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Technical Advance

Betaine, Dimethyl Sulfoxide, and 7-Deaza-dGTP, a Powerful Mixture for Amplification of GC-Rich DNA Sequences

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Currently, polymerase chain reaction is the most used technique in many laboratories for either diagnostic or molecular biology purposes. Despite the large number of DNA sequences that can be easily analyzed, some GC-rich sequences are refractory to amplification due to the formation of secondary intramolecular structures. To overcome this problem, several molecules have been described to improve polymerization. Here we show that a combination of three additives—betaine, dimethyl sulfoxide, and 7-deaza-dGTP—was essential to achieve amplification of DNA sequences of three disease genes showing a GC content ranging from 67 to 79%. ***(J Mol Diagn 2006, 8:544–550; DOI: 10.2353/jmoldx.2006.060058)***

The polymerase chain reaction (PCR) is a powerful technique to accomplish amplification of DNA sequences that can be used for many purposes, such as sequencing for molecular diagnosis or cloning into vectors and for protein expression or promoter studies. The majority of DNA sequences do not require particular conditions to undergo specific amplification, especially when deoxyribonucleotide content is equally distributed among their entire length. However, sequences in the genome with a high content of G and C are often present. When these sequences must be amplified, during the first cycles of amplification, the single-stranded template can form intramolecular stem loops where the *Taq* polymerase jumps this hairpin structure, promoting amplification of shortened PCR products lacking the stem loop sequence.[1] Nevertheless, it often happens that longer nonspecific PCR products may be produced, favored by compatible priming sequence and lower GC contents. To overcome these troubles, organic molecules such as dimethyl sulfoxide (DMSO), glycerol, polyethylene glycol, formamide, betaine, 7-deaza-dGTP, and dITP have been shown to improve the amplification of GC-rich DNA sequences.[2–10] Moreover, the combination of two additives such as betaine and DMSO has also been found to enhance amplification of long PCR products and of a random sequence DNA library.[11,12]

In the attempt to amplify a DNA region of 392 bp with 79% GC content, encompassing the transcription start site of the *RET* tyrosine kinase receptor gene, we obtained high yield of specific PCR product by including in the reaction 1.3 mol/L betaine, 50 μmol/L 7-deaza-dGTP, and 5% DMSO. Afterward, we explored the possibility of amplifying two other GC-rich sequences spanning a region from exons 7 to 8 of the *LMX1B* gene (67.8% GC) and exon 3 of the *PHOX2B* gene (72.7% GC), in which the vast majority of the patients with congenital central hypoventilation syndrome (CCHS) show triplet GCN expansion.[13] In both cases, we obtained specific amplification, demonstrating that these low-cost additives can be used for amplification of a variety of DNA templates with unbalanced content of G and C deoxyribonucleotides.

Materials and Methods

Oligodeoxyribonucleotide sequences used in PCR and cycling conditions are reported in Table 1. PCR reactions were set up in a total volume of 25 μl containing 1.25 units of *Taq* polymerase (Eppendorf-5 Prime, Inc., Boulder, CO), 1× buffer supplemented with 2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 10 nmol of each primer, and 100 ng of genomic DNA from the IMR-32 neuroblastoma cell line. For amplification of exon 3 of the *PHOX2B* gene

