Subcycling-PCR for Multiplex Long-Distance Amplification of Regions with High and Low GC Content: Application to the Inversion Hotspot in the Factor VIII Gene

BioTechniques 25:1022-1028 (December 1998)

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ABSTRACT

Previously we described a PCR protocol for detecting the inversion in the factor VIII gene, which is a common cause of Hemophilia A. This PCR assay is challenging due to the size of the amplification (10–12 kb), the varying GC content (30%–80%) and the multiplex PCR products involved (four for carrier female). Efficient amplification of the four segments depends on three unusual modifications to standard long-distance PCR protocols: (i) very high concentrations of dimethyl sulfoxide, (ii) addition of deazadGTP and (iii) high concentration of Taq and Pwo DNA polymerases. One of the segments was amplified much more efficiently than the others under standard three-temperature cycling conditions (12 s at 94°C, 30 s at 65°C and 14 min at 68°C). To facilitate the uniform amplification of the multiple regions, subcycling-PCR (S-PCR) was developed. In S-PCR, the combined annealing/elongation step is composed of subcycles of shuttling between a low and a high temperature, e.g., shuttling four times between 60° and 65°C. S-PCR produces consistent robust amplification of the various segments produced by wild-type, mutant and carrier individuals. S-PCR is a simple generalization of PCR, which generally may be advantageous in three contexts: (i) amplification of long segments in which the GC content varies within the segment, (ii)

multiplex amplification of long segments and (iii) multiplex amplification of short segments in which the GC content varies among the segments.

INTRODUCTION

Hemophilia A is one of the most common coagulation disorders, with an incidence of about one in 5000 males. The disease is caused by mutations in the factor VIII gene. Inversions within the factor VIII gene cause almost half of all cases of severe hemophilia A. A 9.5-kb region of intron 22 (*Int22h1*) is repeated twice near the Xq telomere (*Int22h2* and *Int22h3*). These repeated sequences are more than 99% identical with one another (12). The inversion occurs by intra-chromosomal homologous recombination between *Int22h1* and an extragenic homolog (either *Int22h2* or *Int22h3*, known as Types I and II inversions, respectively) (1,7,11). The inversions are detected routinely by time-consuming, costly Southern blots using a probe from *Int22h1*. Successful polymerase chain reaction (PCR) amplification spanning these regions has not been reported, presumably because the regions contain a 3.5-kb GC island of 65% GC content and a 1-kb region of 79% GC content within the GC island (Figure 1).

A single-tube PCR assay was reported that combines overlapping PCR (9) with long-distance PCR (2,5) to differentiate the wild-type, affected males and female carriers (Figure 2). Herein, we supplement the initial clinical report

(8) by analyzing parameters affecting amplification.

Multiplex PCR is a rapid and convenient method, but uneven amplification is common (4). Efforts have been made to achieve uniform amplification. Since primer concentration is often difficult to optimize, a simplified optimization procedure was developed based on the use of chimeric primers (14). Each primer contains a 3' region complementary to sequence-specific recognition sites and a 5' region made up of a universal 20-nucleotide (nt) sequence. Each individual PCR was first optimized by adjusting primer concentrations, cycling times and annealing temperatures. In another approach, two detergents, dimethyl sulfoxide (DMSO) and betaine, were combined to achieve uniform amplification for three templates differing in GC contents (3). Additional approaches include adjusting the annealing temperature, KCl (salt) concentration and primer concentration for each locus encountered in developing multiplex PCR of small sizes (6). Herein, we describe subcycling-PCR (S-PCR), a novel method for this purpose.

MATERIALS AND METHODS

Primer Design of the PCR Assay

A major difficulty in developing the PCR assay was lack of sufficient flanking sequences to design the PCR primers. Fifty base pair of 5' flanking sequence of *Int22h1* in intron 22 were

Table 1. Oligonucleotide Positions and Sequences

Name/Position	Sequence No.	GenBank Accession No.
P(-1212)	gcc cTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC	AF062514
Q(+1334)	ggc ccT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA	X86012
A(-167)	CAC AAG GGG GAA GAG TGT GAG GGT GTG GGA TAA GAA	AF062515
B(+118)	ccC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CATA	AF062516
Note: The high GC tails are indicated as lower case letters. The primer's first base at 5' end of the sequence-specific region is assigned, - = before the homolog and + = after the homolog.		

