REF 9945

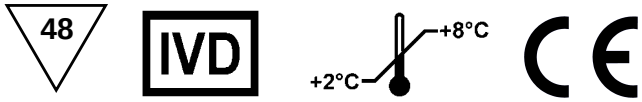
PRONTO DIAGNOSTICS

PRONTO® CFTR 5T

INSTRUCTIONS FOR USE

For the detection of the

5T mutation in the CFTR gene



INTENDED USE

The PRONTO® CFTR 5T kit is a Single Nucleotide Primer Extension Assay, determined by ELISA, intended for the qualitative in vitro detection of the 5T mutation of the human Cystic Fibrosis Transmembrane Regulator (CFTR) gene in amplified DNA.

For *In Vitro* Diagnostic Use.

BACKGROUND

Cystic Fibrosis (CF) is an autosomal recessive disorder common among Caucasians, characterized by progressive lung disease, pancreatic dysfunction, elevated sweat electrolytes and male infertility. The disease is caused by defects in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene, which encodes for a protein that functions as a chloride channel. Defects in the CFTR gene cause abnormal chloride concentration across the apical membrane of epithelial cells in the airways, pancreas, intestine and vas deferens.

The frequency of CF disease in the general Caucasian population is 1:2,500 live births, which corresponds to an estimated carrier frequency of 1:25 on the average. The actual frequency of the disease and the frequency of the different mutations that cause it, vary considerably between different ethnic groups.

CFTR mutations and male infertility

Almost all CF male patients are infertile due to congenital bilateral absence of the vas deferens (CBAVD). CBAVD is also found in infertile men who do not present additional clinical characteristics of CF.

REFERENCES

1. The Metabolic Basis of Inherited Disease, 7th ed., 3799-3877 (1995).
2. J. Urol.: 106(4): 568-574 (1971).
3. Am. J Hum. Genet: 61: 1200-1202 (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.

Two steps are carried out prior to the use of this PRONTO® kit:

1 DNA EXTRACTION: Genomic DNA is extracted from whole blood.

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

The following steps are carried out using the PRONTO® kit:

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

4 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.

5 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.

6 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

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 (conc. 100x)	1 x Vial	(450 µL)
TMB- Substrate	1 x Bottle	(40 mL)
PRONTO® CFTR 5T Plate	1 x individually pouched Plate	
Detection Plate …	1 x Coated Plate	

STORAGE AND STABILITY

- **Do not freeze**.
- Kit reagents are stable until the expiration date (check box label) if stored at 2-8º C.
- 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.**
- Do not use the kit beyond its expiration date (check box label).

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

Two initial steps must be carried out prior to this assay:

Step 1 - DNA Extraction:
Using Pronto's DNA Extraction kit (REF: 9925) or other validated DNA purification procedures.
Step 2 - DNA Amplification:
According to the instructions for DNA Amplification supplied with the kit.

1 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. **Use** the remaining 10 µL of the amplified DNA sample (in the labeled test tube or Thermowell plate) to continue with the Post Amplification Treatment. Make sure the required volume is used as indicated in the following table.
2. **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 of reagents in the Post Amplification Mix

Solution	Volume for one sample	Volume for ___ samples
PRONTO® Buffer 2	30.0 µL	_____µL
Solution C	1.5 µL	_____µL
Solution D	1.0 µL	_____µL

3. **Mix** gently by pipetting this solution in and out five times. **Do not vortex**.
4. **Dispense** 32 µL of the Post Amplification Mix into each Thermowell plate well or tube containing 10 µL of the amplified DNA.
   Make sure that the solution you add becomes well mixed with the sample by inserting the tip under the oil, down to the bottom of the well or tube and mixing the two solutions by pipetting.
5. **Top** off with one drop of *ColoRed™ oil* to each well. Be sure that the oil drops along the side of the 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 cover the solution with the red oil.*
6. **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.

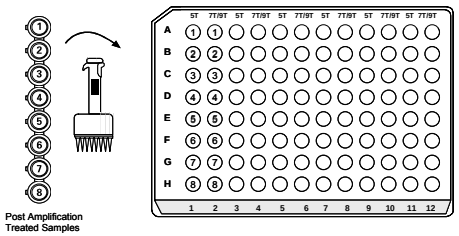
2 PRIMER EXTENSION REACTION

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

Cycle	Temperature	Time
Start:	94ºC	15 sec.
20 cycles:	94ºC	10 sec.
	63ºC	30 sec.
End:	18° C - 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 two 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® CFTR 5T 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. **Place** the PRONTO® plate in a previously programmed 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.

3 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.

PREPARATIONS

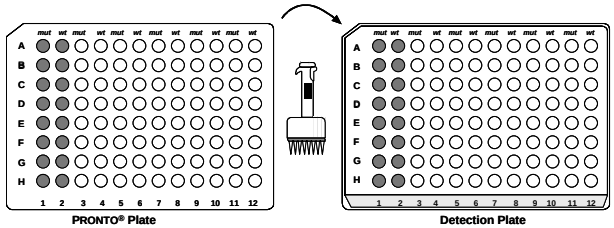
All components used in the detection step should reach room temperature before starting the assay.

