

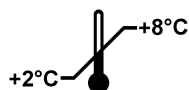
PRONTO[®] Connexin[™] kit

For the detection of the following mutations:

Connexin 26 (GJB2)	35delG
.....	167delT
Connexin 30 (GJB6)	del(GJB6-D13S1830)

Instructions for Use

REF 9968



INTENDED USE

The PRONTO[®] Connexin kit is a single nucleotide primer extension ELISA procedure, intended for the qualitative *in vitro* detection of the following three mutations in amplified human DNA: 35delG (sometimes called 30delG) and 167delT in the Connexin 26 gene (GJB2), and the 342 Kb deletion (delGJB6-D13S1830) in the Connexin 30 gene (GJB6).

For *in vitro* diagnostic use.

BACKGROUND

Congenital deafness occurs in approximately 1 in 1,000 births, of which more than 60% are thought to have a genetic origin. Mutations in the Connexin 26 (GJB2) and Connexin 30 (GJB6) genes cause nonsyndromic deafness. The 35delG mutation in the Connexin 26 gene is the most common cause of autosomal recessive deafness in Caucasians, with a carrier rate of 1/35 in Southern Europe, 1/40 in the US population of European descent and 1/135 in the Ashkenazi Jewish population. Another GJB2 mutation, 167delT, occurs almost exclusively in the Ashkenazi Jewish population at a carrier frequency of 1/25. A 342 Kb deletion in GJB6 (delGJB6-D13S1830), is found in compound heterozygosity with a GJB2 mutation in about 2% of the patients, with higher frequencies reported in Spain, France, Italy and Israel.

REFERENCES

1. Storm et al. Hum Mutat. 1999;14(3):263-6.
2. Kelsell et al. Nature. 1997 May 1;387(6628):80-3.
3. del Castillo et al. N Engl J Med. 2002 Jan 24;346(4):243-9.
4. Cohn et al. Cohn et al., Pediatrics 1999; 103:546-50.

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.

- 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 then takes place in the presence of the 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

Connexin Amplification Mix.....	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)
Stop Solution (1M H ₂ SO ₄)	1 bottle	(30 mL)
Connexin PRONTO [®] Plates.....	3 individually pouched plates	
Detection Plates	3 Streptavidin-coated ELISA plates	

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 a 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 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 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 Connexin	18.0 µL
Taq DNA polymerase (5 u/µL)	0.2 µL

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

- BioLabs Cat. # M267S
- PEQ LAB Cat. # 01-1020
- Sigma Cat. # D6677
- Fermentas Cat. #EP0709

3. **Dispense** 18 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 essential to use oil.

5. **Place** the thermoplate well or tube in a thermocycler previously programmed with the following protocol:

Cycling protocol

- | | | | |
|----|-------|------------|-------------|
| 1. | 94° C | 5 minutes | |
| 2. | 94° C | 30 seconds | } 35 cycles |
| 3. | 60° C | 30 seconds | |
| 4. | 72° C | 30 seconds | |
| 5. | 72° 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:

Gene	Mutations	Fragment size
Connexin 26 (GJB2)	35delG 167delT	580 bp
Connexin 30 (GJB6)	del(GJB6-D13S1830)	372 bp (291 bp)*

* The third fragment (291 bp) appears only in the presence of the Connexin 30 deletion.

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 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	47.0 µL
Solution C	2.0 µL
Solution D	1.5 µL

- 2 **Mix** gently by pipetting this solution in and out five times. Do not vortex.
- 3 **Add** 50 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:

Cycle	Temperature	Time
Start:	94°C	15 sec.
20 cycles:	94°C	30 sec.
	62°C	10 sec.
End:	18-25°C - Cool down to room temperature	

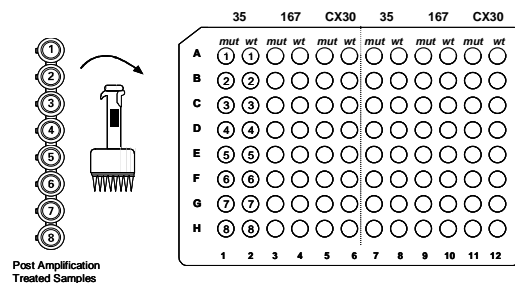
2 Take a PRONTO® Plate out of its pouch. Notice the color at the bottom of the wells. For each mutation tested, use a pink well (*mut*) and a blue well (*wt*). Mark the plate with the ID numbers of your test.

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 six 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 Connexin 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 procedure 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 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.