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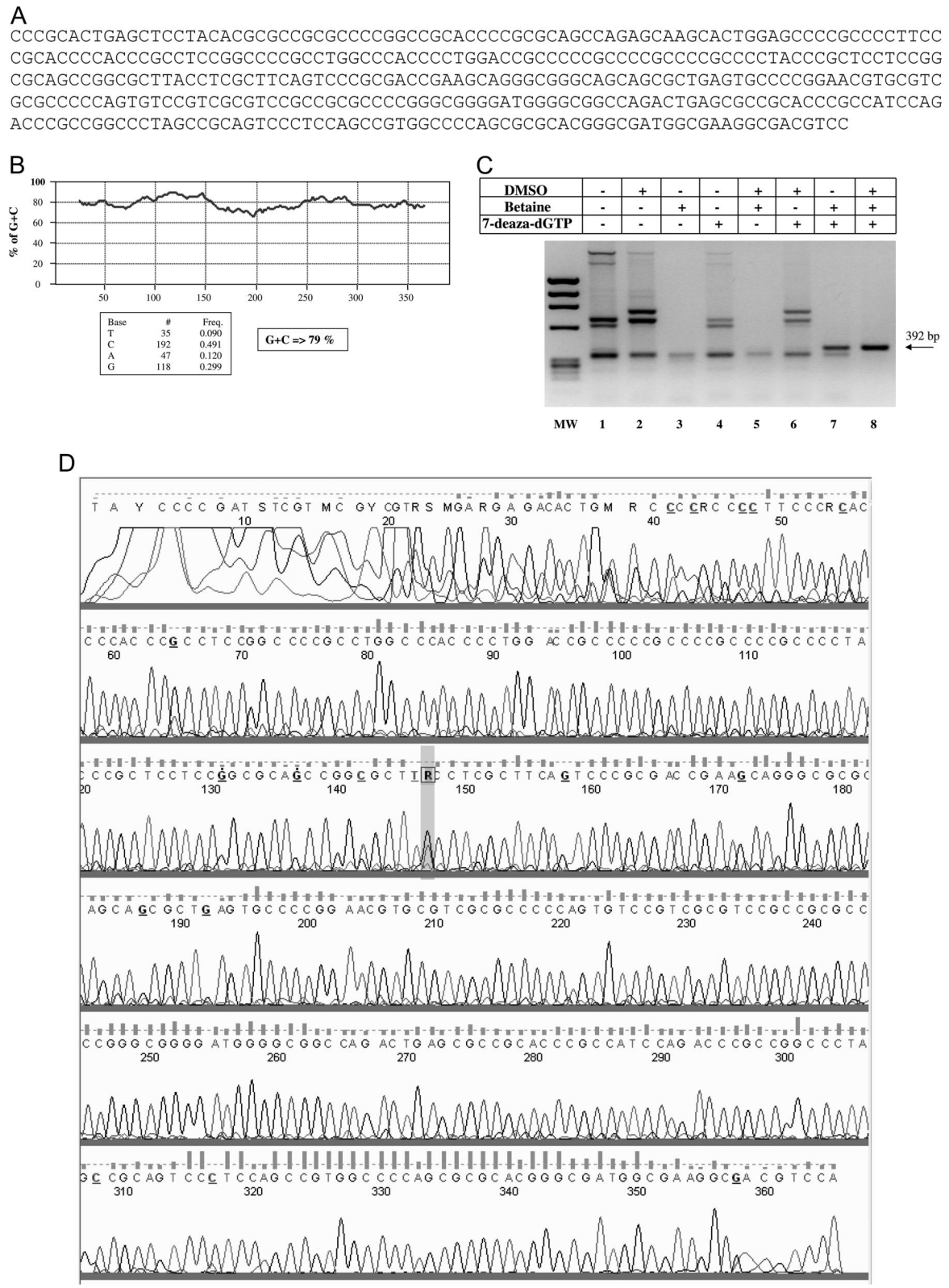

Figure 1. Amplification of *RET* promoter region. **A:** Nucleotide sequence (392 bp) spanning from −179 to +213 with respect to the transcription start site of *RET* gene. **B:** Base composition plot of GC content of the sequence. The amount and frequency of each deoxyribonucleotide and the percentage of GC are also indicated. **C:** Agarose gel electrophoresis. Ten μl from each PCR product obtained under different conditions, namely single additives or their combinations at a final concentration of 1.3 mol/L betaine, 5% DMSO, and 50 mmol/L 7-deaza-dGTP, were loaded as indicated (**lanes 1** to **8**). PhiX174 *Hae*III digested (New England Biolabs) is indicated as a molecular weight marker. **D:** Chromatogram of the DNA sequence obtained with the RET f primer. Nucleotide number 147, corresponding to −5 promoter SNPs (G/A), shows the presence of two different alleles in the IMR-32 cell line, as expected.

