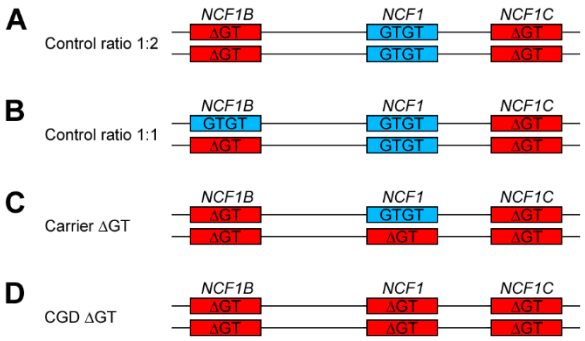
OMTM, Volume *13*

Supplemental Information

Novel Diagnostic Tool for p47^{phox}-Deficient Chronic Granulomatous Disease Patient and Carrier Detection

Dominik Wrona, Ulrich Siler, and Janine Reichenbach

Supplemental Information
Supplemental Figures
Figure S1



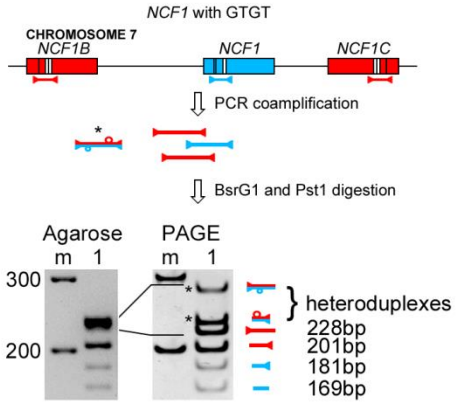
The scheme of the genetic configuration of GTGT and ΔGT sequences located in *NCF1* and pseudogene alleles on chromosome 7

(A) A majority of healthy individuals exhibit the GTGT sequence in both alleles of the *NCF1* gene, and the ΔGT mutation in all four alleles of the *NCF1* pseudogenes (*NCF1B* and *NCF1C*), resulting in a ratio of GTGT to ΔGT carrying *NCF1* alleles of 1:2. (B) A minority of healthy individuals exhibit three GTGT carrying *NCF1* alleles, two located on both *NCF1* gene alleles and one present on one of the pseudogenes, while the ΔGT mutation is shared by the remaining three *NCF1* pseudogene alleles. Hence, in these healthy individuals the ratio of GTGT to ΔGT carrying *NCF1* alleles is 1:1. (C) In carriers of the ΔGT mutation, GTGT is present in only one of the *NCF1* gene alleles, while (D) in ΔGT p47^{phox} CGD patients, all *NCF1* loci carry the ΔGT mutation, resulting in a ratio of 1:5 and 0:6, respectively.

Introduction to Figures S2 to S6

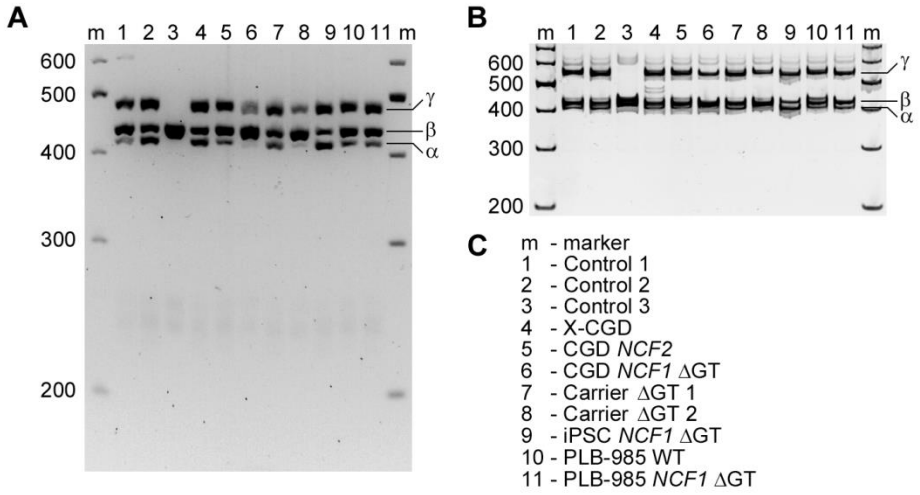
The following figures describe the origin of heteroduplex PCR products that result from simultaneous PCR co-amplification of the *NCF1*, *NCF1B* and *NCF1C* sequences.

