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Multiplex PCR for Screening of Microdeletions on the Y Chromosome

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Purpose: *The aim of this study was to develop a multiplex PCR protocol, which could be suitable for screening of microdeletions in the three azoospermia factor (AZF) regions on the Y chromosome.*

Methods: *In the screening protocol, 36 known sequence tagged site (STS) primer pairs were first tested in single PCR reactions and thereafter combined in multiplex PCR to test for specificity and sensitivity in order to develop a stable and reliable multiplex PCR assay to detect Y microdeletions.*

Results: *Of the 36 primers tested, 11 turned out not to be specific or produced PCR products that were too weak, and they were therefore not used in the multiplex PCR. The remaining 25 STSs were selected on the basis of their ability to be reproducibly amplified with each other using identical amplification conditions. Five multiplex sets, each consisting of five primer pairs, were established in the multiplex PCR setup.*

Conclusion: *The multiplex PCR protocol presented in this study is an easy and reliable method for detection of Y chromosome microdeletions and could be used for screening of infertile men to allow genetic counseling about the risk of transmitting infertility from father to son.*

KEY WORDS: azoospermia factor; male infertility; microdeletions; multiplex PCR; Y chromosome.

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INTRODUCTION

Infertility affects about 15% of all couples attempting pregnancy, with a male factor being responsible in approximately 35–40% (1–3). The pathophysiology of the underlying male-factor infertility often remains uncertain. However, several recent reports have established the relationship between severe male-factor infertility and partial deletions of the Y chromosome (4–9). Cytogenetic and molecular deletion analyses of azoospermic and oligozoospermic males have suggested the existence of an azoospermia factor (AZF) region coinciding with three functional regions (AZFa, AZFb, and AZFc) associated with spermatogenic failure. The AZF region resides in intervals 5 and 6 of the human Y chromosome (10–12).

Initial discoveries of the Y chromosome deletions in infertile men were made through karyotype analysis where deletions of Yq11 were observed (13). However, deletions on the Y chromosome are often too minute to be detected by standard karyotype evaluation. Today PCR application using sequence tagged sites (STSs) has made it possible to identify microdeletions on the Y chromosome. The number of STSs used for detection of Y chromosome microdeletions varies in different studies from 1 to 131 (7,8,14–23). The microdeletion frequency has been found to range between 1 and 58.3% in infertile men (7,8,14–26). This wide range of Y chromosome microdeletions may be partly explained by the different numbers of primers and methods that have been used in these studies. However, although numerous clinical papers have been published on the frequency of Y chromosome

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microdeletions, methodological details are usually not clearly described.

In the present paper we report the design and construction of five unique multiplex PCR sets, which include 25 Y-linked STSs for screening of microdeletions on the Y chromosome. Furthermore, we evaluate the various conditions which can improve this multiplex PCR setup.

MATERIALS AND METHODS

Genomic DNA was extracted from whole blood and processed using digestion of cellular proteins and subsequent salting-out of the proteins with sodium chloride and ethanol precipitation of DNA (27).

Multiplex PCR Conditions

The following describes the optimized PCR conditions. The parameters marked with an asterisk have been varied to obtain maximum specificity and sensitivity; see Results.

Five hundred nanograms of genomic DNA* was added to a mixture of 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl (Perkin Elmer, NJ), 1.5 m*M* $MgCl_2$ (Perkin Elmer), 0.4 m*M* each dNTP (Boehringer Mannheim, Germany), 5 IU of Ampli Taq Gold DNA polymerase* (Perkin Elmer), and an 0.8 μM concentration of each primer* and adjusted with redistilled H_2O to a final volume of 50 μl. Amplifications were carried out on a Thermocycler (Gene Amp 9700; Perkin Elmer) with the following program: initial denaturation at 95°C for 10 min and subsequent series of 45 cycles at 94°C for 45 sec (denaturation), 60°C for 1 min (annealing), and 72°C for 2 min (extension). A final extension was carried out at 72°C for 7 min. Samples were electrophoresed on a 2% agarose gel (Agarose MS; Boehringer Mannheim) prepared in 1 × TBE buffer containing ethidium bromide at a concentration of 1 μg/ml (Sigma, St. Louis, MO) with 100 V for 90 min at room temperature. Gels were photographed with the Gel Doc 2000 gel documentation system (Bio-Rad, CA).