A

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TGCCTGGGGAGTCCCAAGGAGATCAGAAGGGGAGGCTGCTGGGGTGTAGCTGGGAGGGCGTGGACCAGGCCAGGGGGTGA
AGGCTCACTGTGCCCCCAGAGGTCCTGTCCAGCCGCATGGAGGGCATGATGGCTTCCTACACGCCGCTGGCCCCACCACA
GCAGCAGATCGTGGCCATGGAACAGAGCCCCTACGGCAGCAGCGACCCCTTCCAGCAGGGCCTCACGCCGCCCCAAATGC
CAGGTGACCACATGAACCCCTATGGTAAGCCGCCCTACCCCCACCCGCCCGCCCCAGCACAGCCCCTGCCCCCTGCCAGG
CCAGGCAGGCCTGGGGCCAGGACAGCCACCTGCTGCCCTAGGGCTCAGGCAGCATGGCCAGCACAGCCCTCCTGCCCCTG
CTGACAGGAAGGGCCCCAGTCCTTCTTGGCCGGCACTAGTCAGCAGGCCATCCTGTCTCTCCCTGGCCTCCAGTCACACA
GCCTACAGGGCAAACAGGGGGCCCCCAGGAGCCCCAGCCTGTACCCCGGTCCTGACACCCCTTCTGCCCCCCAGGGAACG
ACTCCATCTTCCATGACATCGACAGCGATACCTCCTTAACCAGCCTCAGCGACTGCTTCCTCGGCTCCTCAGACGTGGGC
TCCCTGCAGGCCCGCGTGGGGAACCCCATCGACCGGCTCTACTCCATGCAGAGTTCCTACTTCGCCTCCTGAGAGCCAGC
CAGGCGCACGGACGCTT
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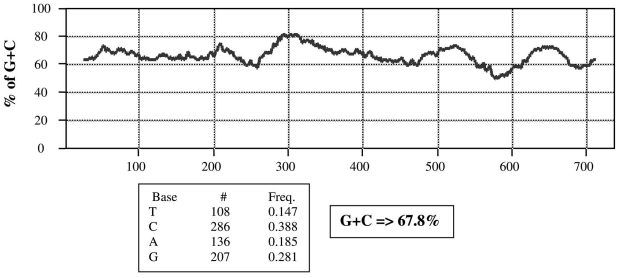
B



C

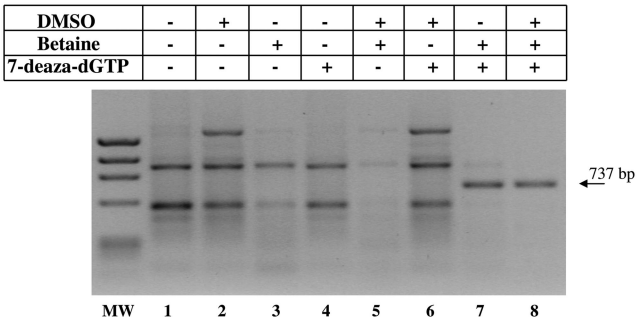


Figure 2. Amplification of *LMX1B*. **A:** Nucleotide sequence (737 bp) spanning from intron 6 to intron 8 of *LMX1B* gene. **B:** Base composition plot of GC content; the amount and frequency of each deoxyribonucleotide and the percentage of GC are also indicated. **C:** Agarose gel electrophoresis. Five μl of each PCR, performed by using single additives or their combination at a final concentration of 1.3 mol/L betaine, 5% DMSO, and 50 mmol/L 7-deaza-dGTP, were loaded as indicated (**lanes 1** to **8**). PhiX174 *Hae*III digested is indicated as a molecular weight marker. **D:** Chromatogram of the DNA sequence obtained with the LMX1B f primer.