Table 2. The Key Parameters

Component Changed ^a		Amplifications
DSMO (%)	10.5	-
	9	++
	7.5	+++
	6	++
	4.5	-
	0	-
deaza-dGTP:dGTP (%:%)	50:50	+++/ (AB/PQ) ^b
	37.5:62.5	+++/ +++
	25:75	++/ ++
	0:100	-/-
	10	+++/ (AB/PQ)
<i>Taq</i> / <i>Pwo</i> (U/25 µL)	5	+++/ +++
	2.5	+++/ ++
	1.25	+++/ +
	0.625	++/-
	0.3125	-/-
	0	-/-
^a Only one component was changed. The effect was tested with the four primers (each at 0.2 µM) at 65°C for the annealing/elongation.		
^b AB and PQ segments are indicated in order.		

available from GenBank® (Accession No. X86011). A 6-kb PCR was amplified spanning the exon 22 and 5' end of *Int22h1* with the Expand™ Long Template PCR System (Boehringer Mannheim, Indianapolis, IN, USA). An additional 1319 bp of the 5' flanking sequence were obtained by automatic sequencing (GenBank Accession No.

AF062514). Only 50 bp of both 5' and 3' flanking sequences were known for *Int22h2* and *Int22h3* (12). A 6-kb region including *Int22h2* flanking sequences was amplified by inverse-PCR (13). Additional 5' and 3' flanking sequences of *Int22h2* (695 and 412 bp, respectively) were obtained (GenBank Accession Nos. AF062515 and AF06-

2516) and were confirmed to be identical in *Int22h3* by PCR amplification of patients with Type I and Type II inversions, as previously determined by Southern blot analysis.

The primers P, Q, A and B were designed with the aid of OLIGO™ Version 5 software (National Biosciences, Plymouth, MN, USA). The melting temperature (T_m) value of each PCR segment was estimated by the formula of Wetmur: $T_{m\text{ product}} = 81.5 + 16.6 \log [K^+] + 0.41\% (\text{GC}) - 675/\text{length} (16)$. The GC content of the AB segment is 51% on average, and the T_m is 81°C. The T_m values of the primers were estimated by the nearest-neighbor method at 50 mM KCl and 250 pM DNA with the formula: $T_{m\text{ primer}} = \Delta H / \{ \Delta S + R \times \ln(C/4) \} + 16.6 \log [K^+] - 273.15$. Each primer was designed to have a T_m that was 10°C lower than the average T_m of the PCR products (73°C). Primer lengths varied from 36–40 nt. For primers P, Q and B, a high GC tail of 2–5 nt was added to achieve a T_m value of 73°C. Formation of primer dimers was minimized by designing the 3' end of each primer to have no more than 4 nt of intra-strand and inter-strand complementarity. Finally, there were no more than six bases of complementarity at the 3' end of any primer to any of the PCR products. The positions and sequences of the oligonucleotides are described in Table 1.

The PCR Assay

The PCR was performed from human genomic DNA isolated from white blood cells. Unless stated, the PCR mixtures contained a total volume of 25 µL: 50 mM Tris-HCl, pH 9.2, 2.25 mM

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Table 3. Comparison of Different Enzyme Systems

DNA Polymerases ^a	Optimal Parameters ^b (I+II+III)	Single Parameter Changed ^c			Standard Conditions ^d (-I-II-III)
		2% DMSO (-I)	Only dGTP (-II)	Standard Enzymes (-III)	
<i>Taq</i> / <i>Pwo</i>	+++/ ++ (AB/PQ)	-/- (AB/PQ)	-/- (AB/PQ)	+++/ + (AB/PQ)	-/- (AB/PQ)
<i>Taq</i> / <i>Vent</i>	+++/ ++	-/-	-/-	+++/ +	-/-
ELONGASE	+++/ ++	-/-	-/-	++/ ±	-/-
<i>Tth</i> / <i>Pwo</i>	-/-	-/-	-/-	-/-	-/-
<i>Tth</i> / <i>Vent</i>	-/-	-/-	-/-	-/-	-/-
<i>Tfl</i> / <i>Pwo</i>	++/ +	-/-	-/-	++/ -	-/-
<i>Tfl</i> / <i>Vent</i>	++/ +	-/-	-/-	-/-	-/-
Bio-X-ACT	-/-	-/-	-/-	-/-	-/-

^aThe ratio of *Taq*/*Vent*, *Tth*/*Pwo*, *Tth*/*Vent*, *Tfl*/*Pwo* and *Tfl*/*Vent* were 30:1 (U:U). The segments were amplified from the carrier DNA with four primers (each 0.2 μM) at 65°C for the extension. AB and PQ segments are indicated in order.

^bOptimal parameters: I = 7.5% DMSO, II = 37.5% deaza-dGTP and III = 2.5 U of enzymes.