Multiplex Sets and Primer Sequences

PCR amplification of 25 STSs was performed in 5 multiplex sets, each including 5 primer pairs. The same amplification condition for each multiplex set was used as described above. A set of 25 specific STSs that span the euchromatic region of Y chromosome was combined to cover the AZFa, -b and -c regions reported previously to be associated with spermatogenetic defects. All the STSs have been described previously (4,7,8,28–31). The locus designation, PCR primer sequence, and PCR product size for each STS are listed in Table I.

Primers in each multiplex set were combined so that the amplified products differed in size from adjacent STSs by at least 10 bp.

Internal Quality Control

Female genomic DNA, which controls for specificity and for contamination, was used as a negative control. To control for reagent contamination a sample with PCR reaction mixture without DNA added was used. As a positive control a DNA sample was used which was known to present all 25 PCR products after amplification in the five multiplex PCR sets. Furthermore, to distinguish a negative result from a technical failure, a STS for SRY (sY14), which is localized in Yp, was used as a control primer.

A patient sample was considered positive for the given STS when a PCR product of the expected size was present but considered negative if a product of the expected size was not obtained after three individual PCR attempts.

RESULTS

Optimization of Multiplex PCR Conditions

For the establishment of a multiplex PCR, 36 different primer pairs were tested separately to determine specificity and sensitivity. Eleven primer pairs were either not sensitive or not specific enough for further use to establish a multiplex PCR. The remaining 25 primer pairs were used to construct 5 multiplexes, each consisting of 5 primer pairs, which should fulfill the following criteria: (a) the reaction conditions for each of the 5 multiplex sets should be identical, and (b) PCR products of the 5 STSs in each multiplex set should give strong and specific bands well separated from each other.

To fulfill the criteria, many multiplex sets were tested. While developing our method, multiplex PCR sets with four primer pairs were easily managed. However, it was rather difficult to find a suitable fifth primer pair for each multiplex set. We tried many primer pairs to develop five multiplex PCR sets with five primer pairs, which were working under the same amplification conditions. Part of this experiment is presented in Fig. 1. Only one set of multiplex PCR

Table I. Multiplex PCR Analysis Scheme Used for Detection of Y Microdeletions

Multiplex set	STS	Left primer	Right primer	Base pairs	Location
PCR I	sY14	GAATATTCCCGCTCTCCGGA	GCTGGTGCTCCATTCTTGAG	472	1
	sY132	GAGAGTCATAATGCCGACGT	TGGTCTCAGGAAGTTTTTGC	143	6A
	sY84	AGAAGGGTCTGAAAGCAGGT	GCCTACTACCTGGAGGCTTC	326	5C
	sY152	AAGACAGTCTGCCATGTTTA	ACAGGAGGGTACTTAGCAGT	125	6C
	sY272	GGTGAGTCAAATTAGTCAATGTCC	CCTTACCACAGGACAGAGGG	93	6E
PCR II	sY269	CTCTGGGACAAGTGTTCCTTG	CATTGGCATGAATGTGTATTCA	94	6E
	sY139	TTCAGAGGAATCATGTGGGT	AATGTTTCATCACCATTATCCC	120	6B
	sY153	GCATCCTCATTTTATGTCCA	CAACCCAAAAGCACTGAGTA	139	6C
	sY155	ATTTTGCCTTGCATTGCTAG	TTTTTAAGCCTGTGACCTGG	349	6D
	sY138	CACATGAAGCACTGGAACTG	AGGGCCTGAGTCTCCAGG	170	6B
PCR III	sY160	TACGGGTCTCGAATGGAATA	TCATTGCATTCCTTTCCATT	236	7
	sY143	GCAGGATGAGAAGCAGGTAG	CCGTGTGCTGGAGACTAATC	311	6B
	sY144	TCATCTGCCACCATCAACAT	ACGTGTTTCTACACCTGCCC	143	6C
	sY255	GTTACAGGATTCGGCGTGAT	CTCGTCATGTGCAGCCAC	126	6D
	sY254	GGGTGTTACCAGAAGGCAAA	GAACCGTATCTACCAAAGCAGC	350	6D
PCR IV	sY243	GTTTCTTCATAAGCAACCAAATTG	CAGATTATGCCACTGCCCTT	118	6E
	SPGY	TTTCACATACAGCCATTAAGTTTAGC	CAATTTTGATAGTCTGAACACAAGC	400	6D
	RBM1	ATGCACTTCAGAGATACGG	CCTCTCTCCACAAAACCAACA	800	6B
	sY273	GGTCTTTAAAAGGTGAGTCAAATT	AGACAGAGGGAACTTCAAGACC	93	6E
	sY164	AATGTGCCCACACAGAGTTC	TGGAAGACCAGGATTTCATG	590	6A
PCR V	sY117	GTTGGTTCCATGCTCCATAC	CAGGGAGAGAGCCTTTTACC	262	5Q–6A
	sY166	GAACTCCAATCATTCCCTGA	TTGGCTCTACTTTTCCCCTT	115	6F
	sY150	GGGAGAGTCACATCACTTGG	TTGAATTATCTGCCTGAGTGC	158	6C
	sY277	GGGTTTTGCCTGCATACGTAATTA	CCTAAAAGCAATTCTAAACCTCCAG	310	6D
	sY158	CTCAGAAGTCCTCCTAATAGTTCC	ACAGTGGTTTGTAGCGGGTA	231	6D

