Long Distance PCR in Detection of Inversion Mutations of *F8C* Gene in Hemophilia A Patients

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Abstract. In the present paper, the experience with detection of intron 22 inversion of *F8C* gene in severe hemophilia A patients using a recently described long-distance PCR (LD-PCR) method was reported. To test the sensitivity and the specifity of the LD-PCR, analysis of 46 DNA samples of patients and their family members, previously tested by Southern hybridization, was performed. In addition, 16 DNA samples of severe hemophilia A patients in which causative mutation was unknown, were included in analysis. Four-primers, P, Q, A&B, which are able to differentiate between the affected males with or without the inversion, and in female carriers, were used in LD-PCR. Two primers, P&Q, are located within the *F8C* gene flanking int22h1. Two further primers, A&B, flank int22h2 and int22h3, extragene homologs of int22h1. Nine combinations of four primers were used to identify the optimal one. Four-primers (P, Q, A&B), three-primers (P, Q&B; P, A&B; A, B&Q; P, Q&A) and two-primers (A&B; P&Q; A&Q; P&B) PCR amplifications were performed in the hemophilia A patients as well as in obligate carriers DNA samples. Successful amplification required introduction of some modifications of the original protocol. The most reproducible and uniform results were obtained using two-primers PCR, performed in four single reactions. Thus, a total of 46 DNA samples, 22 were hemizygous for inversion, 6 without the inversion, 14 carriers and 4 non-carriers of inversion. Perfect correlation between genotypes determined using Southern hybridization and LD-PCR was achieved. The optimalized two-primers LD-PCR protocol was used for analysis of 16 DNA samples of severe hemophilia A patients with unknown mutation. Ten cases of inversions and six cases without them were detected. Thus in additional 10 severe hemophilic patients DNA diagnosis was completed. The most successful and reproducible results were obtained performing four single LD-PCR reactions with combinations of two-primers A&B; P&Q; A&Q, and P&B in each DNA sample and this approach is recommended for routine using in clinical practice.

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Key words: Hemophilia A — Inversion — Long distance – PCR — *F8C* — DNA diagnosis

Introduction

Hemophilia A is an X-linked recessive bleeding disorder, caused by a deficiency in an activity of a coagulation factor VIIIC (*F8C*) gene, affecting 1 in 5,000 to 10,000 male births (Antonarakis et al. 1985). *F8C* gene (located at band Xq28) is 186 kb long and has 26 exons that code for the FVIII protein, consisting of 2332 amino acids (Gitschier et al. 1984). Unrelated patients were generally expected to carry different mutations, and supporting this, a variety of *F8C* gene defects has been described (Tuddenham et al. 1994; Antonarakis et al. 1995; HGMD 2003). Most of the mutations are private, they have been found in one or only in a few unrelated families. Recent studies have disclosed a large DNA inversion resulting from a homologous intrachromatid or intrachromosomal recombination between a 9.5 kb region in intron 22 (int22) of *F8C* gene (int22h1) and one of two almost identical copies (int22h2 and int22h3) located about 500 and 600 kb telomeric to the *F8C* gene (int22h2 and int22h3 are more than 99 % identical to each other). Recombination produces an inversion because the extragenic homologs are in the opposite orientation relative to int22h1 (Lakich et al. 1993; Naylor et al. 1993a,b, 1995). The inversions can be detected by Southern hybridization analysis after *Bcl*I digestion of genomic DNA and by probing with the 0.9 kb *EcoR*I/*Sst*I fragment of plasmid p482.6, region derived from int22 and containing part of the int22h sequence (Levinson et al. 1990, 1992). Several studies using this assay have confirmed that the *F8C* gene inversions are present in about 50 % of patients with severe hemophilia A (Higuchi et al. 1991; Lakich et al. 1993; Naylor et al. 1993 a,b; Antonarakis et al. 1995; Becker et al. 1996; Poláková et al. 1998).