^cOne of the three parameters was changed, assigned as -I, -II and -III. Standard enzyme = 1.2 U.

^dStandard conditions = 2% DMSO, dGTP and 1.2 U of enzymes per 25 μL reaction.

Figure 1. Map plot of GC content of segment AB. The Y-axis indicates the GC content of the sequential windows of 300 nt. The solid line plots the GC content of segment AB, and the dotted line plots the GC content of segment AB after random shuffling. Composition analysis of symbol number range -166–9623 using a sliding analysis window of 300 symbols and smoothed by a 21-symbol moving average filter. The solid line denotes the AB segment, and the broken line denotes the AB segment shuffled.

MgCl₂, 16 mM (NH₄)₂SO₄, 7.5% DMSO, 500 μM of dGTP and deaza-dGTP (62.5%:37.5% or 50%:50%), 500 μM of each of the other dNTPs and 250 ng of genomic DNA. Three types of cycling conditions were utilized: three-temperature-, two-temperature- and S-PCR. The cycling conditions for three-temperature PCR were 94°C for 12 s, 65°C for 30 s and 68°C for 14 min for the first 10 cycles (GeneAmp® PCR System 9600; PE Applied Biosystems, Foster City, CA, USA). The remaining 20 cycles were performed by adding an extra 20 s to the elongation per cycle.

Conditions for two-temperature PCR were: 94°C for 12 s and 65°C for 15 min for the first 10 cycles, with an extra 20 s added to the elongation per cycle for the remaining 20 cycles. The conditions for S-PCR for the first 10 cycles were: 94°C for 12 s, followed by four subcycles of annealing/elongation that involve 60°C for 120 s and 65°C for 120 s for each subcycle. The remaining 20 cycles were modified by the addition of an extra 3 s per cycle for each step of the annealing/elongation step. An additional 2 min of denaturation was utilized in the first cycle. The en-

zyme amount and primer concentration depended on the types of cycling conditions for Expand Long Template DNA Polymerase (Boehringer Mannheim), amounts of enzyme and primers (P, Q, A and B) are as follows: (i) for three-temperature PCR, 3.3 U and 0.4, 0.4, 0.12 and 0.12 μM, (ii) for two-temperature PCR, 2.5 U and 0.2, 0.2, 0.15 and 0.15 μM and (iii) for S-PCR, 2.5 U and 0.2, 0.2, 0.2 and 0.2 μM. Ten microliters of the reaction were mixed with an equal amount of 2× loading buffer (5% Ficoll® 400, 5 mM MgCl₂, 25 mM NaCl, 10 mM Tris-HCl, pH 7.9) and then incubated at 37°C for 5 min. Samples were electrophoresed on a 0.6% agarose gel and then stained with ethidium bromide for UV photography by a charge-coupled device (CCD) camera (Gel Doc™ 1000; Bio-Rad, Hercules, CA, USA).

RESULTS

Utility of DMSO, deaza-dGTP and High Concentrations of DNA Polymerase

A PCR-based assay for the inversion was developed to distinguish among the wild-type, inversion and carrier states. Herein, we report an analysis of

Figure 2. Schematic of the PCR assay. At the top, the locations of four primers (P, Q, A and B) are represented by arrows. The upper box represents *Int22h1*, and the dashed lines indicate the flanking sequences. The lower box represents *Int22h2* and *Int22h3*, and the wavy lines indicate the flanking sequences. Deleterious inversions can occur by recombination between *Int22h1* and either *Int22h2* or *Int22h3* (dotted lines). The PCR amplifies overlapping and multiplex segments from genomic DNA with four primers P, Q, A and B to generate four potential segments PQ, AB, PB and AQ. Two primers, P and Q, are specific to the flanking sequences of *Int22h1*, located at -1212 bp before and at +1334 bp after the homolog; two primers, A and B, are specific to the flanking sequences of *Int22h2* and *Int22h3*, located at -167 bp before and at +118 bp after the homologs. P and Q anneal at different distances from A and B to differentiate PQ (12 kb), AB (10 kb) and PB+AQ (11 kb) segments on agarose gels. Genomic DNA from wild-type, male patient or female carrier samples produces three patterns of amplification. PQ is produced in a wild-type sample, PB+AQ is generated in males with the inversion and both PQ and PB+AQ are present in the carriers. AB is always produced and serves as a positive control, because at least one copy of *Int22h2* and *Int22h3* remains intact. S-PCR was performed with each primer at 0.2 μM. The relative copy numbers of segments PQ, AB and PB+AQ before amplification are indicated at the bottom, although some individuals have one or more extra copies of *Int22h2* or *Int22h3* located on the X chromosome or autosome.