Supplemental Figure S2



PCR co-amplification and RFLP products of the *NCF1* gene and its pseudogenes developed in agarose and polyacrylamide gels.
The heteroduplex PCR products (marked with asterisks) form during the PCR reaction by annealing of single-stranded PCR products derived from the *NCF1* gene and its pseudogenes. These heteroduplexes migrate differently in polyacrylamide and agarose gels, as compared to the homoduplex PCR products, even after restriction digestion. PCR-RFLP product of size 63 bp is not shown in the gels.

Figure S3



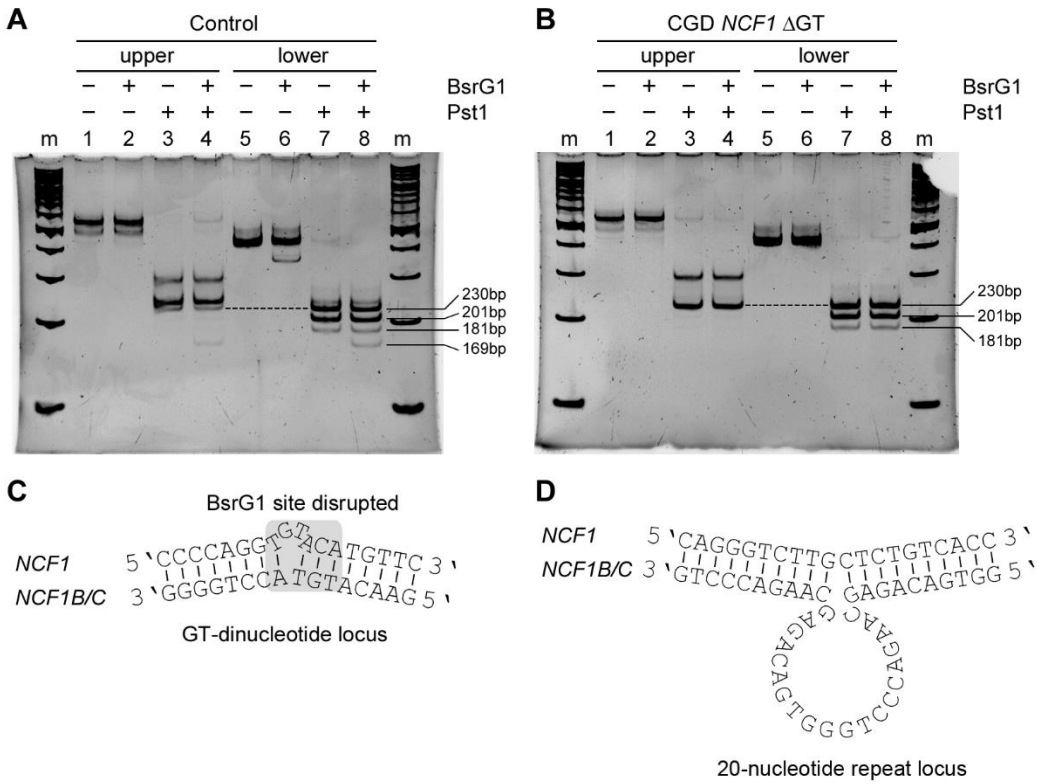
PCR products of co-amplified *NCF1, NCF1B,* and *NCF1C* sequences in agarose (A) and polyacrylamide (B) gels

Sequences around the *NCF1* exon 2 were PCR co-amplified utilizing one pair of primers, and representative PCR products from eleven individuals (C) were developed in a 5 % agarose (A) or a 7.5 % polyacrylamide (B) gel. Migration of bands "α" and "β" indicate PCR product sizes between 400 and 450 bp in both, the agarose gel (A) and the polyacrylamide gel (B). The band "γ" migrates differently: in the agarose gel it is located below the 500 bp band of the marker, whereas in the polyacrylamide gel it is located between 500 bp and 600 bp.