from two healthy individuals, one wild type and one heterozygous for a polymorphic 21-bp deletion, or from a CCHS patient carrying an 18-bp expansion, 1.25 units of Gold *Taq* polymerase (Applied Biosystems, Foster City, CA), and 2 mmol/L $MgCl_2$ were instead used. In each case, additives were added at the following final concentration: 1.3 mol/L betaine (Sigma-Aldrich, St. Louis, MO), 5% DMSO (Sigma), and 50 μmol/L 7-deaza-dGTP (Roche Diagnostics, Indianapolis, IN). Amplifications were performed in a 2700 Applied Biosystems thermal cycler. PCR products (5 μl) were separated in 1.2% agarose gel or, in the case of *PHOX2B* exon 3-digested products, on a 6% polyacrylamide gel. For enzyme digestion, 10 μl of each PCR product was ethanol precipitated and cut with 5 units of *Ava*II (New England Biolabs, Ipswich, MA) for 16 hours at 37°C. For DNA sequencing reactions, 5 μl of each PCR product was treated at 37°C for 45 minutes with 1 μl of Exo-Sap (Appied Biosystems), followed by 15 minutes inactivation at 80°C. Two μl of this mixture were directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Amplification was performed in a 2700 Applied Biosystems thermal cycler through a denaturation step of 3 minutes at 94°C and 25 cycles consisting of 10 seconds at 94°C and 3 minutes at 68°C. Samples were analyzed on an ABI 3100 DNA Sequencer (Applied Biosystems). The base composition plot of GC content was obtained using MacVector 3.5 software (Accelrys, San Diego, CA).

Results

Amplification of the RET Promoter Region

The *RET* proto-oncogene encodes a tyrosine kinase receptor whose gain- or loss-of-function mutations are involved in medullary thyroid carcinoma and Hirschsprung disease, respectively. The amplification of the *RET* promoter region (Figure 1A), a DNA sequence contained in the BAC clone RP11-351D16 (GenBank GI no. 19919985), has been performed previously in our laboratories using glycerol and 7-deaza-dGTP to genotype and reconstruct haplotype(s) associated with Hirschsprung disease.[14] However, the specific 392-bp PCR product was often difficult to obtain because of a 79% GC content, with a peak between nucleotides 100 and 150 that reaches 90% (Figure 1B). As shown in Figure 1C, at least five major nonspecific PCR products were amplified in the absence of additives (lane 1). Following the addition of DMSO and 7-deaza-dGTP, either separate or in combination, some nonspecific bands disappeared, but no amplification of the specific product was obtained (lanes 2, 4, and 6). Betaine by itself drastically reduced nonspecific background, allowing amplification of a band with a slightly faster electrophoretic mobility with respect to the specific product (lane 3). An overlapping result was obtained when betaine was used in combination with DMSO (lane 5), demonstrating that betaine can

