PRONTO® 4 GeneScreen™

PRONTO® 4 GeneScreen™	REF **9953**	Σ 24
PRONTO® Canavan	REF **9906**	Σ 48
PRONTO® Bloom/Fanconi	REF **9931**	Σ 48
PRONTO® FD Screen	REF **9963**	Σ 48

Instructions for Use

REF **9953**

PRONTO DIAGNOSTICS

+2°C +8°C CE IVD

INTENDED USE

The PRONTO® 4 GeneScreen™ kit is a Single Nucleotide Primer Extension Assay, determined by ELISA, intended for the qualitative *in vitro* detection of the following six mutations: 693C>A and 854A>C in the ASPA gene, 6-bp del/7-bp ins in the BLM gene, IVS4+4 A>T in the FACC gene, 2507+6 T>C and R696P in the IKBKAP gene, in amplified human DNA.

Two assay formats are available:

Option A - simultaneous detection of all six mutations (PRONTO® 4 GeneScreen™ kit).

Option B - separate detection (PRONTO® Canavan or PRONTO® Bloom/Fanconi or PRONTO® FD Screen kits).

For *in vitro* diagnostic use.

BACKGROUND

Similar to some ethnic populations, the Ashkenazi Jewish population has a higher prevalence of certain genetic disorders. These diseases are inherited in an autosomal recessive pattern. Affected individuals have inherited two copies of the mutated gene, one from each parent.

The following table shows the carrier frequency in the Ashkenazi Jewish population and the detection rate of the conditions tested by the PRONTO® 4GeneScreen™ kit panel.

Disease	Mutation	Carrier Frequency	Disease Frequency	Detection Rate
Familial Dysautonomia	2507+6 T>C R696P	1/30	1/3,600	98%
Canavan Disease	693C>A 854A>C	1/41	1/6,724	98%
Fanconi Anemia C	IVS4+4 A>T	1/80	1/25,600	99%
Bloom Syndrome	6-bp del/7-bp ins	1/110	1/48,400	99%

REFERENCES

Bloom Syndrome

1. German G., et al. Bloom's syndrome. VI. The disorder in Israel and an estimation of the gene frequency in the Ashkenazim. Am. J. Hum. Genet; 29(6):553-62. (Nov. 1997).
2. Oddoux C., et al. Prevalence of Bloom syndrome heterozygotes among Ashkenazi Jews. Am. J. Hum. Genet; 64(4):1241-3. (Apr. 1999).
3. Peleg L., et al. Bloom Syndrome and Fanconi's Anemia: Rate and Ethnic Origin of Mutation Carrier in Israel. IMAJ 4:95-7. (2002).

Fanconi Anemia C

1. Whitney M.A., et al. A common mutation in the FACC gene causes Fanconi anemia in Ashkenazi Jews. Nat. Genet; 4(2):202-5. (Jun. 1993).
2. Whitney M.A., et al. The Ashkenazi Jewish Fanconi anemia mutation: incidence among patients and carrier frequency on the at risk population. Hum. Mutat. 3(4):339-41. (1994).

Canavan Disease

1. Kronn D., et al. Prevalence of Canavan disease heterozygotes in the New York metropolitan Ashkenazi Jewish population. Am. J. Hum. Genet. (5): 1250-2. (Nov. 1995).
2. Matalon R. Canavan disease: diagnosis and molecular analysis. Genet Test. 1(1):21-5. Review (1997).

Familial Dysautonomia (FD)

1. Anderson S.L., et al. FD is caused by mutations of the IKAP gene. Am. J. Hum. Genet.; 68(3): 753-758. (Mar. 2001).
2. Blumenfeld A., et al. Comparison of two methods for routine accurate determination of the two mutations in the IKBKAP gene causing FD. Abstract presentation at the ASHG 52nd Annual Meeting. (2002).

WARNINGS AND 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 assay.
Two steps are carried out prior to the use of this PRONTO® kit:

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 TEATMENT:** 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 reaction 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 in 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 biotin-labeled extended primer 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 takes place in the presence of the substrate - TMB.

6. **INTERPRETATION OF THE RESULTS:** The results are determined either visually (substrate remains clear or turns blue) or colorimetrically (substrate color 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.

MATERIALS PROVIDED

4GeneScreen amplification mix	1 x vial	(0.95µL)
PRONTO® Buffer 2	1 x bottle	(3 mL)
Solution C	1 x vial (yellow cap)	(130 µL)
Solution D	1 x vial (red cap)	(100 µL)
ColoRed™-Oil	1 x dropper bottle	(13 mL)
Assay Solution	1 x bottle (green solution)	(100 mL)
Wash Solution (conc. 20x)	1 x bottle	(100 mL)
Conjugated HRP	1 x vial	(450 µL)
TMB- Substrate	1 x bottle	(40 mL)
Stop Solution (1M H_2SO_4)	1 x bottle	(30 mL)
Detection Plates	3 x Streptavidin-coated plates	
PRONTO® 4GeneScreen™	3 x individually pouched plates	

Alternatively (Option B):

Detection Plates ..... ……	2 x Streptavidin-coated plates
PRONTO® Plates ……	2 x Canavan or Bloom/Fanconi or FD Screen 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 Master Mix shortly before dispensing the Mix. Gently mix by pipetting in and out several times.

