PRONTO® CF OPEN kit

For the detection of the following mutations in the CFTR gene:

S549R, G85E, D1152H, W1089X, 1717-1G>A, 405+1G>A, Q359/T360K, and 4010delTATT

REF **9920**

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INSTRUCTIONS FOR USE

PRONTO DIAGNOSTICS

IVD +2°C +8°C CE

INTENDED USE

The PRONTO® CF Open kit is a Single Nucleotide Primer Extension Assay, determined by ELISA, intended for the qualitative in vitro detection of the following mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene: S549R, G85E, D1152H, W1089X, 1717-1G>A, 405+1G>A, Q359/T360K, and 4010delTATT in amplified human DNA

For *in vitro* diagnostic use.

BACKGROUND

Cystic fibrosis is the most common severe autosomal recessive genetic disorder among Caucasians (1). A defect in the Cystic Fibrosis Transmembrane conductance Regulator protein (CFTR) interferes with the transport of chloride ions across the membrane of epithelial cells lining of certain organs.

Since the identification of the gene and the major mutation responsible for CF, more than 800 mutations and DNA sequence variations have been identified in the CFTR gene (2). The predominant CFTR gene mutation, ΔF508, is observed almost in all populations, while its prevalence varies among different ethnic groups. The highest carrier frequency is observed in Caucasian populations, where it accounts for approximately 70% of CF alleles. Some 15-20 other common mutations account for 2-15% of CF alleles, depending on the ethnic composition of the patient group studied. Most of the remaining mutations are rare (3).

In addition, mutations in the CFTR gene have been found to cause congenital bilateral aplasia of the vas deferens (CBAVD) leading to male infertility (4).

REFERENCES

1. Weish MJ. Tsui L-C Boat TF, Beaudet AL Cystic fibrosis In: Scriver CR, Beaudet et al. (eds) The Metabolic and Molecular Basis of Inherited Disease. New York, McG (1994)
2. Pediatrics Fed: 107 (2): 280-6. (2001)
3. Mum. Mol. Genet: 1993.2:355.
4. Am. J. Hum. Genet: 60:87 – 94.(1997)

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.
- 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 reaction is carried out in a 96-well thermoplate or strip. 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, 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 using ELISA Reader.

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

Amplification Mix CF Open	2 x vials	(950 µL)
PRONTO® Buffer 2	2 x bottles	(3 mL)
Solution C	2 x vials (yellow cap)	(130 µL)
Solution D	2 x vials (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 (conc. 100x)	1 x vial	(450 µL)
TMB- Substrate	1 x bottle	(40 mL)
PRONTO® CF Open Plates or strips	2/24 x individually pouched plates/strips	
Detection Plates	2 x Strepatividin-coated 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

- 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 and & 200-1,000 µL)
- Filtered tips
- ELISA reader with a 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.

Master mix

Solution	Volume for: 1-2 mutations	3-4 mutations	4-6 mutations
Amplification mix CF OPEN	13.0 µL	17.5 µL	22.5 µL
Taq DNA Polymerase *(5 u/µL)	0.25 µL	0.4 µL	0.5 µL

*Not supplied.

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

- PHARMACIA Cat. No. 27-0799
- SIGMA Cat. No. D-1806
- ROCHE Cat. No. 1-146-165
- PROMEGA Cat. No. M-1661
- BIOLINE Cat. No. M95801B
- PERKIN ELMER Cat. No. M801-0060

3. **Dispense** the total volume as indicated in the table above 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. When using a thermocycler with a hot lid, it is not essential to use oil.
5. **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	5 cycles
3.	60ºC	30 seconds	
4.	72ºC	60 seconds	
5.	94ºC	30 seconds	5 cycles
6.	58ºC	30 seconds	
7.	72ºC	60 seconds	
8.	94ºC	30 seconds	30 cycles
9.	56ºC	30 seconds	
10.	72ºC	60 seconds	
11.	72ºC	5 minutes	

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

Mutations	Position	Fragment size
S549R, 1717-1 (G>A)	Exon 11	220 bp
G85E, 405 + 1 (G>A)	Exon 3/ Intron 3	310 bp
4010 del TATT	Exon 21	400 bp
Q359K / T360K	Exon 7	410 bp
D1152H	Exon 18	470 bp
W1089X	Exon 17b	560 bp

Limitation of the test:

Different Taq DNA polymerases and thermocyclers may influence the amplification yield dramatically. Use a validated Taq DNA polymerase and a calibrated thermocycler.