Although Southern hybridization is reliable in detecting the inversions, this method is time consuming and labor intensive. Until 1998 successful polymerase chain reaction spanning the 9.5 kb region of int22h has not been reported, presumably because the regions contain a 3.5 kb island of 65 % GC content and a 1 kb region of 79 % GC content within the GC island (Liu and Sommer 1998). In addition, the major difficulty was the insufficient knowledge of flanking sequences to design the polymerase chain reaction (PCR) primers. Additional DNA sequences flanking int22h2 and int22h3 were reported by Liu and Sommer (1998) and Liu et al. (1998). Four-primers P, Q, A&B were designed. P&Q is specific for 5′- and 3′-flanking region of int22h1 of *F8C* gene, respectively. A&B is specific for 5′- and 3′-flanking regions of int22h2 and int22h3, respectively. They developed PCR technique based on combination of long distance PCR (LD-PCR) (Cheng et al. 1994) and overlapping PCR (Liu et al. 1997) to replace Southern hybridization. Figure 1 A. shows schematically the relative position of the primers. Primers P&Q produced 12 kb segment, A&B 10 kb segment and P&B and A&Q 11 kb (each) segments on agarose gels. A&B segment was always produced and served as a positive control,

because at least one copy of int22h2 and int22h3 remains intact. LD-PCR protocol for detection *F8C* inversion differs from standard long range PCR protocols. Modifications include the addition of high concentration of dimethyl sulphoxide (DMSO) and incorporation of 7-deaza-dGTP to enable reading through the high GC content region upstream of the factor *F8C* gene, and substantially increased amounts of Taq and Pwo DNA polymerases. Additional modifications included adjusting cycling conditions and other components of PCR reaction (Liu and Sommer 1998; Liu et al. 1999).

At the present time, several reports were published to examine *F8C* gene inversion mutations by recently described PCR in severe hemophilia A patients (Liu and Sommer 1998; Liu et al. 1998, 1999; Vidal et al. 2001; Xuenfeng et al. 2001; Liu et al. 2002). However, LD-PCR protocols used in many laboratories differ each other and for these reasons implementation of this method for routine clinical usage requires some modifications. In this paper we present the results of the detection of inversion mutations by LD-PCR method in the sample of severe hemophilia A patients. The aim of this report was to establish the optimal LD-PCR protocol to be used for routine detection of the inversions in hemophilia A patients, in carriers testing, as well as in the prenatal diagnosis in severe hemophilia A families.

Materials and Methods

The 28 unrelated patients and their 18 family members, previously typed by Southern hybridization, were included in the analysis of int22 inversions by the LD-PCR method. The patients included in the study were of Slovak origin and suffered from severe hemophilia A. Twenty-two of them were positive for the int22 inversion, and 6 were without it, as detected by Southern hybridization. Family members included 14 carriers and 4 non-carriers of inversion. Analysis also included additional 6 female DNA samples coming from non-hemophilic families that served as a control.

After optimization of reaction conditions and introducing the optimal PCR protocol, detection of int22 inversion for 16 unrelated severe hemophilia A patients was performed in which causative mutation was unknown. Six were familial and 10 sporadic cases without family history.

Blood samples were provided by Institute of Hematology and Blood Transfusion in Bratislava and by Departments of Clinical Genetics in Slovakia.

DNA samples

Genomic DNA was extracted from EDTA anticoagulated peripheral blood by phenol chloroform extraction according to Kunkel et al. (1977). Included were only DNAs with fragment length of 50 kb or more. The quality and length of each DNA was evaluated spectrophotometrically and by agarose gel electrophoresis.