with 5 primer pairs worked well (lane 3) among 11 primer pairs tested as being the fifth primer in the multiplex PCR. This is included in our method as multiplex set 1 in Table I. The other four multiplex sets with five primer pairs presented in Table I (multiplex sets 2–5) were generated by experiments similar to that shown Fig. 1. In conclusion, we managed to develop five multiplex PCR sets, which worked successfully with 25 primer pairs.

An example of multiplex PCR screening of DNA extracted from peripheral blood of two oligozoospermic men (from a larger series to be published) using the five multiplex sets presented in Table I is shown in Fig. 2. Twenty-five bands representing 25 amplified DNA fragments from the AZF region by multiplex PCR were observed testing the DNA of the first oligozoospermic man, indicating that no Y microdeletions were present (Fig. 2A). In the second oligozoospermic man 12 of 25 amplified DNA fragments from the AZF region (represented by asterisks) were absent, indicating microdeletions on the Y chromosome (Fig. 2B). The microdeletion spanned the region from 6A to 6D.

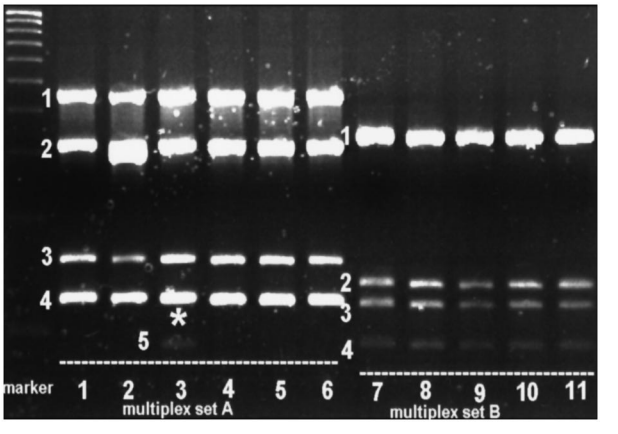

Fig. 1. Finding a suitable fifth pair for each multiplex set. Two sets of multiplex PCR (A—sY152, sY132, sY84, sY14; B—sY269, sY139, sY153, sY155), each consisting of four different oligonucleotide primers, were successfully performed. Six and five different primer pairs were tested as an additional fifth primer pair for multiplex sets A and B, respectively, to increase the combination of primer pairs in the multiplex PCR sets. The added fifth primer pair did not work in the quadruplex groups except in lane 3 (sY272), represented by the asterisk. The DNA ruler, which shows 10 bands ranging from 100 to 1000 bp, with 100-bp increments, was used as a marker.

Amount of DNA Template

DNA template concentrations of 250 and 500 ng were tested in the multiplex PCR. The best results were obtained with the DNA template concentration of 500 ng, and this concentration was used in further studies.