2 POST-AMPLIFICATION TREATMENT

! Set aside 5 µL of every amplified sample now.
It is recommended to use this 5 µL to visualize the amplified DNA by Agarose gel electrophoresis.

1. **Prepare** a post-amplification mix shortly before use. Combine in a single test tube PRONTO® buffer 2, Solution C and Solution D according to the volumes appearing in the following table. Multiply the volume by the number of tested samples, plus one spare volume.

Volumes for the Post-Amplification Treatment

Solution	Volumes for 1-2 mutations	Volumes for 3 mutations	Volumes for 4-6 mutations
PRONTO® Buffer 2	30.0 µL	50.0 µL	100.0 µL
Solution C	1.5 µL	2.0 µL	4.0 µL
Solution D	1.0 µL	1.5 µL	3.0 µL
Total volume	32.5 µL	53.5 µL	107.0 µL

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

3. **Add** 65 µL of the post-amplification mix into each well or tube containing 10 µL of each amplified DNA sample..

   Ensure that the solution you add becomes well mixed with the DNA sample by inserting the tip under the oil, down to the bottom of the tube and mixing the two solutions by pipetting.

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

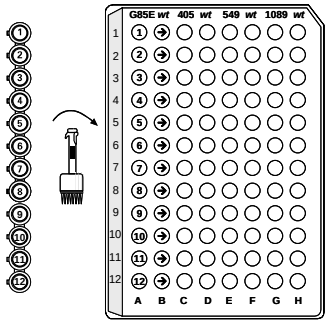
3 PRIMER EXTENSION REACTION

1. **Program** the thermocycler to the following protocol:

Cycle	Temperature	Time
Start:	94ºC	15 sec.
20 cycles:	94ºC	10 sec.
	50ºC	30 sec.
End:	18°C - 25°C (Cool down to room temperature)	

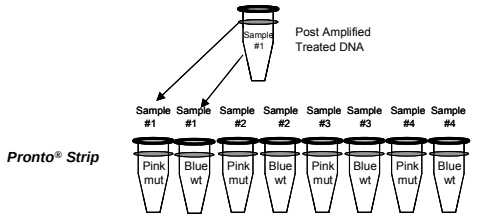
2. **Take** a PRONTO® Plate or strip out of its pouch. Notice the color at the bottom of the wells. For each mutation tested, use a pink well (*mut*) and a blue well (*wt*). Mark the plate with the ID numbers of your test.
   If you intend to use less than a full plate or strip, you can cut it and return 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 eight wells in the PRONTO® plate (row 1: A-H) as shown in Fig. 1 or PRONTO® Strip as shown in Fig. 2. Continue with the remaining samples. It is possible to transfer up to twelve 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: **Dispensing of the Post-Amplified treated DNA into the PRONTO® CF OPEN 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.

Figure 2: **Dispensing of the Post-Amplified treated DNA into the PRONTO® CF Open Strip**



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. When using a thermocycler with a hot lid, it is not essential to use oil.
5 **Turn on** the thermocycler and start the cycling protocol. Insert the PRONTO® Plate (or Strip) 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.

3 ELISA ASSAY - COLOR DEVELOPMENT

The ELISA assay consists of the following steps:

- **Binding** the biotin-labeled extended primers to the Streptavidin-coated plate.
- **Washing** away unbound primers.
- **Incubating** with the HRP conjugate.
- **Washing** away 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 measuring the absorbance, using an ELISA reader at a wavelength of 620 nm.

PREPARATIONS

All components used in the detection step should reach room temperature before starting the assay.

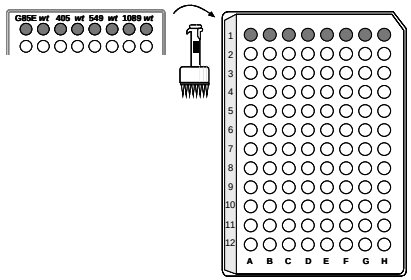
- **Dilute** the 20x Wash Solution to 1x with deionized water.
  Dilute solution may be kept at 18-25º C for up to one month.
- **Peel** off the plastic cover of the Detection Plate. Mark the side of the plate with the kit name and test number.
- **Place** the PRONTO® Plate (or Strip) and the Detection 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 (or Strip), with a multichannel pipette. Mix the Assay Solution thoroughly with the solution in the wells.
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 or strip has turned green by inspecting them from below.

