PRONTO® Gaucher Screen

For the detection of the 84GG, IVS2+1, N370S, L444P, V394L and R496H mutations

REF **9900-1**

Instructions for Use

24 **Tests**

PRONTO DIAGNOSTICS

+2°C +8°C

CE IVD

INTENDED USE

The PRONTO® Gaucher Screen kit is a single nucleotide primer extension ELISA test intended for the qualitative *in vitro* detection of the following six mutations: 84GG, IVS2+1, N370S (1226G), L444P (1448C), V394L (1297T) and R496H (1604A) in amplified human DNA.

For *In vitro Diagnostics* use

BACKGROUND

Gaucher disease is a lysosomal storage disorder. It is inherited in an autosomal recessive manner and results in deficiency in the lysosomal enzyme glucocerebrosidase. Due to the accumulation of the glucocerebroside substrate, the liver and the spleen may become severely enlarged and the bones may be subject to fractures. Central nervous system involvement is seen in rare cases of the disease (Gaucher Type II and Type III). The most common variant of the disease is Type I which has an incidence rate of 1 in 40,000 and carrier frequency of 1 in 100. In the Ashkenazi Jewish population the carrier rate is 1 in 10 and about 1 in 450 persons is affected.

REFERENCES

1. Human Mutation 12:240-244 (1998).

WARNINGS AND PRECAUTIONS

- Reagents supplied within this kit contain up to 0.1% sodium azide that is very 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 sink and plumbing with large quantities of water.

- TMB Substrate solution is an irritant material to 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 pre-defined polymorphisms in DNA sequences, using a single nucleotide primer-extension 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**: 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

Gaucher Amplification Mix	1 x vial	(0.95mL)
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	1 x vial	(450 µL)
TMB - Substrate	1 x bottle	(40 mL)
Detection Plates	3 x Streptavidin-coated plates	
PRONTO® Gaucher Screen	3 x individually pouched plates	

*370Rec Allele Identifier package……supplied upon request

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)
- 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 & 200-1000 μL)
- Filtered tips
- ELISA reader with a 620 nm filter
- Polaroid camera and color film to record results (optional)
- Automated microtiter plate washer or a wash 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 thermoplate well or tube.
2. **Prepare** a Master Mix in a sterile vial, according to the volume indicated in the table below, plus one spare reaction volume. Add the

Taq DNA polymerase 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 Gaucher	22.5 µL
Taq DNA Polymerase * (5 u/µL)	0.5 µL

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

- PHARMACIA Cat. # 27-0799
- SIGMA Cat. #D-1806
- ROCHE Cat. # 1-146-165
- PROMEGA Cat. # M-1661
- BIOLINE Cat. # M95801B
- PERKIN ELMER Cat. # M801-0060

3. **Dispense** 23 µL Master mix to each 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:

1.	94º C	2 minutes	
2.	94º C	30 seconds	35 cycles
3.	60º C	30 seconds	
4.	72º C	45 seconds	
5.	72º C	5 minutes	

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

Sizes of amplified fragments

Mutation	Fragment size
84GG, IVS 2+1	350 bp
N370S, D409H*, L444P, V394L (*R496H in Gaucher Screen™)	1250 bp

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.

2 POST-AMPLIFICATION TREATMENT

! Only 20 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	100.0 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. **Dispense** 107 µL of the post-amplification mix into each thermowell plate well or tube containing 20 µL of the amplified DNA.
   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 4 hours.

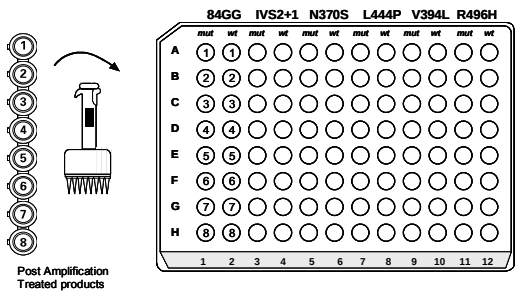
3 PRIMER EXTENSION REACTION

1. Program the thermocycler as follows:

Cycle		Temperature	Time
Start:		94ºC	15 sec.
20 cycles:	{	94ºC	30 sec.
	{	60ºC	10 sec.
End:		18ºC -25ºC (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 six wells in row A (Fig. 1). Continue with the remaining samples. It is possible to transfer up to eight samples simultaneously using a multichannel pipette.