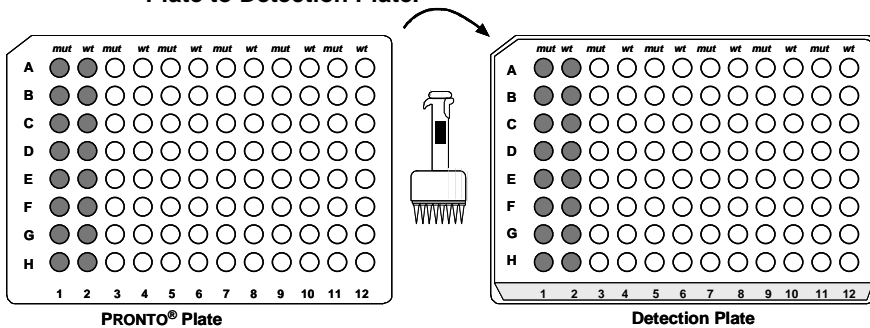
Dilute solution may be kept at 18-25°C for up to one month.

- **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 / trough with the green colored Assay Solution. About 11 mL will be required for a 96-well plate.
- 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.
- 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

Procedure	Visual Detection (Blue color)	Colorimetric Detection (Yellow color)
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. This solution should be freshly prepared each time the test is run.	Dilution: 1:100 (110 µL of Conjugated HRP into 11 mL Assay Solution)	Dilution: 1:200 (55 µL of Conjugated HRP into 11 mL Assay Solution)
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.	√	√
8 Add 100 µL of freshly diluted Conjugated HRP to all the wells, with a multichannel pipette.	√	√
9 Incubate at room temperature.	10 minutes	10 minutes
10 Wash the plate as in step 7.	√	√
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	15 – 30 minutes	15 minutes
12 Add 100 µL of Stop Solution to each well with a multichannel pipette. The solution will turn yellow immediately.	—	100 µL
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).	Agitate the plate gently and read results at O.D. 620 nm	—
14 Within two hours read the absorbance using an ELISA reader (single wavelength setting)	—	O.D. 450 nm

VALIDATION OF THE RESULTS

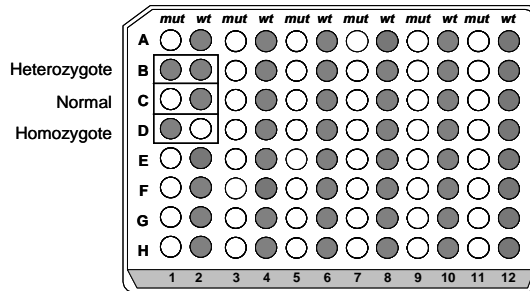
For Visual Detection:

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



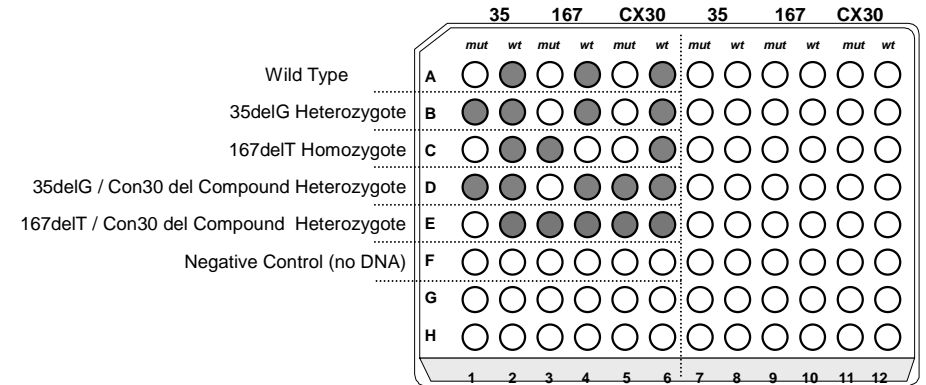
INTERPRETATION OF RESULTS

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:

Genotype	<i>mut</i> well (O.D. 450)	<i>wt</i> well (O.D. 450)	<i>mut</i> / <i>wt</i> ratio
Normal	O.D. ≤ 0.35	O.D. ≥ 0.5	ratio < 0.5
Heterozygote	O.D. ≥ 0.5	O.D. ≥ 0.5	$0.5 < \text{ratio} < 2.0$
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® Connexin - PROCEDURE SUMMARY

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

DNA AMPLIFICATION:

Volumes per reaction: 2 µL Template DNA + 18 µL Amplification Mix + 0.2 µL Taq Polymerase.

Cycling protocol:

94°C 5 min → 35 cycles of {94°C 30 sec. / 60°C 30 sec. / 72°C 30 sec.} → 72°C 5 min.

POST-AMPLIFICATION PROCEDURE:

▪ Volumes per reaction:	PRONTO® Buffer 2	47.0 µL
	Solution C	2.0 µL
	Solution D	1.5 µL
		50.5 µL

- **Pipette** in and out to mix.
- **Add** 50 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 six 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. / 62°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.

	Visual Detection	Colorimetric Detection
Add 100 µL of Conjugated HRP to every well and incubate for 10 minutes at RT.	Dilution 1:100	Dilution 1:200
Empty the wells and wash four times with 350 µL of 1x Wash Solution.	√	√
Add 100 µL of TMB Substrate to each well and Incubate at RT for:	15 – 30 minutes	15 minutes
Add Stop Solution	—	100 µL per well
Read O.D. at:	620 nm	450 nm

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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