Intron 22 inversion PCR

LD-PCR was performed using Expand long template PCR kit (Roche, Mannheim, Germany) by modifications of the manufactures instructions and the original protocol of Liu et al. (1998). Primers P, Q, A&B (Life Technologies, Wien, Austria) according Liu and Sommer (1998) and Liu et al. (1998, 1999) were utilized to amplify the patients and carriers DNA. Sequences of the primers are shown in Table 1. In the experiments nine PCR reactions *per* DNA sample were done, in which the different combinations of two (A&B, P&Q, A&Q and P&B), three (P, Q&B; P, Q&A; P, A&B and A, B&Q) or four (P, Q, A&B) primers were used in single-tube PCR reaction. A total of 25 μl reaction volume contained 100 ng genomic DNA, 2.5 μl of buffer 2 (Expand long template PCR kit), 7.5% DMSO (dimethyl-sulphoxide), 310 μmol/l of dGTP (Life Technologies) and 190 μmol/l of 7-deaza-dGTP (Amersham, Biosciences, Wien, Austria), 500 μmol/l of each of the other dNTPs (Life Technologies). In three and four-primers LD-PCR the concentration of the primers P&Q and A&B was 0.4 μmol/l and 0.12 μmol/l, when three-temperature PCR was performed, respectively. In the case of subcycling PCR the concentration of primers was 0.2 μmol/l of each. Performing two-primers LD-PCR in four single reactions, primers concentration was 0.2 μmol/l of each, either three-temperature or subcycling PCR was done. The amount of enzyme used (Expand long template DNA polymerases) was 1.75 units when two-primers LD-PCR, and 2.5 units when three or four-primers LD-PCR was used, independently on the type of cycling conditions. Two separate mixes were prepared on ice, one containing distilled water, template DNA, primers, nucleotides and DMSO and the another containing water, buffer and Taq mixture. All components of PCR, reaction mixes and samples were kept on ice. The two mixes were subsequently combined in an amplification tube on ice, overlaid with mineral oil and transferred to a pre-heated thermal cycler (95°C) and immediately amplified. Subcycling PCR (S-PCR) and three-temperature PCR according to Liu and Sommer (1998) and Liu et al. (1998) was performed in a thermocycler Biometra (Biotron, Göttingen, Germany), using 0.2 ml thin-wall tube. Conditions for S-PCR were: initial denaturation at 95°C for 2 min; the first 10 cycles were: 95°C for 30 s followed by four subcycles within

Table 1. Oligonucleotide sequences

Name	**Sequence No.**	
P	5′ GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC 3′	38 mer
Q	5′GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA3′	39 mer
A	5′ CAC AAG GGG GAA GAG TGT GAG GGT GTG GGA TAA GAA 3′	36 mer
B	5′ CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT 3′	40 mer

the annealing/elongation step alternating temperature between 60 ℃ for 2 min and 65 ℃ for 2 min for each subcycle. The remaining 20 cycles included the addition of extra 3 s *per* cycle for each annealing/elongation step. The cycling conditions for three-temperature PCR were: 95 ℃ for 2 min initial temperature, followed by 10 cycles of 95 ℃ for 30 s, 65 ℃ for 30 s and 68 ℃ for 14 min. The remaining 20 cycles included adding 20 s to each elongation step. A final extension at 68 ℃ for 7 min was performed. About 15 μl of the PCR product was loaded onto a 0.6 % ethidium bromide stained agarose gel, and electrophoresed at 70 V for 18 to 20 hours to check for the presence of the desired product. The length of the gel was 22 cm.

Results and Discussion

Analysis of int22 inversions should be the first-line test in DNA diagnostics, carrier detection and genetic counseling for severe hemophilia A, and could be particularly useful when there is no affected male family member, or when intervening family members are unavailable for testing. Until recently the detection of int22 inversions was performed by standard Southern hybridization. Recently LD-PCR has been described. As mentioned earlier, the published LD-PCR protocols used at many laboratories differ significantly each other. For this reason, at the beginning of the present work, our first aim was to test the specifity of LD-PCR. After checking the length of the DNA by agarose gel electrophoresis, 46 DNA samples, earlier tested by Southern hybridization were included in the testing of the specifity of LD-PCR. Twenty-two male patients were hemizygous for the inversion, in 6 male patients there was no inversion detected, 14 females were carriers, and 4 were non-carriers, as determined by standard Southern hybridization method (Poláková et al. 1998). Six additional non-hemophilic DNA samples served as a negative control. At the beginning, LD-PCR was realized according to the original protocol of Liu and Sommer (1998) using Expand long template PCR kit. Four primers P, Q, A&B in single-tube PCR were used utilizing the three-temperature PCR. Although, sticking to the described protocol, no reliable results were obtained (high back-ground smear or no product). As mentioned in the previous papers the LD-PCR appeared sensitive to the characteristics of the thermocycler; thus optimal protocols would differ according to the types of cyclers. It is recommended to use a thermal cycler that measures temperature in the reaction tube, rather than in the heating block. LD-PCR described in this report was performed in the cycler that measures the temperature in the heating block. For these reason, at first we optimalized the denaturation temperature and the amount of enzyme. Several DNA polymerase combinations were tested. The best results were achieved with Expand long template DNA polymerases. However, titration of the enzyme amount (between 1.75 and 3.5 units was performed – data not shown) was necessary to get optimal results. The best results were achieved at following conditions: raising the initiation denaturation temperature from 94 ℃ for 30 s to 95 ℃ for 2 min and

cycling denaturation temperature from 94 ℃ for 12 s to 95 ℃ for 30 s, lowering the amount of the enzyme for three-temperature PCR from 3.3 units to 1.75 when two-primers PCR, and to 2.5 units when three or four-primers LD-PCR was performed. In addition, 0.2 μmol/l each of primers was used when two-primers LD-PCR was done, either using three-temperature or S-PCR conditions. As reported by Liu and Sommer (1998), the amount of template DNA influences LD-PCR. In the present work amount of template DNA of good quality in range from 50 to 250 ng did not influence the relative yields of the products. Optimal results were achieved with 100 ng DNA of good quality.

