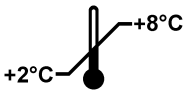
PRONTO® ClopidoRisk™ Kit

For the detection of the following variant alleles:

Cytochrome P450 2C19 Gene	***2**	**c.681G>A**
	***3**	**c.636G>C**
	***4**	**c.1A>G**
	***5**	**c.1297C>T**
ABCB1 Gene		**c.3435C>T**

Instructions for Use

REF **9985**



PRONTO DIAGNOSTICS

CE IVD

INTENDED USE

The Pronto® ClopidoRisk kit is a single nucleotide primer extension ELISA procedure, intended for the qualitative *in vitro* detection of the following four variant alleles in the CYP2C19 gene: CYP2C19*2 (681G>A), CYP2C19*3 (636G>A), CYP2C19*4 (1A>G), CYP2C19*5 (1297C>T) and one variant in ABCB1 gene (3435C>T) in amplified human DNA.
For *in vitro* diagnostic use.

BACKGROUND

Clopidogrel is an oral antiplatelet agent that inhibits blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It is a prodrug that requires biotransformation to an active metabolite by cytochrome P-450 (CYP) enzymes found in the liver, including CYP2C19. The gene encoding CYP2C19 enzyme is polymorphic, with common alleles conferring reduced function. Researchers have found that patients carrying any of the CYP2C19 alleles associated with loss of function (*2, *3, *4, or *5) have lower levels of the active metabolite of Clopidogrel, less inhibition of platelets, and a 3.58 times greater risk for major adverse cardiovascular events such as death, heart attack, and stroke. The risk was greatest in CYP2C19 poor metabolizers. ABCB1 gene is responsible for modulating clopidogrel absorption. Carriage of the ABCB1 T3435 variant was also associated with a modestly increased risk of an outcome event compared to wild-type ABCB1.

REFERENCES

1. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, Walker JR, Antman EM, Macias W, Braunwald E, Sabatine MS. Cytochrome P-450 Polymorphisms and Response to Clopidogrel. N. Engl. J. Med. 360(4):354-62 (2009).
2. Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Méneveau N, Steg PG, Ferrières J, Danchin N, Becquemont L; French Registry of Acute ST-Elevation and Non-ST-Elevation Myocardial Infarction (FAST-

MI) Investigators. Genetic determinants of response to clopidogrel and cardiovascular events. N. Engl. J. Med. 360(4):363-75 (2009).
3. Shuldiner, Alan R & O'Connell, Jeffrey R. et al. Association of Cytochrome P4502C19 Genotype with the Antiplatelet effect and Clinical Efficacy of Clopidogrel Therapy. JAMA, 2009; 302(8): 849-857.

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.
- 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 procedure.

1 TARGET DNA AMPLIFICATION: The DNA fragments that encompass the tested variants 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 polymorphic 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 polymorphism: the first well of each pair tests for the presence of one allele (i.e., the rare allele - well A) while the second well tests for the presence of the other allele (e.g., the normal or common allele - well B). The biotinylated nucleotide is added to the primer in the course of the reaction - or not added, depending on the genotype of the tested individual.

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.

DISCLAIMER

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.

CONTENTS OF THE KIT

ClopidoRisk Amplification Mix	1 vial	(0.5 mL)
ProntoTaq™	1 vial	(20 µL)
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)
PRONTO® ClopidoRisk Plates	3 individually pouched plates	
Detection Plates	3 Streptavidin-coated ELISA plates	

STORAGE AND STABILITY

- Store the ProntoTaq™ at -20°C.
- Keep the kit 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 & 200-1,000 µL)
- Filtered tips
- ELISA reader with 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 100 ng/µL) to a Thermowell plate 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 ProntoTaq™ 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 ClopidoRisk	13.0 µL
ProntoTaq™	0.3 µL

3. **Dispense** 13 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 recommended to use oil.
5. **Place** the Thermowell plate or tube in a thermocycler previously programmed with the following protocol:

Cycling protocol

1.	95ºC	2 minutes	
2.	95ºC	15 seconds	35 cycles
3.	60ºC	30 seconds	
4.	72ºC	40 seconds	
5.	72ºC	2 minutes	

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

Sizes of amplified fragments:

Gene	Allele variant	Fragment size
CYP2C19	*2 (681G>A)	220
	*3 (636G>A)	459
	*4 (1A>G)	418
	*5 (1297C>T)	353
ABCB1	(3435C>T)	113

Limitation of the test:
Different thermocyclers may influence the amplification yield dramatically. It is recommended to use 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	93.0 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** 100 µL of the post-amplification mix into each Thermowell or tube containing 10 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:

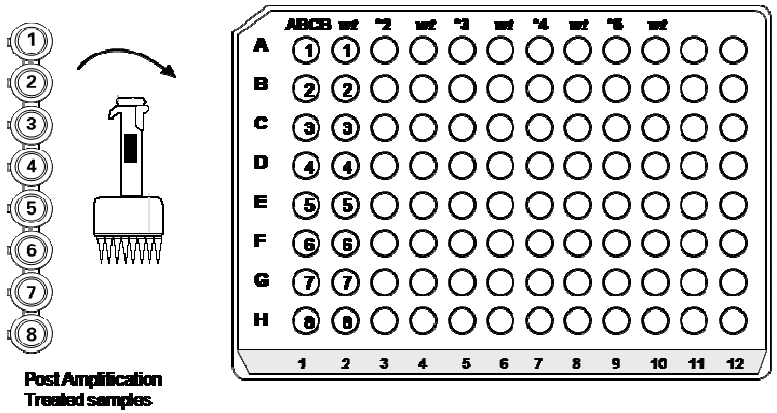
Cycle		Temperature	Time
20 cycles:	{	95ºC	30 sec.
		60ºC	30 sec.
End:		18-25º C - Cool down to room temperature	

2 **Take** a PRONTO® Plate out of its pouch. Notice the color at the bottom of the wells.
For each polymorphic site tested, use a pink well (well A) and a blue well (well B). 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® ClopidoRisk 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 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, or store the reaction products in the refrigerator and carry out the visualization steps within 24 hours.

4 ELISA ASSAY - COLOR DEVELOPMENT

The ELISA 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 measuring the absorbance using an ELISA reader at a wavelength of 620 nm.

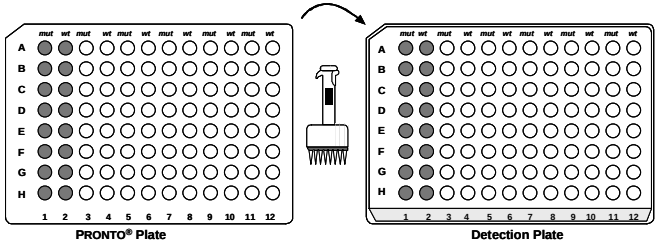
PREPARATION

- 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).

DETECTION BY ELISA

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).

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 10-15 minutes at RT (18-25°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 polymorphic site tested, at least one of the wells should develop a deep blue color. Otherwise, the results are invalid for the relevant polymorphism (see Fig. 4).

For Colorimetric Detection:
For every polymorphic site tested, at least one of the two wells should produce an O.D. ≥ 0.50 reading.

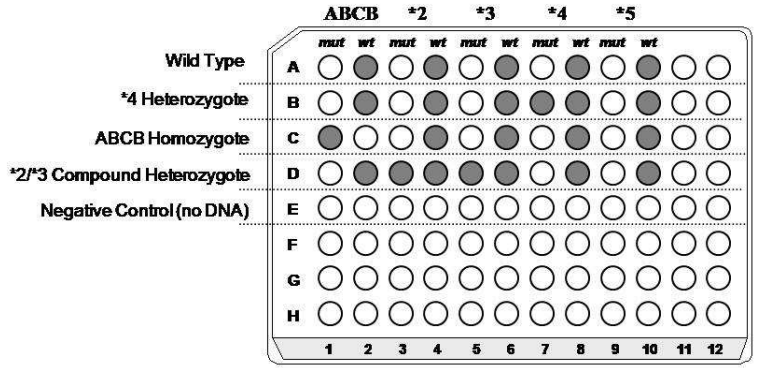
INTERPRETATION OF RESULTS

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. 3).

Figure 3: **Examples of genotype assignment according to visual inspection of test results**



Criteria for Colorimetric Interpretation (O.D. 620)

For each sample, the genotype in each polymorphic site is determined according to two criteria (see example in Fig. 5):

1. The O.D. values of each of the wells
2. The ratio between the O.D. values of those two wells.

For each polymorphic site tested, calculate the ratio (*A/B)* between the two wells by dividing the O.D. of well *A* by the O.D. of well *B*.
Identify the correct genotype according to the following table:

Genotype	Well A (O.D. 620)	Well B (O.D. 620)	A/B ratio
Normal	O.D. $\leq$ 0.35	O.D. $\geq$ 0.5	ratio ≤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 ≥ 2.0

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

PRONTO® ClopidoRisk PROCEDURE SUMMARY

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

DNA AMPLIFICATION:

Volumes per reaction: 2 µL template DNA **+** 13 µL Amplification Mix + 0.3 µL ProntoTaq™

Cycling protocol:

95°C 2 min→35 cycles of {95 °C 15 sec. / 60 °C 30 sec. / 72 °C 40 sec.} →72 °C 2 min.

POST-AMPLIFICATION PROCEDURE:

- Volumes per reaction:

PRONTO® Buffer 2	93.0 µL
Solution C	4.0 µL
Solution D	3.0 µL

- **Pipette** in and out to mix.
- **Add** 100 µL into each well containing 10 µL amplified product, mix well.
- **Add** 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 ten wells of the PRONTO® Plate.
- **Add** one drop of ColoRed™ oil.
- **Start** the cycling protocol:
- 20 cycles of {95 °C 30 sec. / 60 °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 10-15 minutes.

For a 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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