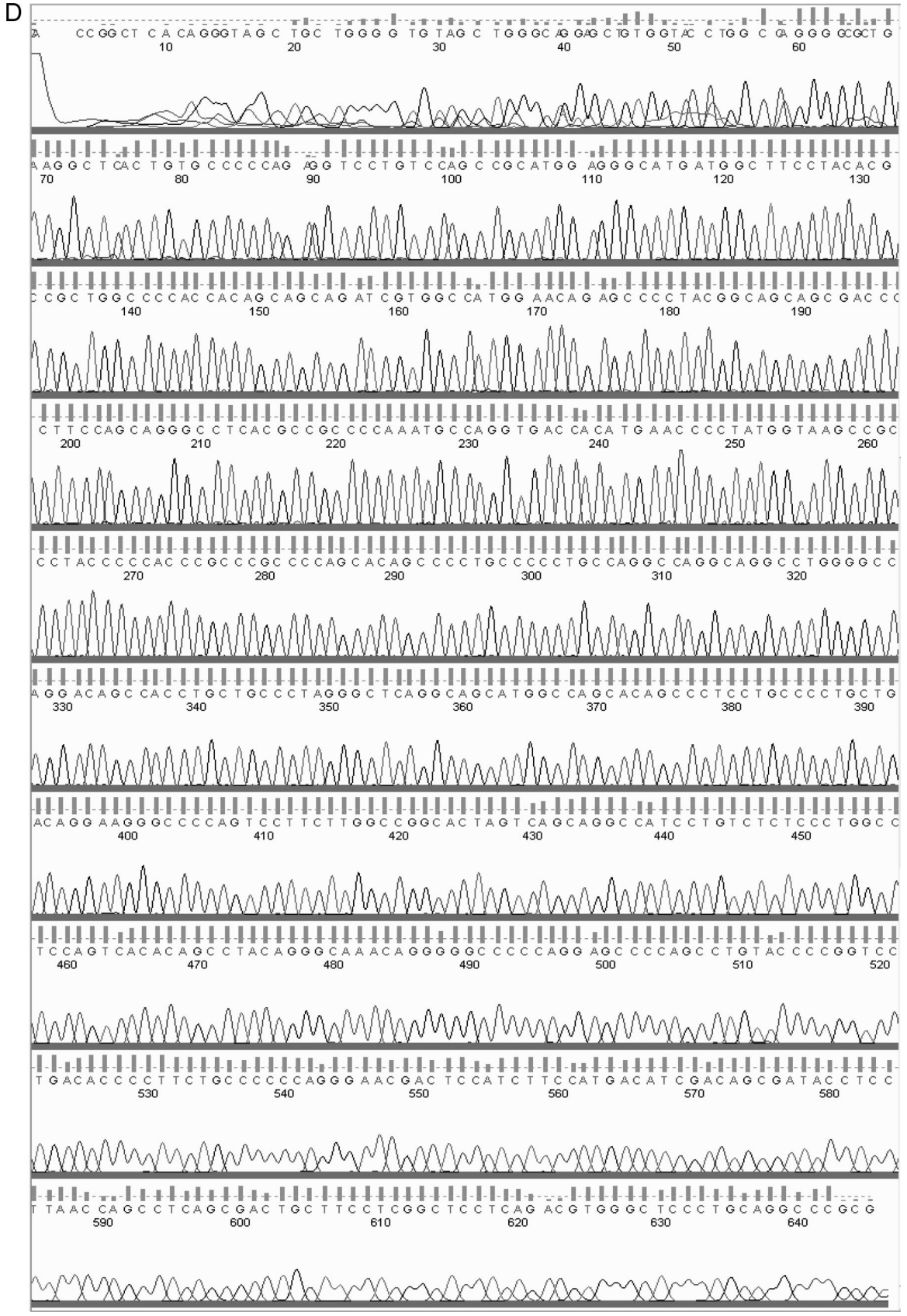


Figure 2—*continued*

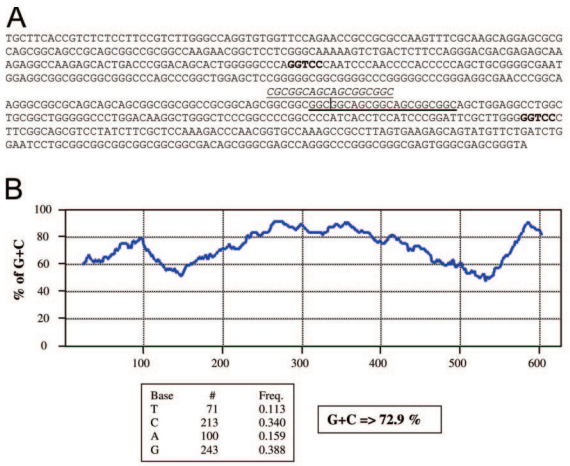
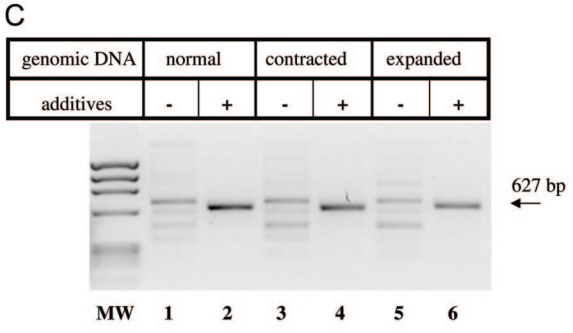
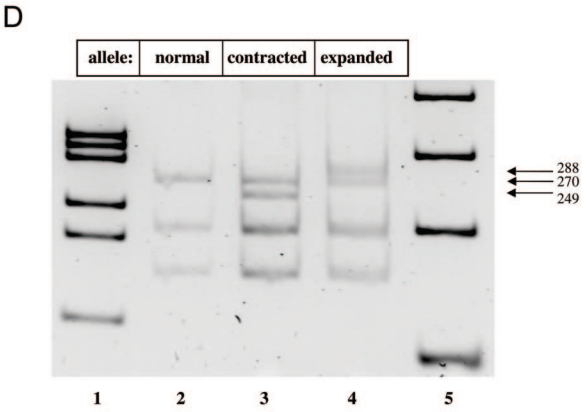