- **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 and the Detection Plate side by side, oriented in the same direction (see Figure 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.
   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 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 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.

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

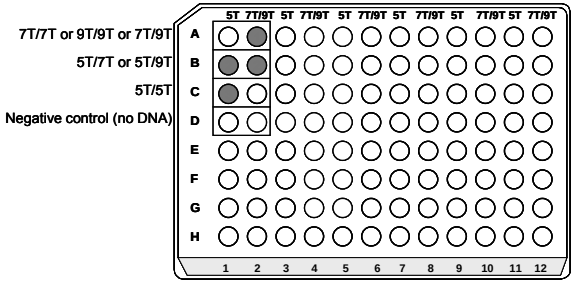
INTERPRETATION OF RESULTS

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 (figure 3).

Figure 3: **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® CFTR 5T – PROCEDURE SUMMARY

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

DNA AMPLIFICATION: according to the Amplification Mix instructions supplied with the kit

POST AMPLIFICATION PROCEDURE:

- Volumes per reaction: PRONTO® Buffer 2 30.0 µL; Solution C 1.5 µL; Solution D 1.0 µL
- **Pipette** in and out to mix.
- **Add** 32 µL 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.
- **Top** off with one drop of ColoRed™ oil.
- **Start** the cycling protocol:
  94°C 15 sec→20 cycles of {94°C 10 sec. / 63°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 15 minutes.

TROUBLESHOOTING GUIDE

PROBLEMS	POSSIBLE CAUSES	SUGGESTED SOLUTIONS
Weak signal throughout the entire plate	Poor quality of Amplified DNA due to:	
	Poor quality of template DNA	Re purify template DNA
	Too much or too little DNA used	Use ~150 ng genomic DNA per reaction
	Poor quality of Taq polymerase	Use Taq from an validated supplier
	Exonuclease activity of Taq polymerase:	Use Taq lacking 3'-5' exonuclease activity
	• Wrong PCR program	• Repeat PCR
	• Thermocycler is not calibrated	• Call a technician
	• HRP conjugate inactive	• Use freshly diluted HRP conjugate
	• ELISA Reader is not calibrated	• Check calibration
Weak signal or no signal at all in both wells of one mutation	Poor quality of PCR products	See above
	Failure in Post Amplification Treatment (PAT):	
	• PAT sample was not transferred into the PRONTO® plate/strip	• Inspect the PRONTO® plate from below to ensure the presence of PAT sample
	• PAT sample did not come into contact with the PRONTO® well	• Ensure that the PAT sample is at the bottom of each well (no air bubbles, no oil bottom)
	• PRONTO® products were not transferred to the detection plate	• Inspect plate from below to confirm transfer
No signal throughout the entire plate	Failure in PCR reactions	Check PCR products on gel
	Failure in Post Amplification Treatment (PAT)*	Repeat PAT on a new PCR sample
	Post Amplification mix was not prepared correctly (e.g., solution C or D is missing)	Review instruction for PAT
	HRP conjugate was not added	Wash plate four times, and repeat from HRP addition

* PAT – Post Amplification Treatment

TROUBLESHOOTING GUIDE

PROBLEMS	POSSIBLE CAUSES	SUGGESTED SOLUTIONS
High background signal or a false positive result	Wrong DNA polymerase used for PCR	Use a thermostable DNA polymerase lacking exonuclease activity (see Amplification Mix instructions)
	Inefficient mixing of PAT mix with PCR product	Make sure PCR product gets well mixed with PAT mix
	PCR reactions contaminated	Take measures to eliminate PCR product contamination:
		• Carry out DNA extraction and set up PCR reactions using filtered tips on dedicated pipettes, in a PCR-free room
		• Include a control (no DNA) every time you carry out PCR
	Inefficient washing in a few wells	Check the plate washer
High background signal or false positive signals throughout the plate	Substrate contaminated (appears blue prior to use)	• Change reagent reservoirs regularly
		• Do not return surplus substrate to bottle
		• Prevent exposure of substrate to light
	Inefficient plate washing	• Check washer
	All PCR reactions contaminated	• Take measures to eliminate PCR product contamination
		• Carry out DNA extraction and set up PCR reactions using filtered tips on dedicated pipettes, in a PCR-free room
		• Include a control (no DNA) every time you carry out PCR
	Mistake in PAT mix preparation	Strictly follow indicated volumes

Note on the use of PCR: Since PRONTO® is a post amplification mutation detection system, the tested gene fragment needs to be amplified. The user of this kit has to procure a suitable amplification system, and to obtain a license for its use, where appropriate. A license for the use of PCR can be obtained by contacting the PCR Licensing Manager, F. Hoffmann-La Roche Ltd., Building 222/350, CH-4500 Basel, Switzerland.

PRONTO is a Registered Trademark and COLORED is a Trademark.

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

Manufactured for Pronto Diagnostics Ltd.
by Hy Laboratories Ltd.
Park Tamar, Rehovot, 76326, Israel
Tel : 972-8-9366475
Fax: 972-8-9366474

Customer Service: info@prontodiagnostics.com

Authorized EU representative:
MedNet GmbH,
Borkstrasse 10, 48163 Muenster,
Germany
Tel: +49 251 32266-0
Fax: +49 251 32266-22

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