After introducing mentioned modifications, LD-PCR was repeated, using the same DNA samples and four-primers P, Q, A&B, in a single-tube reaction utilizing three-temperature PCR. From a total of 46 DNA samples, 22 showed a smear extending down the gel or no product was observed. PCR products of desired length were noticed in only 24 DNA samples. However, in all of them, occasionally preferential amplification of one of three alleles was identified, overlapping the other alleles. Preferential amplification is often a problem in multiplex PCR, particularly if regions with divergent GC content are amplified (Henegariu et al. 1997). It is the region of int22h with 30 %–80 % varying GC content that makes the amplification of long fragments very difficult. In the multiplex PCR, interactions among the primers affect both the yield and the specifity of the reaction. In general, the greater the number of primers added to the reaction, the lower the yield of the individual fragments. The optimal concentrations of the primers P&Q and A&B recommended by Liu and Sommer (1998) and Liu et al. (1998, 1999) are 0.4 μmol/l and 0,12 μmol/l, respectively. In addition, the type of genomic template used in the reaction may influence the efficiency of accumulation and the final amount of product produced. In the samples of non-carriers, individual yields were greater in comparison to the carriers (Cheng et al. 1994; Shuber et al. 1995; Henegariu et al. 1997; Liu et al. 1997). In an attempt to find conditions for obtaining even amplification of four segments of overlapping sequences, Liu et al. (1998) developed S-PCR. According to the authors under the cycling conditions of S-PCR the number and concentrations of the primers much less affected the efficiency of amplification multiplex and overlapping segments. An attempt to achieve uniform amplification of desired fragments all DNA samples were retested using S-PCR. The results of four-primers LD-PCR in single-tube using thermal conditions of subcycling LD-PCR are seen in Figure 1B. The analyzed DNAs were derived from two hemophilic patients, one hemizygote for the inversion, one without it and two carriers of the inversions. Using all four-primers simultaneously in a single-tube PCR, genomic DNA without the inversion, male patients or female carriers samples produced three patterns of amplification. An upper 12 kb band, and a lower 10 kb band only meant that the inversion was not present (lane 2). Middle 11 kb band, and a lower 10 kb band meant that the inversion was present in an affected male (lane 1). Bands 10 kb, 11 kb and 12 kb indicated a female carrier of the inversion (lanes 3 and 4).

Although more uniform results were obtained, in the majority of DNA samples without inversion, besides the 10 kb and 12 kb fragments, also a nonspecific