Figure 3: **Transferring the primer extension products from the PRONTO® Plate (or Strip) to Detection Plate.**



4. **Repeat** this procedure using a new set of tips for each remaining row. It is important to maintain the order of the samples.
   Note: 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 RT (18 to 25º C).
6. While the incubation takes place, **dilute** the Conjugated HRP 1:100 in Assay solution (green solution). About 11 mL are needed for a 96-well Plate. This solution must be freshly prepared each time the test is run.
7. **Empty** the Plates, **wash** four times with 350 µL 1x Wash Solution. Ensure that the Plates are dry after the last wash step.
8. With a multichannel pipette add 100 µL **freshly-diluted** Conjugated HRP to all the wells.
9. **Incubate** for 10 minutes at RT.
10. **Wash** as in step 7.
11. **Add** 100 µL TMB substrate to each well with a multichannel pipette and incubate for 10-30 minutes at RT (18 to 25°C) until the blue color appears sufficiently strong.
12. For Visual Detection: Results may be documented by a standard Polaroid camera with color film (for example – Fuji FP-100C).

13. For Colorimetric Detection: **Agitate** the Plate gently to homogenize the color in the wells. **Read** the results in an ELISA reader using a 620 nm filter (single wavelength setting).

VALIDATION OF THE RESULTS

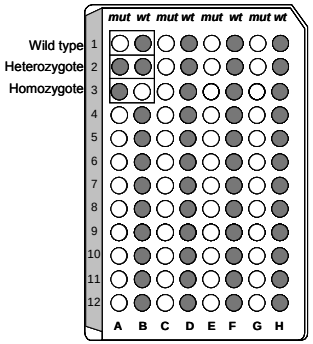
For Visual Detection:

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.

Figure 4: Visual Interpretation of Genotypes



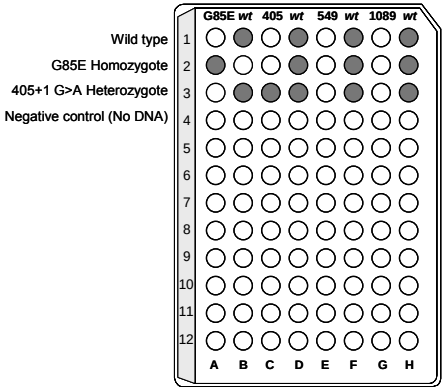
INTERPRETATION OF RESULTS

Important: Positive results (corresponding to a heterozygote or homozygote genotype) should be confirmed by re-testing the sample. 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 (Fig. 5).

Figure 5: Example of genotype assignment according to visual inspection of test results



Criteria for Colorimetric Interpretation (O.D. 620)

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 $\leq$ ratio $\leq$ 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.

PRONTO® CF OPEN – PROCEDURE SUMMARY

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

DNA AMPLIFICATION:

		2 Mutations	3 Mutations	4-6 Mutations
Volumes per reaction:	Amplification Mix	13.0 µL	17.5 µL	22.5 µL
	Template DNA	2.0 µL	2.0 µL	2.0 µL
	Taq Polymerase	0.25 µL	0.4 µL	0.5 µL

Cycling protocol: 94°C 5 min→5 cycles of {94°C 30 sec. / 60°C 30 sec./ 72°C 60 sec.} →5 cycles of {94°C 30 sec. / 58°C 30 sec./ 72°C 60 sec.} →30 cycles of {94°C 30 sec. / 56°C 30 sec./ 72°C 60 sec.} →72°C 5 min.

POST-AMPLIFICATION PROCEDURE:

		2 Mutations	3 Mutations	4-6 Mutations
▪ Volumes per reaction:	PRONTO® Buffer 2	30.0 µL	50.0 µL	100.0 µL
	Solution C	1.5 µL	2.0 µL	4.0 µL
	Solution D	1.0 µL	1.5 µL	3.0 µL

- **Pipette** in and out to mix.
- **Add** the post-amplification mix into each well containing **10 µL** amplified DNA sample and mix well.
- **Top** with one drop of ColoRed™ Oil.
- **Incubate** 30 minutes at 37°C, then 10 minutes at 95°C.

PRIMER EXTENSION REACTION:

- **Dispense** 8 µL of each post-amplification treated DNA into two wells of the PRONTO® Plate / Strip.
- **Top** off with one drop of ColoRed™ Oil.
- **Start** the cycling protocol:
  94°C 15 sec→20 cycles of {94°C 10 sec. / 50°C 30 sec.} →Cool.
- **Insert** the PRONTO® Plate in the thermocycler when the temperature has reached is 90°C.

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.
- **Add** 100 µL 1:100 Conjugate HRP to every well; incubate for 10 minutes at RT.
- **Wash** the wells again.
- **Add** 100 µL Substrate to each well; incubate at RT for 10-30 minutes.

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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