# PRONTO® CF Basic+

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

**ΔF508, G542X, W1282X, N1303K, 3849+10Kb and D1152H**

REF 9943

## Instructions for Use

24 Tests

PRONTO DIAGNOSTICS

+2°C +8°C

CE

IVD

## INTENDED USE

The Pronto® CF Basic+ kit is a Single Nucleotide Primer Extension ELISA Assay, intended for the qualitative *in vitro* detection of the following mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: ΔF508, G542X, W1282X, N1303K, 3849+10Kb and D1152H in amplified human DNA.
**For *in vitro* diagnostic use.**

## BACKGROUND

Cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride channel and controls the regulation of other transport pathways. Mutations in the CFTR gene have been found to cause cystic fibrosis (CF) and congenital bilateral aplasia of the vas deferens (CBAVD).
In addition to functioning as a chloride channel, CFTR controls the regulation of other transport pathways. For example, patients with CF and the homozygous CFTR-deficient mouse have enhanced sodium ion absorption; this enhanced sodium ion absorption is corrected by addition of a wild type copy of CFTR.

## REFERENCES


1. Weish M.J., Tsui L-C., Boat T.F., Beaudet A. Cystic Fibrosis in: Scriver CR, Beaudet et al. (eds). *The Metabolic and Molecular Basis of Inherited Disease*. New York, McGraw Hill (1994).
2. *Paediatrics Fed*., 107(2): 280-6 (2001).
3. *Mum. Mol. Genet*., 2:355 (1993).
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.
- 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 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, 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 | 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) |
| Conjugated HRP (conc. 100x) | 1 vial | (450 µL) |
| TMB- Substrate | 1 bottle | (40 mL) |
| PRONTO® CF Basic+ 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

- 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** 2µL 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 one sample |
|---|---|
| Amplification mix CF Basic+ | 22.5 µL |
| Taq DNA Polymerase * (5 u/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

3. **Dispense** 23 µL master mix to each well or tube.
4. **Add** one drop of ColoRed™-Oil to each tube. 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 | 5minutes | |
| 2. | 94ºC | 30 seconds | 35 cycles |
| 3. | 55ºC | 30 seconds | |
| 4. | 74ºC | 45 seconds | |
| 5. | 74ºC | 5 minutes | |

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

**Sizes of amplified fragments:**

| Gene | | Fragment Size |
|---|---|---|
| G542X | Exon 11 | 220 bp |
| W1282X | Exon 20 | 300 bp |
| ΔF508 | Exon 10 | 350 bp |
| N1303K | Exon 21 | 400 bp |
| 3849+10Kb | Intron 19 | 450 bp |
| D1152H | Exon 18 | 470 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

1. **Prepare** a post-amplification 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.

**Volumes for the post-amplification treatment:**

| Solution | Volume for one sample |
|---|---|
| PRONTO® Buffer 2 | 100.0 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** 107 µL of the post-amplification mix into each well or tube containing the remaining 20 µL of the 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 4 hours.**

# 3 PRIMER EXTENSION REACTION

1. **Program** the thermocycler as follows:

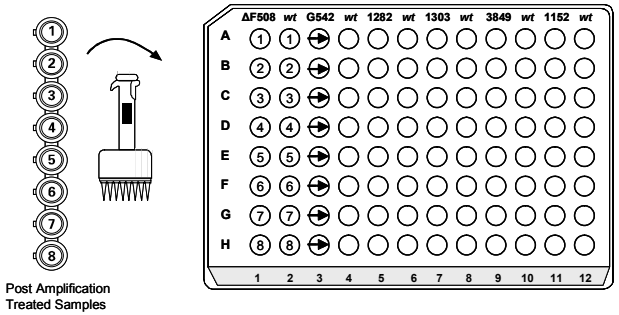
| Cycle | Temperature | Time |
|---|---|---|
| Start: | 94ºC | 15 sec. |
| 20 cycles: | 94ºC | 10 sec. |
| | 50ºC | 30 sec. |
| End: | 18°-25° C (Cool down to room temperature) | |

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 twelve 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:** **Dispensing of the Post-Amplified DNA into the PRONTO® CF Basic+ 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. 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.
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 primers 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 measuring the absorbance, using an ELISA reader at a wavelength of 620 nm.