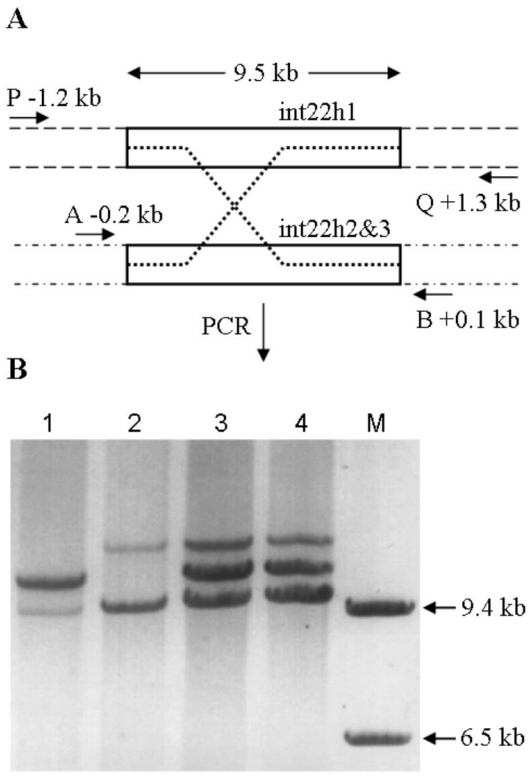

Figure 1. Scheme of primer location and length of LD-PCR products. **A.** The upper box represents int22h1, the lower box represents int22h2 and int22h3. The dashed lines indicate the flanking sequences. Primers P&Q are specific to the flanking sequence of int22h1 and produce 12 kb segment in DNA of male patients without the inversion and also in DNA of carriers and non-carriers. Primers A&B are specific to the flanking sequences of int22h2 and int22h3 and produce 10 kb segment. A&B segment is always produced and serves as positive control, because at least one copy of int22h2 and int22h3 remains intact. Inversion can occur by recombination between int22h1 and either int22h2 or int22h3 (dotted lines) and thus each P&B and A&Q produce 11 kb segments resulted from the inversion recombinant gene from male patients or female carrier samples (from Liu and Sommer 1998). **B.** Patterns of four-primers LD-PCR in single tube reaction by cycling conditions of S-PCR. Lane 1 shows 10 kb and 11 kb fragments from male patient with inversion; lane 2 shows 10 kb and 12 kb fragments from male patients without the inversion; lane 3 shows 10 kb, 11 kb and 12 kb fragments from DNA of female carrier; lane 4 represents a control female carrier of inversion; M, marker the 9.4 and 6.5 kb bands from lambda DNA /HindIII digest.

slight 11 kb long fragment was observed. Such result prevented the precise genotyping, especially among the carriers of inversions. The following testing included three-primers PCR, which also differentiated DNA without the inversion, with the inversion and carrier states in a single-tube reaction. All DNA samples were submitted to an amplification using primers of the following combinations: P, A&B and A, B&Q and P, Q&A and P, Q&B, under the conditions of S-PCR. Included were also DNA samples, which did not amplify, in four-primers LD-PCR. Primers P, Q&A and P, Q&B gave 2 bands in carriers and one band in hemizygotes (11 kb and 12 kb and 11 kb, respectively, data not shown), while both P, A&B and A, B&Q produced 10 kb and 11 kb bands in patients as well as in carriers, respectively (Figure 2, lanes 1, 2 and 3). Although Liu et al. (1999) recommended as an initial

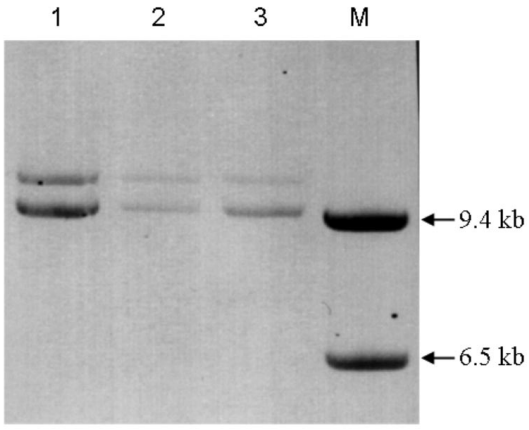

Figure 2. Results of LD-PCR with three-primers. Figure demonstrates results of three-primers LD-PCR of male patients. Only results of PCR with primers A, P&B are shown. Three-temperature LD-PCR was performed. Lanes 1–3 show 10 kb and 11 kb fragments indicating that inversion is present; M, marker the 9.4 kb and 6.5 kb bands from lambda DNA/HindIII digest.

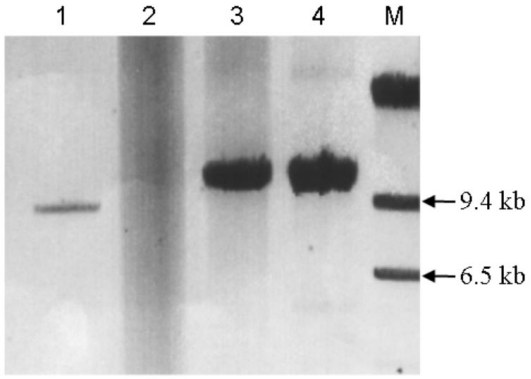

Figure 3. Pattern of two-primers LD-PCR and genomic DNA from a patient with *F8C* gene inversion. In lane 1, LD-PCR was performed with primers A&B and produced 10 kb fragment, in lane 2 with primers P&Q and no product was observed (P&Q primers flank the int22h1 region which is involved in recombination), in lane 3 with primers A&Q and in lane 4 with primers P&B, 11 kb fragments were produced in both reactions; M, marker the 9.4 kb and 6.5 kb bands from lambda DNA/HindIII digest. S-PCR thermal conditions were performed.

step in diagnosis to use three primers (P, Q&A and P, Q&B), in the majority of our DNA samples, only one of two alleles was clearly detectable in the gel.

