**INTENDED USE**

The PRONTO® kit is a single nucleotide primer extension ELISA Assay, intended for the qualitative *in vitro* detection of one of the following three mutations: Factor V Leiden G1691A (R506Q), Prothrombin G20210A and Methylene-tetrahydrofolate Reductase (MTHFR) C677T, in amplified human DNA.

*For in vitro diagnostic use*

**BACKGROUND**

**Factor V**

Activated Protein C (APC) limits clot formation by proteolitic inactivation of Factor V. The G1691A mutation in the Factor V gene results in a protein that is resistant to inactivation by APC. The mutation is present in a frequency of about 4% to 10% in Western populations. It is the most common inherited abnormality in patients with thromboembolic disease, occurring in 20% of patients with a first episode of venous thrombosis and in up to 50% of those with recurrent venous thrombosis.

**Prothrombin**

Prothrombin is the precursor of the serine protease thrombin, a key enzyme in the processes of hemostasis and thrombosis. It exhibits procoagulant, anticoagulant and antifibrinolytic activities. Prothrombin is encoded by a 21 Kb-long gene (localized to chromosome 11). A mutation in the 3' untranslated region of the Prothrombin gene (G20210A) is present in 2% of the population and is associated with elevated plasma Prothrombin concentrations. Increased Prothrombin activity was associated with an increased risk of thrombosis. While Factor V Leiden increases the risk of thrombosis 3-8 fold, Prothrombin G20210A appears to be a slightly milder risk factor. They are both found in a large number of patients with venous thrombosis (10% - 20%) and therefore their presence explains a large proportion – up to 25% - of all thrombotic events in the population. Also, the G20210A Prothrombin gene mutation and Factor V Leiden individually are associated with an increased risk of venous thromboembolism which is a leading cause of morbidity and mortality during pregnancy. The risk among women with both mutations is disproportionately higher than that among women with only one mutation.
MTHFR

Hyperhomocysteinaemia has been identified as a risk factor for cerebrovascular, peripheral vascular and coronary heart disease. Elevated levels of plasma homocysteine can result from genetic or nutrient related disturbances in the pathways for homocysteine metabolism. The C677T mutation in the MTHFR gene causes severe MTHFR deficiency leading to elevated levels of plasma homocysteine. This mutation is the most common inborn error of folate metabolism.

REFERENCES

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.
- 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: Results are determined either visually (substrate remains clear or turns blue), or colorimetrically (substrate 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.
CONTENTS OF THE KIT

Thromborisk Amplification Mix...........2 vials (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)
HRP Conjugate ............................... 1 vial (450 µL)
TMB Substrate ................................. 1 bottle (40 mL)
Stop Solution (1M H2SO4 ) ................. 1 bottle (30 mL)
PRONTO® Plates* .............................. 2 individually pouch plates
Detection Plates ............................. 2 Streptavidin-coated ELISA plates
* (Factor V or Prothrombin or MTHFR)

STORAGE AND STABILITY

- Store at 2-8°C. 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 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 Taq DNA Polymerase to the amplification mix shortly before dispensing the mix. Mix gently by pipetting.

<table>
<thead>
<tr>
<th>PCR Master mix</th>
<th>Volume for one sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification mix ThromboRisk™</td>
<td>14.0 µL</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5 u/µL)</td>
<td>0.2 µL</td>
</tr>
</tbody>
</table>

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

- PHARMACIA
- SIGMA
- ROCHE
- PROMEGA
- BIOLINE
- PERKIN ELMER

3. **Dispense** 14.0 µL of the 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 essential to use oil.
5. **Place** the thermoplate well or tube in a thermocycler previously programmed with the following protocol:

<table>
<thead>
<tr>
<th>Cycling protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 94ºC 5 minutes</td>
</tr>
<tr>
<td>2. 94ºC 30 seconds</td>
</tr>
<tr>
<td>3. 66ºC 20 seconds</td>
</tr>
<tr>
<td>4. 72ºC 20 seconds</td>
</tr>
<tr>
<td>5. 72ºC 2 minutes</td>
</tr>
<tr>
<td>6. 58ºC 30 seconds</td>
</tr>
<tr>
<td>7. 72ºC 30 seconds</td>
</tr>
<tr>
<td>8. 72ºC 30 seconds</td>
</tr>
<tr>
<td>9. 72ºC 5 minutes</td>
</tr>
</tbody>
</table>

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

**Sizes of amplified fragments:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td>677C&gt;T</td>
<td>200 bp</td>
</tr>
<tr>
<td>Factor V</td>
<td>1691G&gt;A</td>
<td>220 bp</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>20210G&gt;A</td>
<td>420 bp</td>
</tr>
</tbody>
</table>

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.

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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.

<table>
<thead>
<tr>
<th>Post-Amplification Mix</th>
<th>Solution Mix</th>
<th>Volume for one sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRONTO® Buffer 2</td>
<td>20.0 µL</td>
<td></td>
</tr>
<tr>
<td>Solution C</td>
<td>1.0 µL</td>
<td></td>
</tr>
<tr>
<td>Solution D</td>
<td>0.7 µL</td>
<td></td>
</tr>
</tbody>
</table>

2. **Mix** gently by pipetting this solution in and out five times. Do not vortex.
3. **Add** 21 µL of the post-amplification mix into each well or tube containing 5 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 37°C, then for 10 minutes at 95°C in a thermocycler.