PCR Master Mix

Solution	Volume for one sample
Amplification Mix 4GeneScreen™	13.0 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

1. **Dispense** 13.5 µL Master Mix to each sample.
2. **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 recommended to use oil.
3. **Place** the thermoplate well or tube in a thermocycler previously programmed with the following protocol:

Cycling protocol			
1.	94ºC	5 minutes	
2.	94ºC	30 seconds	35 cycles
3.	60ºC	30 seconds	
4.	72ºC	30 seconds	
5.	72ºC	5 minutes	

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

Gene	Mutation	Fragment size
ASPA	693 C>A	162 bp
FACC	IVS4+4A>T	253 bp
ASPA	854 A>C	285 bp
BLM	6-bp del/7-bp ins	660 bp
IKBKAP	2507+6 T>C	802 bp
IKBKAP	R696P	802 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

! Only 5 or 10 µL of each amplified DNA sample will be used to carry out this assay

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

Components	Option A (6 mutations) PRONTO® 4 GeneScreen™ Kit	Option B (2 mutations) PRONTO® Canavan Kit PRONTO® Bloom/Fanconi Kit PRONTO® FD Screen Kit
Amplified DNA	**10.0 µL**	**5.0 µL**
PRONTO® Buffer 2	90.0 µL	45.0 µL
Solution C	4.0 µL	2.0 µL
Solution D	3.0 µL	1.5 µL
Total Volume	**97.0 µL**	**48.5 µL**

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

3 **Add** 48.5 or 97 µL of the Post-Amplification mix into each well or tube containing 5 or 10 µL of each amplified DNA sample according to the table above.
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:		94ºC	15 seconds
20 cycles:	{	94ºC	30 seconds
	{	57ºC	10 seconds
End:		Cool down to 25ºC (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.

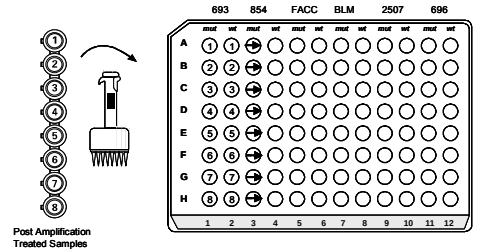
3 Primer extension can be carried out in one of two ways:
Option A: using the combined PRONTO® 4 GeneScreen™ plate for simultaneous detection of six mutations.
Option B: using separate PRONTO® plates for simultaneous detection of two mutations (Canavan or Bloom/Fanconi or FD).

Option A:

Simultaneous test (6 mutations):

Starting from the first sample, dispense 8 µl Post-Amplification treated DNA into each one of the twelve wells in row A as shown in Figure 1. 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. **Co**ntinue with the remaining samples.

Figure 1: Scheme for Dispensing Post-Amplification Treated Samples into the PRONTO® 4 GeneScreen™ Plate.



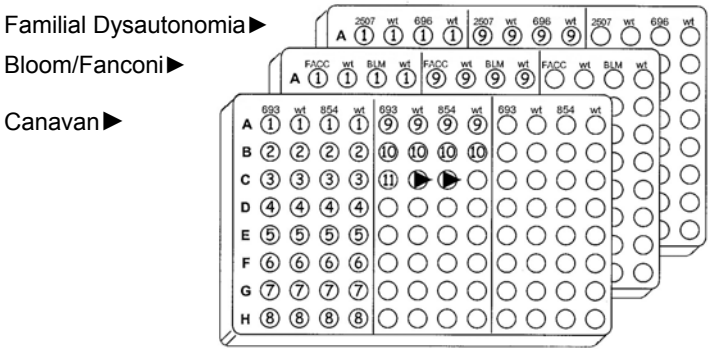
Recommendation:

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.

Option B:

Separate test – for Canavan or Bloom-Fanconi or FD Screen (2 mutations): Starting from the first sample, dispense 8 µl Post-Amplification treated DNA into each one of the four wells in row A as shown in Figure 2. 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. **Co**ntinue with the remaining samples.

Figure 2: Scheme for Dispensing Post-Amplification Treated Samples into Separate PRONTO® Plates.



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. Insert the plate when the temperature has reached 90ºC.

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:

1. **Binding** the biotin-labeled extended primer to the Streptavidin-coated plate.
2. **Washing** away unbound primers.
3. **Incubating** with the HRP conjugate.
4. **Washing** away unbound conjugate.
5. **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.

PREPARATIONS

- 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.
  Diluted 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 3).