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

## PREPARATION

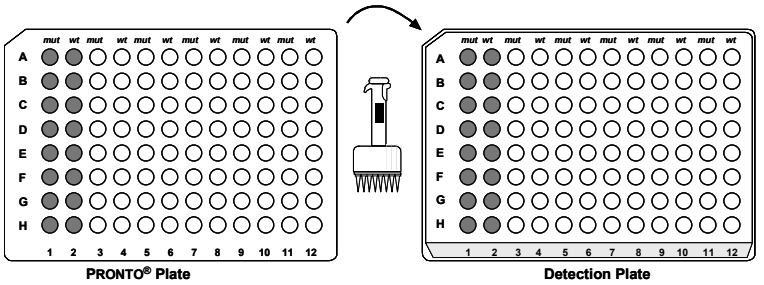
- All components used in the detection step should reach room temperature before starting the assay.
- **Dilute** the 20x Wash Solution to 1x with Deionized water.
  *Dilute solution may be kept at 18-25ºC for up to one month.*
- **Peel** off the plastic cover of the Detection Plate. Mark the side of 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. **Add** 100 µL of Assay Solution to the bottom of each well in column 1 of the PRONTO® Plate, 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. 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 remaining column. It is important 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 RT (18-25ºC).
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 15 minutes at RT (18-25°C) until the blue color appears sufficiently strong (15 minutes).
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

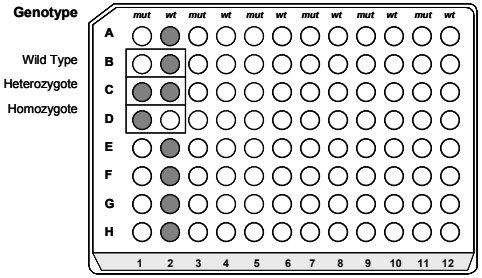
**For Visual Detection:**

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 (see Fig. 3).

**For Colorimetric Detection:**

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

**Figure 3:** **Visual Interpretation of Genotypes**




## INTERPRETATION OF RESULTS

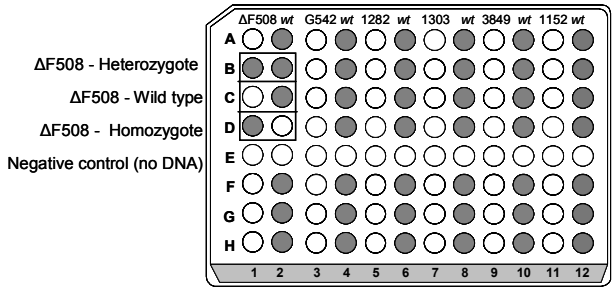
**Important:** Positive results (corresponding to a heterozygote or homozygote genotype) should be confirmed by re-testing the sample. It is recommended to repeat the test with newly extracted DNA.

### Criteria for Visual Interpretation

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



**Figure 4:** **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** (O.D. 450) | ***wt* well** (O.D. 450) | ***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 Basic+ – PROCEDURE SUMMARY

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

**DNA AMPLIFICATION**:
**Volumes per reaction:** 2 µL Template DNA + 22.5 µL Amplification Mix + 0.5 µL Taq Polymerase.
**Cycling protocol:**
94°C 5 min→35 cycles of {94°C 30 sec. / 55°C 30 sec./ 74°C 45 sec.} →74°C 5 min.

**POST-AMPLIFICATION PROCEDURE:**

| | | |
|---|---|---|
| ▪ Volumes per reaction: | PRONTO® Buffer 2 | 100.0 µL |
| | Solution C | 4.0 µL |
| | Solution D | 3.0 µL |

- **Pipette** in and out to mix.
- **Add** 107 µL into each well containing 20 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 twelve wells of the PRONTO® Plate.
- **Add** 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.

**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 15 minutes.



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



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