a positive signal, while negative signals appear as a clear or pale blue-colored well (see Fig. 4).

Figure 4: Genotype assignment according to visual inspection of test results



CRITERIA FOR COLORIMETRIC INTERPRETATION

The genotype of each sample is determined according to two criteria:

1. The O.D. values of the *mut* and *wt* wells.
2. The ratio of *mut / wt* O.D. values.

Calculate the *mut / wt* ratios by dividing the signal of the *mut* well by the signal of the *wt* well.

Identify the correct genotype using the table below:

Genotype	***mut* well** (O.D. 450)	***wt* well** (O.D. 450)	**mut/wt ratio**
Normal	O.D. ≤ 0.35	O.D. ≥ 0.5	ratio < 0.5
Heterozygote	O.D. ≥ 0.5	O.D. ≥ 0.5	0.5< ratio < 2.0
Homozygote	O.D. ≥ 0.5	O.D. ≤ 0.35	ratio >2.0

! Samples with values not included in the above table are considered indeterminate and should be retested.

FMF SCREEN™ - PROCEDURE SUMMARY

DNA EXTRACTION: from human whole blood, using a validated method.

DNA AMPLIFICATION:
Volumes per reaction: 2 µL Template DNA + 18 µL Amplification Mix + 0.5 µL Taq Polymerase.
Cycling protocol:
94°C 2 min→35 cycles of {94°C 30 sec. / 59°C 45 sec./ 72°C 30 sec.} →72°C 5 min.

POST-AMPLIFICATION PROCEDURE:

- Volumes per reaction: PRONTO® Buffer 2 90.0 µL; Solution C 4.0 µL; Solution D 3.0 uL
- **Pipette** in and out to mix.
- **Add** 97 µL into each well containing 15 µL amplified product, mix well.
- **Add** one drop of ColoRed™ oil.
- **Incubate** 30 minutes at 37ºC, then 10 minutes at 95ºC.

PRIMER EXTENSION REACTION:

- **Dispense** 8 µL of each post-amplification treated DNA into ten wells of the PRONTO® Plate.
- **Add** one drop of ColoRed™ oil.
- **Start** the cycling protocol:
  94°C 15 sec→20 cycles of {94ºC 30 sec. / 60ºC 10 sec.} → Cool.
- **Insert** the PRONTO® Plate in the thermocycler when the temperature is 90ºC

DETECTION:

- **Add** 100 µL Assay Solution to each well in the PRONTO® Plate and mix.
- **Transfer** 100 µL from each well of the PRONTO® Plate to the identical position in the Detection Plate. Incubate 10 minutes at RT.
- **Empty** the wells and wash four times with 350 µL of 1x Wash Solution.

	Visual Detection	**Colorimetric Detection**
Add 100 µL of Conjugated HRP to every well and incubate for 10 minutes at RT.	Dilution 1:100	Dilution 1:300
Empty the wells and wash four times with 350 µL of 1x Wash Solution.	✓	✓
Add 100 µL of TMB Substrate to each well and incubate at RT for:	15 minutes	15 minutes
Add Stop Solution	—	100 µL per well
Read O.D. at:	620 nm	450 nm

For troubleshooting guide, please refer to our website:
www.prontodiagnostics.com/ts

The PRONTO® Technology is covered by US patent 5,710,028, by European patent 0648222 and by corresponding national patents.

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