Figure 3. Amplification of *PHOX2B*. **A:** Exon 3 nucleotide sequence (627 bp) of the *PHOX2B* gene. *Ava*II restriction sites are in bold characters, the 21-bp sequence deleted in the control polymorphic variant are underlined, whereas the 18-bp sequence duplicated in the CCHS patient is shown in italic. **B:** Plot of GC content; the amount and frequency of each deoxyribonucleotide and the percentage of GC are also indicated. **C:** Agarose gel electrophoresis. Five μl of each PCR product obtained using genomic DNA from a control individual (**lanes 1** and **2**), from a control individual with the polymorphic deletion (**lanes 3** and **4**) or from a CCHS patient with an expanded allele (**lanes 5** and **6**), without any additive (**lanes 1**, **3**, and **5**) or with betaine, DMSO, and 7-deaza-dGTP (**lanes 2**, **4**, and **6**). **D:** Polyacrylamide gel electrophoresis of *Ava*II-digested PCR products from a control individual (lane 2), a control individual carrying the polymorphic deleted allele (**lane 3**), or a CCHS patient carrying the expanded allele (**lane 4**). Molecular weight markers are PhiX174 *Hae*III digested and a 100-bp ladder (**lanes 1** and **5**, respectively). **Arrows** indicate the *Ava*II fragments containing the 18-bp expansion (288 bp) or the 21-bp deletion (249 bp) and the normal allele (270 bp). **E:** Chromatograms of DNA region involved in deletion or expansion whose origin is indicated by **arrows**.