Figure 1: Scheme for Dispensing Post-Amplification Treated DNA Samples into the PRONTO® Gaucher 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:

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.

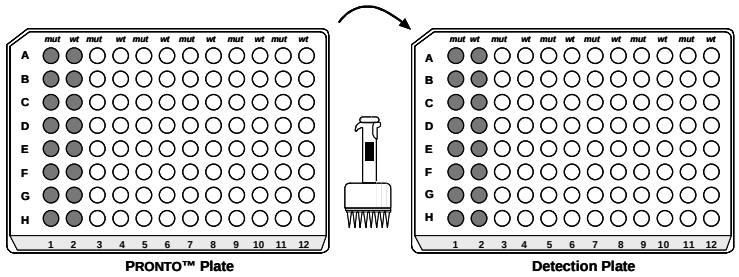
PREPARATIONS

- 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 18-25oC 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 (Fig. 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. 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 (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 10-30 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 (singe wavelength setting).

VALIDATION OF THE RESULTS

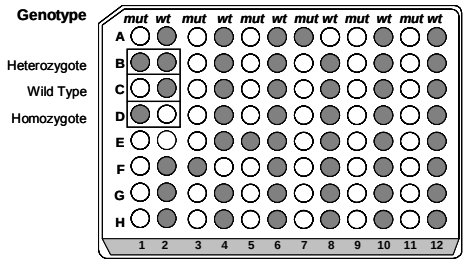
For Visual Detection:

For every mutation site tested, at least one of the wells should develop a deep **blue** color. Otherwise, results are invalid for the relevant mutation (Fig. 3).

For Colorimetric Detection:

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

Figure 3: Visual Interpretation of Genotypes



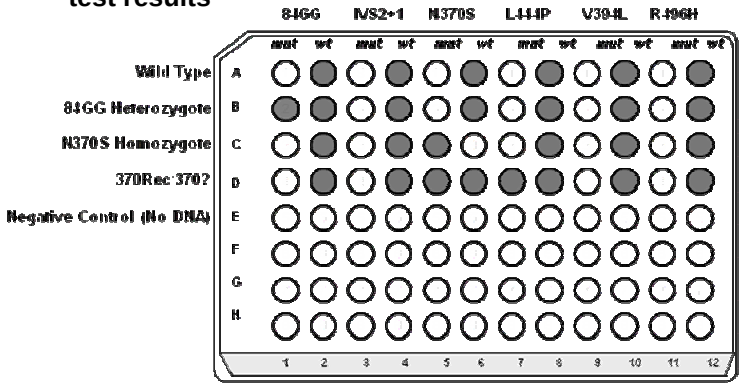
INTERPRETATION OF RESULTS

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 (Fig. 4).

Figure 4: 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.

370Rec Allele identification

A positive result for the two mutations: N370S and L444P (as shown in Fig. 4 on row D) suggests the possibility of the presence of the 370Rec allele. Use the 370Rec Identifier package (Cat. No. 9915) to distinguish between the 370Rec/370 (homozygous for N370S) and 370Rec/N (heterozygous for N370S) genotypes.

PRONTO® GAUCHER SCREEN – PROCEDURE SUMMARY

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

DNA AMPLIFICATION: Volumes per reaction: 2 µL template DNA + 22.5 µL Amplification Mix + 0.5 µL Taq Polymerase.
Cycling protocol:
94°C 2 min→35 cycles of {94°C 30 sec. / 60°C 30 sec./ 72°C 45 sec.} →72°C 5 min.

POST-AMPLIFICATION PROCEDURE:

- Volumes per reaction:

PRONTO® Buffer 2	100.0 µL
Solution C	4.0 µL
Solution D	3.0 µL

- **Pipette** in and out to mix.
- **Add** 107 µL into each well containing 20 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 twelve 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 30 sec. / 60°C 10 sec.} →Cool.

Insert the PRONTO® Plate in the thermocycler when the temperature has reached 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 10-30 minutes.

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

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

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