Figure 3. Schematic of subcycling-PCR. Each cycle of S-PCR involves a denaturation step and an annealing/elongation step. The annealing/elongation step is composed of subcycles to shuttle among low and high temperatures. m = the number of shuttled temperatures within a subcycle ≥ 2 , n = the number of subcycles ≥ 1 , $m \times n > 3$.

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the PCR parameters important for the amplification (Figure 4 and Table 2). Successful amplification required 6%–9% DMSO, 25%–50% deaza-dGTP, and 1.25–10 U enzymes, with optimal results achieved at 5–10 U per 25 μ L reaction.

Analysis of several other DNA polymerase combinations [*Taq* and Vent[®] (Boehringer Mannheim and New England Biolabs, Beverly, MA, USA, respectively), eLONGASE[™] Enzyme mixture, which contains *Taq* and *GB-D* (Life Technologies, Gaithersburg, MD, USA), *Tth* and *Pwo* (Boehringer Mannheim), *Tth* and Vent, *Tfl* (Promega, Madison, WI, USA) and *Pwo*, *Tfl* and Vent and Bio-X-ACT[™] DNA Polymerase (Intermountain Scientific, Kaysville, UT, USA)] indicated that high concentrations of DMSO and deaza-dGTP were critical for all enzymes studied, while high enzyme concentration was helpful but not universally critical (Figure 4 and Table 3).

Subcycling-PCR Achieves Even and Efficient Amplification

Despite substantial efforts at optimization, segment AB was amplified much more efficiently than segments PQ, PB or AQ with standard three-temperature PCR (Figure 4A, lane 1). Preferential amplification is often a problem in multiplex PCR, especially if segments with divergent GC contents are amplified. Although even one nucleotide change in a sequence sometimes causes differential amplification (10), we reasoned that areas of low GC content in the 2.5 kb of the flanking sequences present in segment PQ could account for some or all of the differential efficiency of amplification. Since the optimal extension temperature varies with GC content (15), the annealing/elongation temperature was lowered. Much of the differential amplification was eliminated, but the yields of amplified product were both lowered and inconsistent with a faint spurious segment (Figure 5, lanes 1–6). To obtain high and relatively uniform yields, we developed S-PCR, which is characterized by “subcycling parameters” within the annealing/elongation step (Figure 3). With the subcycling parameters between 60° and 65°C, S-

PCR amplified the four segments more evenly and efficiently with the four primers from the carrier DNA template (Figure 5, lane 7). When the wild-type and inversion templates were used, the

same results were obtained. Similar results were obtained with a four-step subcycling program: two subcycles at 60°, 64°, 68° and 64°C for 2 min each, for a total of 16 min (data not shown).

Figure 4. (A) The effect of DMSO and deaza-dGTP. 0.2 μ M each of primers P, Q, A and B were used to amplify the carrier DNA template. Lanes 1 and 2: standard three-temperature PCR (94°, 65° and 68°C); lanes 3 and 4: three-temperature PCR, but the annealing temperature is lowered from 65° to 60°C; lanes 5–7: two-temperature PCR (94° and 65°C). The samples in the first of each pair of lanes were amplified with optimal concentration of DMSO (7.5%), deaza-dGTP (37.5%) and *Taq* and *Pwo* polymerase (2.5 U per 25 μ L reaction). In lanes 2 and 4, the maximum concentration of DMSO recommended by Boehringer Mannheim (2%), no deaza-dGTP and the maximum enzyme concentration recommended by Boehringer Mannheim (1.2 U). In lanes 6 and 7, amplification was performed as in lane 5, except with 2% DMSO (lane 6) or the absence of deaza-dGTP (lane 7). (B) The effect of enzyme concentration. Lanes 1–6, *Taq* and *Pwo* polymerase at 10, 5, 2.5, 1.25, 0.62 and 0.31 U per 25 μ L reaction, respectively; Lane 7–12, with *Tfl* and *Pwo* polymerase (30:1, U:U). Two-temperature PCR was performed with 7.5% DMSO and 37.5% deaza-dGTP. M = Size standard, only the 9.4-kb segment is shown (200 ng of λ DNA/*Hind*III).

Figure 5. The effect of annealing/elongation temperature and S-PCR. Primers P, Q, A and B were used to amplify the carrier DNA template with each primer at 0.2 μ M concentration. Two-temperature PCR was used with annealing/elongation temperatures varying from 69°–59°C. The PCR was performed with a RoboCycler[®] Gradient Temperature (Stratagene, La Jolla, CA, USA) with slightly modified periods of denaturation and annealing/elongation designed to be equivalent to amplification of the GeneAmp 9600 Thermal Cycler based on the manufacturer’s protocol. In lane 7, S-PCR was performed for comparison.

