PRONTO® 5FU-Risk kit

For the detection of the following variant allele in the Dihydropyrimidine dehydrogenase DPD gene :

IVS14+1 (DPD*2A) G/A

Instructions for Use REF 9984

+2°C +8°C

48 Tests

PRONTO DIAGNOSTICS

CE IVD

INTENDED USE

The PRONTO® 5FU-Risk kit is a single nucleotide primer extension ELISA procedure, intended for the qualitative *in vitro* detection of the following mutation in amplified human DNA: IVS14, G/A, +1 in the DPD (Dihydropyrimidine dehydrogenase) gene.

For *in vitro* diagnostic use.

BACKGROUND

Pharmocogenetics has allowed clinicians to identify associations between an individual's genetic profile and his/her response to drugs. 5-FU, a commonly used chemotherapeutic agent, is a pyrimidine analogue, which is metabolized by the pyrimidine metabolic pathways . It exerts cytotoxic effects on tumor cells via its metabolites. The 5-FU metabolite 5–5-fluoro-dUMP inhibits thymidylate synthase thus impairing DNA synthesis. Additional cytotoxic mechanisms result from 5-FU metabolites being incorporated into RNA and DNA . The amount of 5-FU available for anabolic reactions and subsequent cytotoxicity is determined by its catabolism. Less than 10% of administered 5-FU undergoes renal excretion, whereas 80% is cleared by catabolic degradation. The rate-limiting initial step of 5-FU catabolism is catalyzed by DPD, and deficiency in DPD enzyme activity is correlated with a delay in the clearance of 5-FU from the plasma. Accordingly, toxic side effects of 5-FU have been linked to low levels of DPD enzyme activity in human blood cells. Approximately one-fourth of patients who experience severe 5-FU-related toxicity carry the exon 14-skipping mutation, with the majority of them being heterozygous. These patients are at high risk of experiencing severe to life-threatening toxicity upon 5-FU treatment. On the basis of a prevalence of nearly 1% within the Caucasian population, it therefore appears that routine genetic screening for this common splice-site mutation should be carried out before 5-FU chemotherapy.
However, it should be pointed out that not everybody who is heterozygous for the mutation may actually suffer severe 5-FU side effects. The individual risk of heterozygous patients suffering from severe or life-threatening 5-FU-related toxicity can only be addressed by pharmacokinetic measurements of the

individual 5-FU turnover in heterozygous patients using very low doses of 5-FU. Thus, routine screening for the exon 14-skipping mutation and subsequent individual determination of the 5-FU pharmacokinetics of heterozygous patients provides a concept of individualized therapy and allows the avoidance of undesired treatment toxicity. A high prevalence of the IVS14+1 G/A mutation has been observed in the normal population with a frequency of heterozygotes of 1.8%. Furthermore, the IVS14+1 G/A mutation proved to be the most common mutation present among cancer patients with a partial DPD deficiency; it was detected in 50% of patients suffering from grade IV neutropenia. The important role of DPD in the etiology of 5-FU toxicity, therefore, warrants the analysis of DPD activity and genetic screening for DPD mutations in cancer patients prior to the administration of 5-FU.

REFERENCES

1. *Raida* et al*., Clin. Cancer Res. 2001. Sep; Vol.7: 2832–2839.*
2. *van Kuilenburg* et al*., Clin. Cancer Res. 2001. Sep; Vol.7: 1149–1153.*
3. *van Kuilenburg* et al*., Annals of Oncology 2003. Vol.14 -2: 341–342.*

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 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.

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 5FU-Risk™	1 vial (clear cap)	(1.2 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)
5FU-Risk PRONTO® Plates	3 individually pouched plates	
Detection Plates	3 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 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 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 5FU-Risk™	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.	59ºC	30 seconds	
4.	72ºC	20 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	Variant	Fragment size
Dihydropyrimidine dehydrogenase DPD	DPD*2A G/A	294 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

! Only 5 µL of each amplified DNA sample will be used to carry out this assay

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	24.0 µL
Solution C	1.0 µL
Solution D	0.75 µL

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

3 Add 25 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 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:

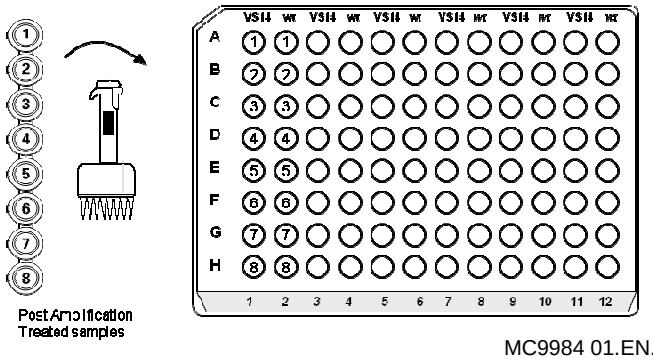
Cycle	Temperature	Time
20 cycles:	94ºC	30 sec.
	62ºC	20 sec.
End:	18-25º C - Cool down to room temperature	

2 **Take** a PRONTO® Plate out of its pouch. Notice the color at the bottom of the wells. For each 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: Scheme for dispensing Post Amplification Treated DNA samples into the PRONTO® 5FU-Risk 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).

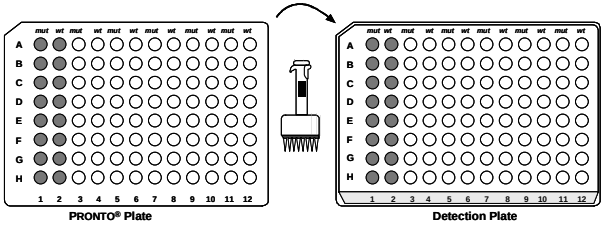
DETECTION BY ELISA

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 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 (singe 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, 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.

INTERPRETATION OF RESULTS

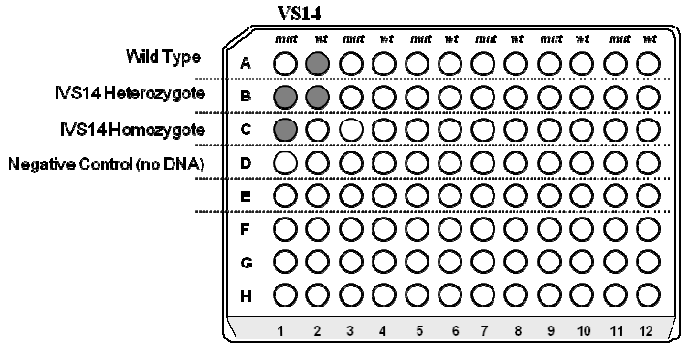
Important: Heterozygous or homozygous mutant results should be confirmed by retesting. It is recommended to repeat the test with newly extracted DNA.

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)

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:

Genotype	*mut* well	*wt* well	mut/wt 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® 5FU-Risk 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. / 59 °C 30 sec. / 72 °C 20 sec.} →72 °C 2 min.

POST-AMPLIFICATION PROCEDURE:

- Volumes per reaction:

PRONTO® Buffer 2	24.0 µL
Solution C	1.0 µL
Solution D	0.75 µL

- **Pipette** in and out to mix.
- **Add** 25 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:
- 20 cycles of {94 °C 30 sec. / 62 °C 20 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 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

Produced by Pronto Diagnostics Ltd.
Kiryat Atidim
Building 3, 3rd floor
POB 58129
Tel Aviv 6158002, Israel
Tel: +972.73.2126155
Fax: +972.73.2126144

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

M9984 01.EN.01