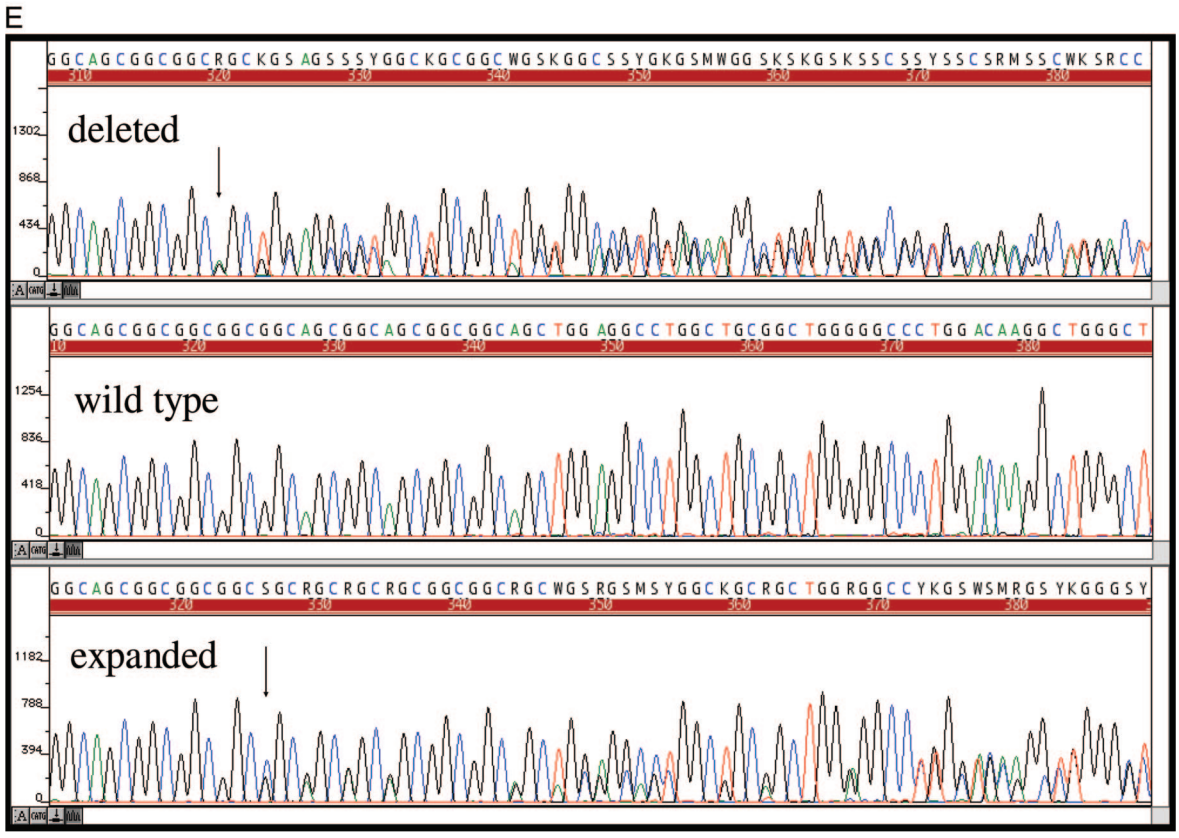


Table 1. Primer Sequences and PCR Cycle Condition

Primers	Sequence 5′ > 3′	PCR cycles
RET f	CCCGCACTGAGCTCCTACAC	94°C (5 minutes)
RET r	GGACGTCGCCTTCGCCATCG	94°C (30 seconds), 60°C (30 seconds), 72°C (45 seconds) × 40 cycles 72°C (5 minutes)
LMX1 f	TGCCTGGGGAGTCCCAAGG	94°C (5 minutes)
LMX1 r	AAGCGTCCGTGCGCCTGGCT	94°C (30 seconds), 60°C (30 seconds), 72°C (45 seconds) × 30 cycles 72°C (5 minutes)
PHOX2B f	TGCTTCACCGTCTCTCCTTCCGT	94°C (5 minutes)
PHOX2B r	TACCCGCTCGCCCACTCGCCCGCC	94°C (30 seconds), 60°C (30 seconds), 72°C (1 minute) × 35 cycles 72°C (5 minutes)

reduce the background of nonspecific PCR products, but it remains insufficient to accomplish the specific amplification. This 344-bp PCR product was sequenced and found to be identical to a DNA sequence residing on chromosome 2 with a GC content of 50.3%, excluding the primer sequences. The flanking sequences were complementary for 10 and 12 consecutive nucleotides with the 3′-end of RET forward (f) and RET reverse (r) primers, respectively. On the other hand, using betaine in combination with 7-deaza-dGTP achieved amplification of the RET promoter sequence, but the nonspecific 344-bp product was still present (lane 7). Finally, when all three additives were included in the reaction, a unique specific PCR product corresponding to the RET promoter region was obtained (lane 8), as shown by DNA sequencing (Figure 1D).

Amplification of LMX1B Region

The *LMX1B* gene encodes the zinc finger protein LIM homeobox transcription factor 1 whose mutations are involved in nail patella syndrome.[15] The DNA sequence reported in Figure 2A, spanning from introns 6 to 8 and contained in BAC clone RP11-489N22 (GenBank GI no. 11691463), has an average GC content of 67.8%, but between nucleotide 278 and nucleotide 358, the GC content reaches 75.6% (Figure 2B). In our hands, this DNA sequence was refractory to amplification because of several nonspecific products that were generated, as shown in Figure 2C (lane 1). Each single additive promoted the amplification of only nonspecific products (lanes 2 to 4), as did DMSO in combination with either betaine or 7-deaza-dGTP (lanes 5 and 6). As observed for the *RET* promoter region, amplification of the specific band was achieved when betaine and 7-deaza-dGTP were included in the reaction mix; however, a trail of the major nonspecific bands was still present (lane 7). Finally, a clean specific product, confirmed by DNA sequencing (Figure 2D), was obtained when all three additives were combined (lane 8).