In healthy controls 1 and 2 (Control 1 and 2; lanes 1 and 2 in A and B) restriction digestion of PCR products with BsrG1 and Pst1 generates a 201 bp fragment derived from the pseudogenes and a 181 bp fragment (see also: **Figure 1B and C,** as well as **Figure 2A and B**) derived from the *NCF1* gene. These fragments differ from one another only by the presence or absence of the second copy of the 20-nucleotide (20-nt) sequence repeat within intron 2.

Interestingly, PCR products of healthy control 3 (Control 3; lane 3 in A and B) do not include PCR products "α" and "γ". Restriction digestion of the PCR products of this control with BsrG1 and Pst1 results in a band pattern where the 181 bp band is absent (see: **Figure 2A, B**). The lack of this band indicates the presence of a double 20-nt repeat sequence within the intron 2 of all six *NCF1* loci. This configuration of the *NCF1* intronic 20-nt repeat has not been linked to the CGD phenotype.

Figure S4



The restriction fragment length polymorphism analysis of upper ("γ") and lower fractions ("α" and "β") of PCR co-amplification products of the *NCF1* loci

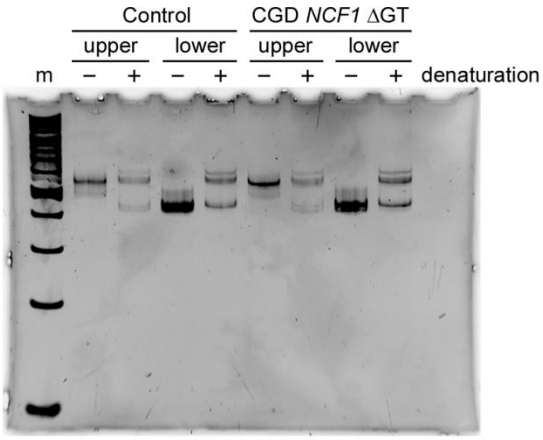
PCR co-amplification products of the *NCF1* loci from a healthy control, and from a ΔGT p47^{phox} CGD patient were separated in an agarose gel (see: **Figure S3A**), followed by gel purification of upper and lower fractions (gel not shown). The lower fraction consisted of bands "α" and "β" (see: **Figure S3A**) and the upper fraction corresponded to the band "γ" (see: **Figure S3A**). To avoid changes in the composition of isolated cross-hybridized PCR heteroduplexes, the temperature has not exceeded 46 °C during purification. The upper (band "γ") and lower (bands "α" and "β") fractions of PCR co-amplification products were subjected to restriction digestion with BsrG1 and Pst1 restriction enzymes. Digestion was performed as described in Methods, without the final denaturation of DNA and without inactivation of the enzymes at 80 °C. The digestion products were visualized in a 7.5 % polyacrylamide gel.

In healthy controls the lower fraction consisting of bands "α" and "β" (**Figure S4A and B, lanes 5 to 8**) can be digested by BsrG1 (**Figure S4A, lane 6**) and by Pst1 (**Figure S4A, lane 7**), whereas double digestion results in the expected band pattern for healthy individuals, with four bands in total (**Figure S4A, lane 8**). In ΔGT p47^{phox} CGD, however, the ΔGT abolishes the BsrG1 restriction site in the lower fraction of PCR products (**Figure S4B, lane 6**), resulting only in 3 bands after digestion with BsrG1 and Pst1 (**Figure S4B, lane 8**). As expected, the smallest 169 bp band, which is the result of BsrG1 digestion of the PCR co-amplification products from the healthy control (**Figure S4A, lanes 6 and 8**), is absent in digestion samples from the ΔGT p47^{phox} CGD patient (**Figure S4B, lane 8**). The 181 bp and 201 bp bands were produced solely by Pst1 digestion of the lower fraction of PCR products of both, the healthy control and the ΔGT p47^{phox} CGD patient (**Figure S4A and B, lanes 7 and 8**).