   **If not used immediately, the treated sample can be kept at 2-8°C for a maximum of four hours.**
3 PRIMER EXTENSION REACTION

1 Program the thermocycler as follows:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start:</td>
<td>94º C</td>
<td>15 sec</td>
</tr>
<tr>
<td>20 cycles:</td>
<td>94º C</td>
<td>30 sec</td>
</tr>
<tr>
<td>52º C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>End:</td>
<td>18-25º C - Cool down to room temperature</td>
<td></td>
</tr>
</tbody>
</table>

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 each one of 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: Scheme for dispensing Post-Amplification Treated DNA samples into the PRONTO® 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 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:

▪ 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.
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.

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 18-25º 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 2).
TRANSFER TO THE DETECTION PLATE

1. Fill a reagent reservoir/through 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. 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 (18-25°C).

DETECTION BY ELISA

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Visual Detection (Blue color)</th>
<th>Colorimetric Detection (Yellow color)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. While the incubation with Assay Solution is taking place, dilute the Conjugated HRP in Assay Solution. For every detection plate used (96 wells), about 11 mL of diluted conjugate is required. <strong>This solution should be freshly prepared each time the test is run.</strong></td>
<td>Dilution: 1:100 (110 µL of conjugated HRP into 11 mL Assay Solution)</td>
<td>Dilution: 1:200 (55 µL of conjugated HRP into 11 mL Assay Solution)</td>
</tr>
<tr>
<td>7. Empty the plate and wash four times with 350 µL 1x Wash Solution. Ensure that the plate is relatively dry after the last wash step.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>8. Add 100 µL of freshly diluted conjugated HRP to all the wells, with a multichannel pipette.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>9. Incubate at room temperature.</td>
<td>10 minutes</td>
<td>10 minutes</td>
</tr>
<tr>
<td>10. Wash the plate as in step 7.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>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</td>
<td>30 minutes</td>
<td>15 minutes</td>
</tr>
<tr>
<td>12. Add 100 µL of Stop Solution to each well with a multichannel pipette.</td>
<td></td>
<td>100 µL</td>
</tr>
<tr>
<td>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).</td>
<td>Agitate the plate gently and read results at O.D. 620 nm</td>
<td></td>
</tr>
<tr>
<td>14. Within two hours read the absorbance using an ELISA reader (single wavelength setting)</td>
<td></td>
<td>O.D. 450 nm</td>
</tr>
</tbody>
</table>
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, the 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. ≥ 0.50 reading.

Figure 3: Visual Interpretation of Genotypes

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 4).

Figure 4: Genotype assignment according to visual inspection of test results

Criteria for Colorimetric Interpretation
The genotype of each sample is determined according to two criteria:
1. The O.D. values of the mut and wt wells.
2. 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 using the table below:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>mut well (O.D. 450)</th>
<th>wt well (O.D. 450)</th>
<th>mut/wt ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>O.D. &lt; 0.35</td>
<td>O.D. &gt; 0.5</td>
<td>ratio &lt; 0.5</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>O.D. &gt; 0.5</td>
<td>O.D. &gt; 0.5</td>
<td>0.5 &lt; ratio &gt; 2.0</td>
</tr>
<tr>
<td>Homozygote</td>
<td>O.D. &gt; 0.5</td>
<td>O.D. &lt; 0.35</td>
<td>ratio &gt;2.0</td>
</tr>
</tbody>
</table>

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

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

DNA AMPLIFICATION:
Volumes per reaction: 2 µL Template DNA + 14 µL Amplification Mix + 0.2 µL Taq Polymerase.
Cycling protocol:
94°C 5 min — 15 cycles of {94°C 30 sec. / 66°C 30 sec. / 72°C 30 sec.} — 72°C 2 min.
— 25 cycles of {94°C 30 sec. / 58°C 30 sec. / 72°C 30 sec.} — 72°C 5 min

POST-AMPLIFICATION PROCEDURE:
▪ Volumes per reaction: PRONTO® Buffer 2 20.0 µL
  Solution C 1.0 µL
  Solution D 0.7 µL
▪ Pipette in and out to mix.
▪ Add 21 µL into each well containing 5 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 two wells of the PRONTO® Plate.
▪ Add one drop of ColoRed™ oil.
▪ Start the cycling protocol: 94°C 15 sec — 20 cycles of (94°C 30 sec. / 52°C 10 sec.) — Cool.
  Insert the PRONTO® Plate in the thermocycler when the temperature 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 identical position in the Detection Plate. Incubate 10 minutes at RT.
▪ Empty the wells and wash four times with 350 µL of 1x Wash Solution.

<table>
<thead>
<tr>
<th></th>
<th>Visual Detection</th>
<th>Colorimetric Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add 100 µL of Conjugated HRP to every well and incubate for 10 minutes at RT.</td>
<td>Dilution 1:100</td>
<td>Dilution 1:200</td>
</tr>
<tr>
<td>Empty the wells and wash four times with 350 µL of 1x Wash Solution.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Add 100 µL of TMB Substrate to each well and Incubate at RT for:</td>
<td>30 minutes</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Add Stop Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Read O.D. at:</td>
<td>620 nm</td>
<td>450 nm</td>
</tr>
</tbody>
</table>

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

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

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