PRONTO® MNUG

For the detection of the following mutations:

Gene	Mutation	Disease
BCKDHB	**R183P**	**Maple Syrup Urine Disease 1b**
NEB	**2,502-bp del**	**Nemaline Myopathy 2**
PCDH15	**R245X**	**Usher Syndrome Type 1f**
G6PC	**R83C**	**Glycogen Storage Type 1a**

REF **9975**

Instructions for Use

Σ **36 or 72**

PRONTO DIAGNOSTICS

+2°C +8°C CE IVD

INTENDED USE

The PRONTO® MNUG kit consists of a single nucleotide primer extension ELISA assay intended for the qualitative *in vitro* detection of the following four mutations from amplified human DNA: the R183P (538G>C) mutation in the BCKDHB gene; the 2,502 bp deletion mutation (R2478_D2521del) in the Nebulin (NEB) gene; the R245X mutation in the PCDH15 gene; and the R83C mutation in the G6PC gene.

Three formats are available :

A. The **MNUG** format (**M**SUD**, N**emaline, **U**sher and **G**lycogen Storage Disease) - for simultaneous detection of four mutations - *36 tests per kit.*
B. The **MN** format (**M**SUD and **N**emaline) - for simultaneous detection of two mutations - *72 tests per kit.*
B. The **UG** format (**U**sher and **G**lycogen Storage Disease) - for simultaneous detection of two mutations - *72 tests per kit.*

For *in vitro* diagnostic use.

BACKGROUND

Maple Syrup Urine Disease (MSUD) type Ib in Ashkenazi Jews is caused by the 538G>C (R183P) mutation in the E1-beta sub-unit of the BCKDHB gene. The major clinical features of MSUD are mental and physical retardation, feeding problems, and a maple syrup odor to the urine. The carrier frequency of the mutant allele was found to be approximately 1 in 113.

Nemaline Myopathy (NEM2) is a neuromuscular disorder in the Ashkenazi Jewish population that is caused by a 2,502-bp deletion in the Nebulin gene (NEB), which includes exon 55 and parts of introns 54 and 55. The absence of this exon results in the generation of a transcript that encodes 35 fewer amino acids. An analysis of the gene frequency of this mutation in a random sample of 4,090 Ashkenazi Jewish individuals has revealed a carrier frequency of 1/108.

Usher Syndrome is an autosomal recessive disorder characterized by bilateral sensorineural deafness and progressive loss of vision due to retinitis pigmentosa. It is the most frequent cause of deafness and concurrent blindness with a prevalence of 1 in 16,000 to 1 in 50,000. The most severe clinical subtype of Usher is type 1f which involves deafness at birth,

progressive blindness and balance problems, due to mutations in the PCDH15 gene. The R245X mutation accounts for a large proportion of cases of the type 1 Usher syndrome in Ashkenazi Jews, with a carrier rate as high as 1/40.

Glycogen Storage Disease Type 1a (Glycogenosis, GSD1a, von Gierke Disease) is caused by mutations in the G6-Pase gene, which affect the activity of glucose-6-phosphatase (G6Pase), a key enzyme in glucose homeostasis. Patients afflicted with GSD1a are unable to maintain this homeostasis. R83C is the most common mutation among Caucasians. It was found in 93% of Ashkenazi Jewish GSD1a patients, 71% of Mediterranean patients, 60% of Turkish patients, 48% of patients from South Europe and 28% of Hispanic patients. GSD1a has a disease rate of one in 100,000 births.

REFERENCES

- Edelmann et al. Maple syrup urine disease: identification and carrier-frequency determination of a novel founder mutation in the Ashkenazi Jewish population. *Am. J. Hum. Genet.*, 69: 863-868 (2001)
- Anderson et al. Nemaline myopathy in the Ashkenazi Jewish population is caused by a deletion in the nebulin gene*. Hum. Genet*., 115: 185-190 (2004)
- Ben-Yosef et al. A mutation of PCDH15 among Ashkenazi Jews with the type 1 Usher syndrome. *N. Engl. J. Med.*, 48:1664-70 (2003)
- Lei et al. Genetic basis of glycogen storage disease type 1a: prevalent mutations at the glucose-6-phosphatase locus. *Am. J. Hum. Genet.*, 57(4): 766-71 (1995)
- Parvari et al. Glycogen storage disease type 1a in Israel: biochemical, clinical, and mutational studies. *Am. J. Med. Genet*., 72: 286-290 (1997)

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.