To compare standard three-temperature PCR with S-PCR, combinations of two, three and four primers were tested on the carrier templates. For standard PCR, virtually none of segments PQ, PB and AQ were amplified in carriers (estimated molar ratios of more than 20:1), when the molar ratios of the primers were equal. Multiple optimization experiments were undertaken, resulting in unequal primer concentrations of 0.4 μ M for primers P and Q, and 0.12 μ M for primers A and B. These results with optimized primer concentrations were compared with S-PCR utilizing (unoptimized) equal molar ratios of 0.2 μ M (Figure 6A). The relative yields with three primers in Figure 6A, lanes 4–8 were the most uneven with fourfold differences among the products, in comparison with two-fold differences in Figure 6A, lanes 14–18. Similar tendencies were observed when wild-type and inversion genomic DNAs were used (data not shown). The results with two-tempera-

ture PCR were intermediate relative to three-temperature PCR and S-PCR (data not shown).

Figure 6B shows the effects on the relative yields when the concentrations of only primers P and Q (lanes 1–6), of only primers A and B (lanes 7–12) or of all the four primers (lanes 13–18) were varied with carrier DNA as template. Small changes in the primer concentration caused dramatic effects on the relative yields of segments PQ, AB and PB+AQ with three-temperature PCR (Figure 6B, Panel a), but not with S-PCR (Figure 6B, Panel b). Again, two-temperature cycling produced results intermediate to three-temperature PCR and S-PCR (data not shown).

DISCUSSION

An analysis of the parameters affecting a multiplex PCR assay for the Hemophilia A inversion hotspot is presented. Efficient amplification of this

Figure 6. The interaction among the primers and the effect of primer concentration. (A) Two, three and four primers were utilized to amplify the carrier template. Standard three-temperature PCR was used in lanes 1–9. Concentrations of the four primers were 0.4, 0.4, 0.12 and 0.12 μ M, respectively. S-PCR was used in lanes 10–18. Each primer concentration was 0.2 μ M. (B) Carrier DNA template was used for all of the reactions. Lanes 1–6, 7–12 and 13–18 show the effect of varying the concentration of only P and Q primers, only A and B primers and all the four primers, respectively. (a) Standard three-temperature PCR. The 1 $\times$ concentration of the four primers were 0.4, 0.4, 0.12 and 0.12 μ M. (b) S-PCR. The 1 $\times$ concentration was 0.2 μ M for each primer.

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challenging region required very high concentrations of DMSO, the addition of deaza-dGTP and very high concentrations of DNA polymerases. An attempt to find robust conditions for obtaining relatively even amplification of the four segments of overlapping sequence led to the development of S-PCR, which is easier to optimize and is much less affected by the number of primers and their concentrations. In the present system, S-PCR efficiently and evenly amplified the multiplex and overlapping segments and was much less influenced by the primers utilized (PQA, PQB, PAB and ABQ vs. PQAB in Figure 6A) and primer concentrations (Figure 6B). The principle underlying S-PCR (subcycling the annealing/elongation temperature) potentially can provide more robust amplification under situations in which the GC content varies. Thus, S-PCR might facilitate (i) amplification of other long seg-

ments in which the GC content varies within the segment and (ii) multiplex amplification of segments in which the GC content of the different segments varies. S-PCR also can be a convenient starting point for optimizing the amplification of long segments of unknown GC content. Further work is required to test this hypothesis.

A few technical points deserve comment. Besides the PCR assay with four primers, two types of three-primer PCR assays, i.e., with primers P, Q and A and with primers P, Q and B also differentiated the wild-type, inversion and carrier states in a single reaction (e.g., Figure 6A). In addition, the amount of template DNA was a factor affecting the relative yields of the products (4). The PQ, AB and PB+AQ segments were amplified from the carrier DNA template from as little as 12.5 ng per 25 μ L of reactions. Finally, a blinded analysis of 40 DNA samples was performed (8). The PCR results were in complete concordance with Southern blot analysis for detection of wild-type, homozygotes and heterozygotes of the factor VIII gene inversion.

ACKNOWLEDGMENTS

We thank Bill Scaringe for developing the shuffling software, which was utilized to demonstrate random variation in GC content (Figure 1). We thank Dianne Kappas for excellent secretarial assistance. This work was partially supported by National Institutes of Health Grant No. RO1-HL39762.

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Received 20 May 1998; accepted 10 August 1998.

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