The upper fraction of the undigested PCR co-amplification products (see: **Figure S3A and B, band "γ"**) can be digested only by Pst1 for both samples, from the healthy individual (**Figure S4A, lanes 1 to 4**) and from ΔGT p47^{phox}-deficient CGD patient (**Figure S4B, lanes 1 to 4**). The resulting two bands are also present in the polyacrylamide gel for samples derived from healthy controls and carriers of the ΔGT mutation (**Figure 2A,** marked with asterisks). Of these two, the smaller digestion product from the ΔGT p47^{phox} CGD patient overlaps with the 230 bp band derived from digestion of the lower fraction with both BsrG1 and Pst1 enzymes (**Figure 2A lanes 6, 9 and 11,** and **Figure S4A and B**; a dashed line indicates the difference in the position of the smaller digestion products of the upper fraction in lanes 3, 4, 7 and 8).

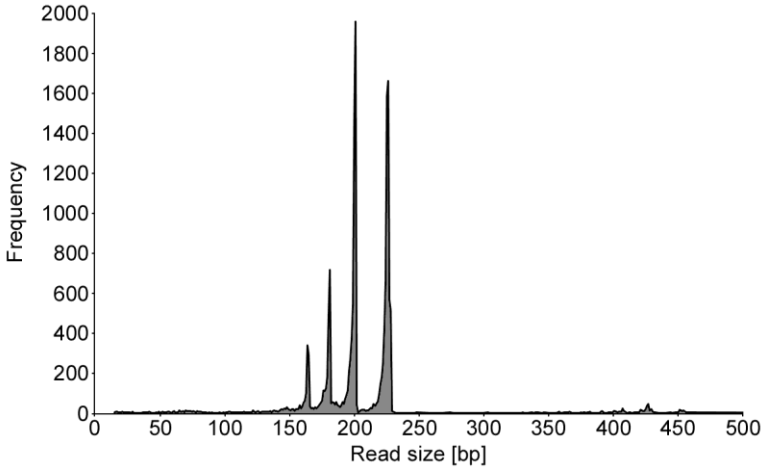
The bending centers responsible for the altered curvature and electrophoresis motility of heteroduplex PCR products are presented in **Figure S4C and D**: the ΔGT mutation in one strand of the GTGT locus (**Figure S4C**) and the 20-nt repeat locus (**Figure S4D**). In healthy controls, the heteroduplexes contain one strand derived from the pseudogenes carrying the ΔGT mutation, and another from the *NCF1* gene with the GTGT sequence, in which the restriction site for BsrG1 is disrupted (**Figure S4C**). These molecules retain their curvature upon restriction digestion with Pst1, as they still migrate slower (**Figure S3A, lane 3 and 4**). Conversely, the sequence of the 5' Pst1 digested fragment for ΔGT p47^{phox} CGD patients is shared between the mutated *NCF1* and the pseudogenes. Because of Pst1 digestion, the 5' digestion fragment of the upper fraction loses its curvature and migrates with the same speed as the 230 bp fragment of the lower fraction (**Figure S3B, lane 3 and 4**). Additionally, the pattern of digested PCR products for healthy control 3, who exhibits two copies of the 20-nt intronic repeat in all *NCF1* alleles (**Figure 2A, lane 3**), does not display the upper of the two additional bands marked with asterisks in **Figure 2A, lane 3.** The lack of this band again can be explained by lost DNA curvature upon Pst1 digestion. As already mentioned, this healthy control presents two copies of the 20-nt repeat sequence in all *NCF1* loci, and Pst1 digestion releases the linear 3' fragment of the heteroduplex that migrates as the regular 201 bp restriction fragment in **Figure 2A, lane 3**.