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

PRONTO® Buffer 2	2 bottles	(3 mL)
Solution C	2 vials (yellow cap)	(130 µL)
Solution D	2 vials (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® Plates	3 individually pouched plates	
Detection Plates	3 Streptavidin-coated ELISA plates	
Amplification Mix MNUG	1 vial	(1.3mL)

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

- 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 620 nm filter (optional)
- 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 DNA AMPLIFICATION

1. **Dispense** 2 µL 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 MNUG	13.0 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
- PERKIN ELMER Cat. No. M801-0060
- BIO LABS Cat. No. M2676
- PEQLAB Cat. No. 01-1020

3. **Dispense** 13 µL Master Mix to each sample.
4. **Add** one drop of ColoRed™ oil to each 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 use oil.
5. **Place** the thermoplate well or tube in a thermocycler that was programmed with the following protocol:

Cycling protocol			
1.	94ºC	5 minutes	
2.	94ºC	30 seconds	35 cycles
3.	52ºC	30 seconds	
4.	72ºC	60 seconds	
5.	72ºC	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	Mutation	Fragment size
BCKDHB	R183P	169 bp
NEB	2,502-bp del	114 bp (312 bp)*
PCDH15	R245X	243 bp
G6PC	R83C	379 bp

* The fifth fragment (312 bp) appears only in the presence of the NEB 2,502 bp deletion.

2 POST-AMPLIFICATION TREATMENT

> **! Only 10 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.

Volumes for the Post-Amplification Treatment

Component	MNUG format 4 mutations	MN / UG format 2 mutations
Amplified DNA	10.0 µL	10.0 µL
PRONTO® Buffer 2	90.0 µL	45.0 µL
Solution C	4.0 µL	2.0 µL
Solution D	3.0 µL	1.5 µL
Total Volume	97.0 µL	48.5 µL

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

4 **Add** the total volume of the post-amplification mix into each well or tube containing 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 four hours.

3 PRIMER EXTENSION REACTION

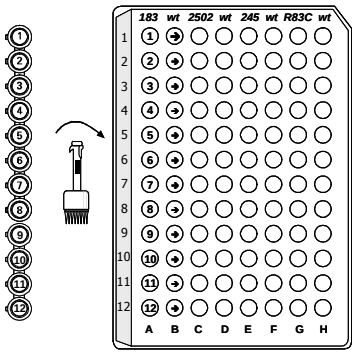
1 **Program** the thermocycler as follows:

Cycle		Temperature	Time
		94ºC	15 sec.
20 cycles:	{	94ºC	10 sec.
		60ºC	30 sec.
End:		18-25ºC - 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 **Primer extension** can be carried out in one of two ways:
MNUG Format using the PRONTO® MNUG plate for simultaneous detection of four mutations.
Starting from the first sample, dispense 8 µl post-amplification treated DNA into each one of the eight wells in row 1 as shown in Fig. 1. Continue with the remaining samples. Using a multichannel pipette it is possible to transfer up to 12 samples simultaneously.
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.

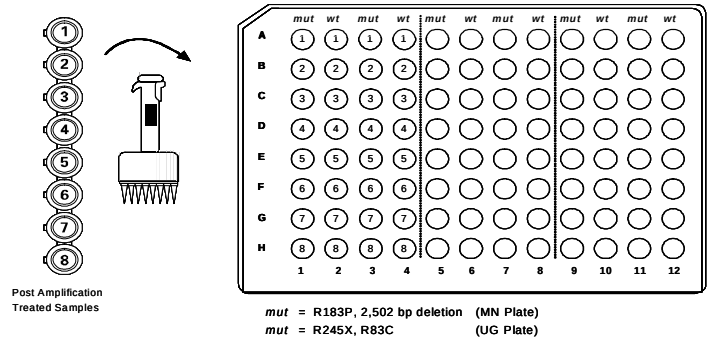
Figure 1 : Scheme for dispensing Post-Amplification Treated DNA samples into the PRONTO® MNUG Plate



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.