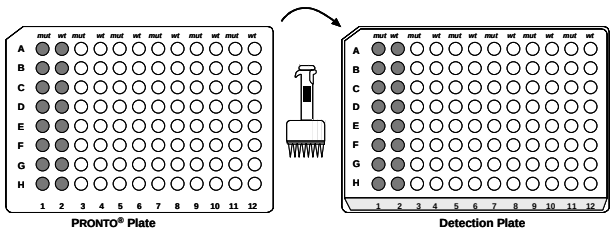
TRANSFER TO THE DETECTION PLATE

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

2 **Add** 100 µL of Assay Solution to the bottom of each well in column 1 of the PRONTO® Plate with a multichannel pipette. 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. 3).
Ensure that the solution at the bottom of all wells of the PRONTO® *plate has turned green by inspecting them from below.*

Figure 3: 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 (18-25°C).

DETECTION BY ELISA

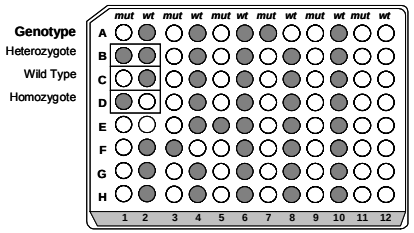
Procedure	Visual Detection (Blue color)	Colorimetric Detection (Yellow color)
6. While the incubation of Assay Solution is taking place, **dilute** the Conjugated HRP in Assay Solution: For every detection plate used (96 well), about 11 mL of diluted conjugate is required. **This solution should be freshly prepared each time the test is run.**	Dilution: 1:100 (110 µL of conjugated HRP into 11 mL Assay Solution per plate)	Dilution: 1:250 (44 µL of conjugated HRP into 11 mL Assay Solution per plate)
7. **Empty** the plate and wash four times with 350 µL 1x Wash Solution. Ensure that the plate is 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- 25°C) 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.	—	100 µL
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).	Agitate the plate gently and read results at O.D. 620 nm	—
14. Within two hours read the absorbance using an ELISA reader (single wavelength setting).	—	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, results are invalid for the relevant mutation (Fig. 4).

For Colorimetric Detection:
For every mutation site tested, at least one of the two wells should yield an O.D. $\geq$ 0.50 reading.

Figure 4: **Visual Interpretation of Genotypes**



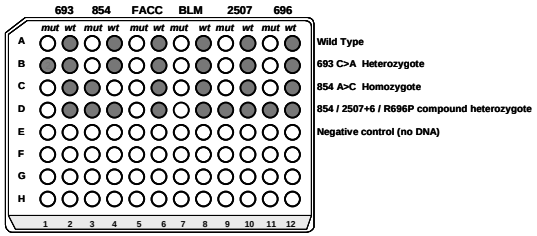
INTERPRETATION OF RESULTS

Important: Heterozygote or homozygote 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 positive signal, while negative signals appear as a clear to pale blue well (see Fig. 5)

Figure 5: **Examples of Genotype assignment according to visual inspection of test results**



Criteria for Colorimetric Interpretation (O.D 450)

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

- The O.D. values of the *mut* and *wt* wells.
- 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 of each mutation using the table below:

Genotype	*mut* well	*wt* well	*mut/wt* ratio
Normal	O.D. $\leq$ 0.35	O.D. $\geq$ 0.5	ratio $\leq$ 0.5
Heterozygote	O.D. $\geq$ 0.5	O.D. $\geq$ 0.5	0.5 < ratio< 2.0
Homozygote	O.D. $\geq$ 0.5	O.D. $\leq$ 0.35	ratio $\geq$ 2.0

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

4 GeneScreen™ PROCEDURE SUMMARY

DNA extraction from human whole blood using a validated method.

DNA Amplification:

Volumes per reaction: 2 µL Template DNA + 13.0 µL Amplification Mix + 0.5 µL Taq Polymerase

Cycling protocol: 94°C 5 min→35 cycles of {94°C 30 sec. / 60°C 30 sec./ 72°C 30 sec.} →72°C 5 min.

Post Amplification Treatment:

		Simultaneous Detection	Separate Detection
▪ Volumes for one reaction	PRONTO® Buffer 2	90.0 µL	45.0 µL
	Solution C	4.0 µL	2.0 µL
	Solution D	3.0 µL	1.5 µL
		97.0 µL	48.5 µL
▪ **Pipette** in and out to mix			
▪ **Add** the mix into each well containing amplified DNA sample		10.0 µL	5.0 µL
▪ **Top** with 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 twelve wells (simultaneous Detection) or four wells (separate Detection) of the PRONTO® Plate.
- **Top** off with ColoRed™ -Oil.
- **Start** the cycling protocol:

  94ºC 15 sec → 20 cycles of {94ºC 30 sec. / 57ºC 10 sec.} → Cool

Insert the PRONTO® Plate in the thermocycler when the temperature has reached 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 respective 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 room temperature.	Dilution 1:100	Dilution 1:250
▪ 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
▪ Results documentation	Photo or O.D. 620 nm	O.D. 450 nm

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

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