When summing up the results of three or four-primers LD-PCR, a total of 46 DNA samples, in only 25 the unambiguous results were obtained.

The remaining 21 unsuccessful DNA samples were subjected to two-primers LD-PCR using combinations of primers A&B, P&Q, A&Q, and P&B, respectively. Figure 3 shows the results of LD-PCR of a male hemophilia A patient. Primers A&B (lane 1) gave 10 kb band (as a control of PCR), P&Q (lane 2) did not give any band; both A&Q and P&B primers (lane 3 and 4) produced 11 kb bands as expected. Successful amplification was observed in 16 of them. Probably, some impurities contained in the remaining non-amplifying DNA samples inhibited LD-PCR.

As described in the published papers, convential phenol-chloroform preparation of genomic DNA will allow amplification of fragments up to 10 kb. For larger targets it is recommended to use ultra pure and high molecular weight DNA (Cheng et al. 1994, Liu et al. 1998, 1999, 2002). In the present work phenol-chloroform extraction of DNA was used in all DNA samples, and is commonly used in our laboratory. In addition, the majority of all analyzed DNA samples were long-term stored and repeatedly frozen and refrozen. As mentioned in several PCR guidelines and publications, freeze-thaw cycles shear DNA, especially very long DNA. It is recommended always store the template DNA at 4℃. Previous facts could contribute to the unsuccessful amplification of mentioned DNA samples. DNA diagnosis of these non-amplifying DNAs was completed by analysis of the DNA of patient's mothers, who were obligate carriers.

Moreover, all amplification protocols accent to use of template DNA with fragments larger than 50 kb. In some of the tested DNA samples, successful amplification was achieved with DNA of lower molecular weight, 40 kb and more, when four two-primers LD-PCR were done. However, such DNAs should be of good quality (data not shown).

In summary, after optimization of conditions for LD-PCR and summing up the analysis of various combinations of primers, the results were completely identical with the results determined by the standard Southern hybridization method.

On the basis of the experience, gained by analysis of four-, three- and two-primers LD-PCR, and including modifications mentioned above, detection of int22 inversion in 16 severe hemophilia A patients, previously untested by Southern hybridization, was carried out by two-primer approach. Four PCR reactions were performed for each DNA sample of the patients, using single combinations of the primers A&B, P&Q, A&Q and P&B. Thermal conditions of S-PCR and reaction mixes described in Materials and Methods were used. All PCR reactions were successful. Analyzing 16 severe hemophilia A patients, in 10 cases the inversions were detected, 6 cases showed no inversions.

In conclusion, tremendous effect on the success of amplification had quality and length of the template DNA. Some DNAs of high molecular weight repeatedly showed a smear, despite of optimal LD-PCR conditions and combinations of primers. Generally, degraded or sheared DNA did not amplify. The results obtained using thermal conditions of S-PCR were more successful, than with three-temperature LD-PCR, either performing four, three or two-primers LD-PCR (data not shown).

As a general rule, in the majority of cases, good results were obtained performing three-primers LD-PCR using three-temperature or S-PCR conditions (Figure 2, lanes 1, 2 and 3). However, following the experience in the first part of the present work, the best and more reproducible results were obtained performing two-primers PCR, rather than four or three-primers PCR. For this reason, two-primers LD-PCR carried out in four PCR reactions *per* patient, following the protocol described above is recommended for the routine usage in a clinical practice.

In conclusion the most important advantage of this method is, that positive

results are unequivocal, providing a definitive diagnosis of hemophilia A. Compared to Southern hybridization, the technique is simple, rapid, less expensive, more sensitive, and does not require to use of the isotopes.

Acknowledgements. This project was partially supported by Slovak Grant Agency VEGA (grant No. 2/120494). The authors are grateful to all Departments of Clinical genetics in Slovakia for their kind cooperation and help by providing the blood samples and to Ildikó Szomolay for technical assistance.

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Final version accepted: March 14, 2003