MN / UG Format using the PRONTO® MN / UG plates for simultaneous detection of two mutations.
Starting from the first sample, dispense 8 µl Post Amplification treated DNA into each one of the first four wells in row A as shown in Fig. 2. Continue with the second sample to dispense in the next four wells in row B and so forth. Continue with the remaining samples. Using a multichannel pipette it is possible to transfer up to 8 samples simultaneously.
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 2 : Scheme for dispensing Post Amplification Treated DNA samples into the PRONTO® MN / UG 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:
- **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.

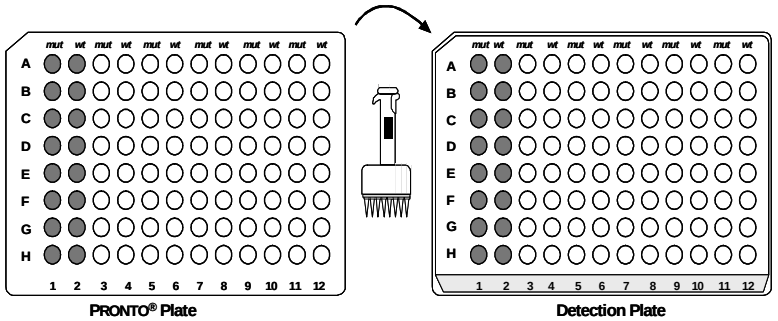
PREPARATION

- 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.
  The 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. 3).

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 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. 3).
Ensure that the solution at the bottom of all wells of the PRONTO® plate has turned green by inspecting them from below.

Figure 3 : 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 RESULTS

For Visual Detection:
For every sample tested, at least one of the two wells should develop a deep blue color. Otherwise, the results are invalid. Results should be interpreted as depicted in Fig. 4.

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

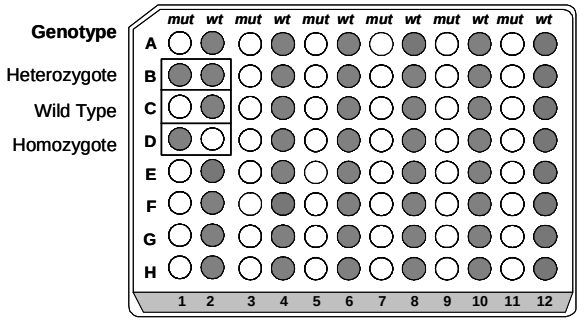
INTERPRETATION OF RESULTS

Important: Positive 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 below)

Figure 4: 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:
- 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. ≤ 0.35	O.D. ≥ 0.5	ratio < 0.5
Heterozygote	O.D. ≥ 0.5	O.D. ≥ 0.5	0.5 < ratio < 2.0
Homozygote	O.D. ≥ 0.5	O.D. ≤ 0.35	ratio > 2.0

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

PRONTO® MNUG - 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 + 0.5 µL Taq Polymerase.
Cycling protocol:
94°C 5 min→35 cycles of {94°C 30 sec. / 52°C 30 sec./ 72°C 60 sec.} →72°C 5 min.

POST-AMPLIFICATION PROCEDURE:

- Volumes per reaction:

	MNUG format	MN / UG Format
Amplified DNA	10.0 µL	10.0 µL
PRONTO® Buffer 2	90.0 µL	45.0 µL
Solution C	4.0 µL	2.0 µL
Solution D	3.0 µL	1.5 µL
Total volume	97.0 µL	48.5 µL

- **Pipette** in and out to mix.
- **Add** the total volume into each well containing amplified DNA, mix well.
- **Add** one drop of ColoRed™ oil.
- **Incubate** 30 minutes at 37ºC, then 10 minutes at 95ºC.

PRIMER EXTENSION REACTION:

- **Dispense** 8 µL of each Post-Amplification treated DNA into eight / four wells of the PRONTO® plate.
- **Add** one drop of ColoRed™ oil.
- **Start** the cycling protocol:
  94ºC 15 sec→20 cycles of {94ºC 10 sec. / 60ºC 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

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

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