Figure S5



Denaturation and renaturation of the upper and lower fractions of the PCR co-amplification products
PCR co-amplification products of the *NCF1* loci from a healthy control and a ΔGT p47^{phox} CGD patient were separated in an agarose gel, the lower fraction consisting of bands "α" and "β" (see: **Figure S3**), and the upper fraction corresponding to band "γ" (see: **Figure S3**) were purified (gel not shown). During isolation the temperature has not exceeded 46 °C. Thereafter, isolated upper and lower fractions were denatured at 95 °C for 3 minutes and renatured at room temperature. The denatured and renatured samples were developed in a 7.5 % polyacrylamide gel along the unprocessed samples. Denaturation of all fractions led to reappearance of the PCR heteroduplexes, indicating that the band "γ" (**Figure S3**) is not an unspecific PCR product, but a product of cross-hybridization of PCR co-amplification products derived from different *NCF1* loci, the *NCF1* gene and the *NCF1* pseudogenes.

Figure S6

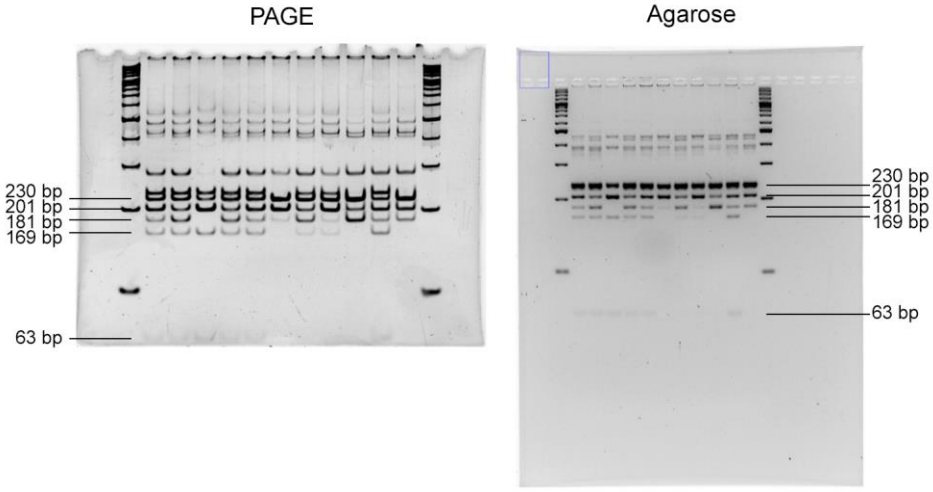


SMRT sequencing of digested PCR co-amplification

PCR co-amplification products of *NCF1* loci consisting of the upper fraction (band "γ") and the lower fraction (bands "α" and "β"), as visualized in **Figure S3**, were digested with BsrG1 and Pst1 resulting in up to seven bands in a polyacrylamide gel (see: **Figure 1B**). All bands except for 63 bp were gel purified and analyzed by SMRT sequencing, as described in Methods. Size distribution of sequenced reads identified four distinct products that correspond to the lengths of expected digestion fragments of the PCR homoduplexes.

Altogether, the results presented in Supplemental Figures S2-S6 suggest that the upper fraction (**Figure S3A and B, band "γ"**) consists of cross-hybridized PCR heteroduplexes, where one strand of the DNA heteroduplex originates from the *NCF1* gene and another from one of its pseudogenes (see: **Figure S2,** marked with asterisks). These heteroduplexes are curved DNA molecules that contain two major bending centers: the GT-dinucleotide deletion in one strand in the GTGT locus (**Figure S4C**) and the 20-nt repeat locus (**Figure S4D**). Due to the curvature, the heteroduplexes migrate slower than expected by their size, as reported before for other curved DNA molecules.[1]

Figure S7



Uncropped PAGE and agarose gels shown in Figure 2A and B of the main text

Supplemental Tables

Table S1

The list of ΔGT p47^{phox}-deficient CGD patients and carriers of the ΔGT mutation tested in the study.

The Table S1 is in the excel file: Supplemental Table S1.

Table S2

The GTGT-content calculated for individual tested in the study, CGD patients, carriers and controls, as determined by restriction length polymorphism visualized in polyacrylamide and in agarose gels, or determined by SMRT sequencing.

The Table S2 is in the excel file: Supplemental Table S2.

Supplemental References

1. Stellwagen, N. C. Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis* **30 Suppl 1,** S188-95 (2009). DOI: 10.1002/elps.200900052