Amplification of PHOX2B Exon 3

The third DNA sequence tested for amplification was exon 3 of the *PHOX2B* gene, reported in Figure 3A (GenBank GI no. 4633283), which is subject to triplet GCN expansion in CCHS patients.[14] Next to the intrinsic technical hitches of amplifying a 72.7% GC sequence (Figure 3B), the additional problem was to obtain amplification of both alleles. In standard PCR conditions using only 7-deaza-dGTP and DNAs heterozygous for two alleles of different lengths,[13] the shortest allele preferentially takes precedence during the first rounds of amplification. This has represented a considerable problem for diagnostic aims because patients heterozygous for expanded allele appear to be homozygous for the wild-type allele. To overcome this problem, it has recently been reported that amplification of both *PHOX2B* alleles can occur through the deamination of template DNA,[16] and we used a kit for GC-rich DNA sequences provided by Roche.[13] This sequence was therefore the ideal final challenge to test the combination of the three reagents. As shown in Figure 3C, using three different genomic DNAs from two control individuals, one wild type (lane 2) and one heterozygous for a polymorphic 21-bp deletion leading to a 7-alanine residue contraction (lane 4), or from a CCHS patient carrying an 18-bp duplication leading to a 6-alanine residues expansion (lane 6), the amplification was perfectly accomplished while several nonspecific PCR products were detected in the absence of the three additives (lanes 1, 3, and 5). These PCR products were then digested with *Ava*II to ensure the amplification of both alleles. As shown in Figure 3D, both alleles were amplified at an equal extent as demonstrated by the intensity of the 270-bp wild-type fragment versus the 249-bp and the 288-bp DNA fragments obtained by digestion of PCR products from deleted or expanded alleles, respectively (lanes 3 and 4). This result was confirmed by DNA sequencing in which heterozygous DNA analysis showed the sequences of two alleles exactly overlapping each other, starting from deletion or insertion points indicated by arrows (Figure 3E).

Discussion

In this report we have demonstrated the powerful effects of betaine, dimethyl sulfoxide, and 7-deaza-dGTP in combination on the amplification of three sequences with a high GC content. These molecules have been shown in the past to enhance amplification separately or in combinations of two, such as with 7-deaza-dGTP-betaine or betaine-DMSO. In our hands, the latter combination was not sufficient to achieve amplification of the tested sequences, whereas the association of 7-deaza-dGTP-be-

taine allowed amplification of target sequences although small amounts of nonspecific products were still detected. Specific and exclusive amplification of the target sequence was obtained with further addition of DMSO. The major result was the achievement of equal amplification of *PHOX2B* exon 3 alleles in heterozygous individuals for expansion or deletion of GCN triplets. This is a common problem for amplification of a variable number of tandem repeat polymorphisms also described for glycoprotein IBα and serotonin transporter genes in which a mixture of two different *Taq* polymerases and the addition of 7-deaza-dGTP could partially prevent the selective amplification of one allele.[17] DMSO and 7-deaza-dGTP have successfully been used for the screening of the Fragile X Syndrome in mentally handicapped children in combination with Expand Long Template PCR system (Roche) in determining the CGG repeat number in males and females for alleles from normal to premutation size range and the detection of full mutations in males.[18,19] In our case, the combination of these two reagents was unable to guarantee a specific amplification, probably due to the different condition in the Expand Long Template PCR system provided by Roche. These molecules, used either alone or in combination with both the Gold *Taq* polymerase (Applied Biosystems) and the *Taq* polymerase provided by Eppendorf (data not shown), gave exactly the same results, suggesting that the combination of the three additives is fruitful with different *Taq* polymerases. All three additives prevent intramolecular stable stem loops in GC-rich template due to the strong G-C pairing, with each chemical acting in a different way. DMSO disrupts base pairing; betaine, which is an isostabilizing agent, equalizes the contribution of GC and AT base pairing to the stability of the DNA duplex[20]; and 7-deaza-dGTP, partially substituting dGTP, reduces the number of hydrogen bonds with complementary dCTP and prevents formation of stable intramolecular G4 quadruplexes and purine-motif triplexes, being incapable of G*G Hoogsteen base pairing.

In this way, each reagent can be used at a concentration that does not inhibit the polymerase chain reaction, providing the additional synergic effect we have demonstrated in three different complicated amplifications of GC-rich DNA sequences. Finally, we want to emphasize that PCR products obtained with the addition of the betaine, DMSO, and 7-deaza-dGTP do not need further purification for TA cloning or automated DNA sequencing, because these molecules do not have any effect on topoisomerase I ligation activity and they can help polymerization in sequencing reaction.

The application of this novel protocol will provide a low cost, general and reliable means to improve the molecular analysis of DNA sequences that are otherwise refractory to amplification. This will ultimately allow proper molecular diagnosis of inherited diseases caused by genetic defects of such GC-rich DNA sequences.

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