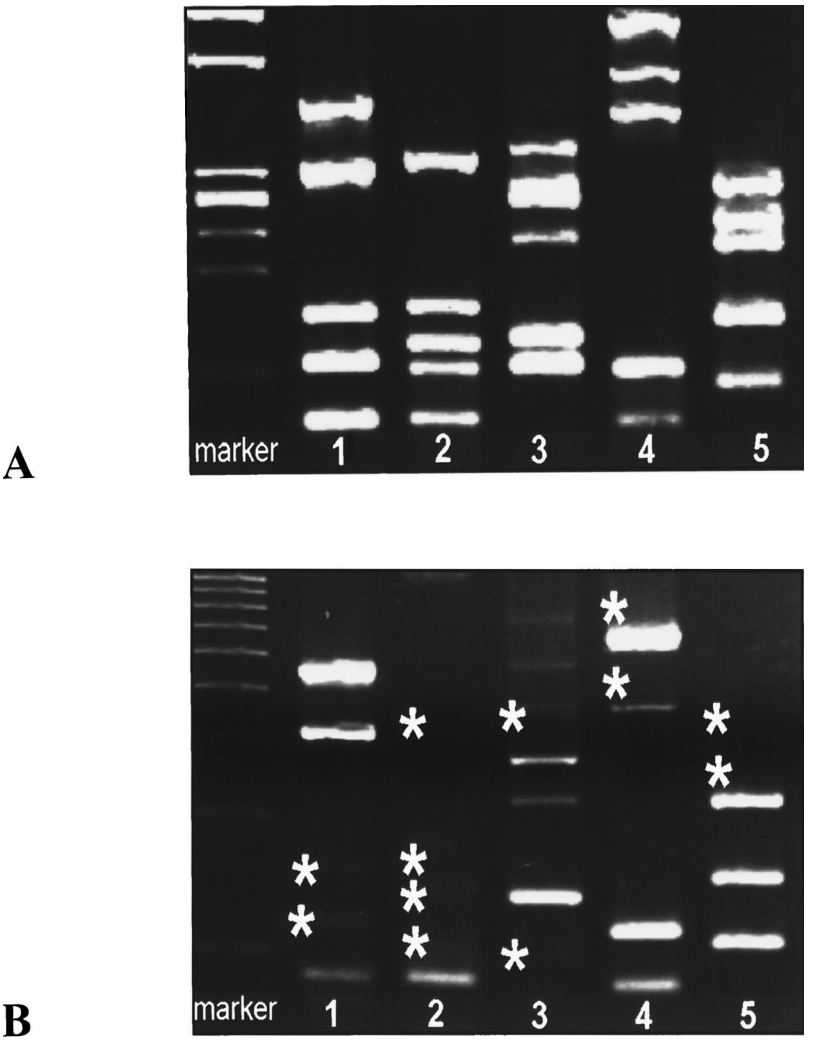

Fig. 2. Multiplex PCR products amplified using primer sets 1–5 as detailed in Table I, in two oligozoospermic men. Twenty-five bands representing 25 amplified DNA fragments from the AZF region by five multiplex PCR sets were demonstrated in the first oligozoospermic man (A). The DNA marker, which shows the seven upper bands, with sizes of 807, 603, 310, 282–271, 234, 194, and 118 bp, was used. In the second oligozoospermic man 12 of 25 STSs (in multiplex set I, sY152 and sY132, in multiplex set II, sY139, sY153, sY138, and sY155; in multiplex set III, sY255 and sY254, in multiplex set IV, SPGY and RBM1; in multiplex set V, sY117 and sY277) were found to be deleted (represented by asterisks) in the five sets of multiplex PCR (Fig. 2B). The DNA ruler, which shows 10 bands ranging from 100 to 1000 bp, with 100-bp increments, was used as a marker.

Primer Concentration

The optimal concentration of the primers used in the multiplex PCR was established empirically. Initially, equimolar primer concentrations of 0.4, 0.8, and 1.6 μM were tested in the multiplex PCR, respectively. Some of the PCR products were barely visible with the primer concentration of 0.4 μM, whereas nonspecific PCR products were obtained with the use of the 1.6 μM primer concentration in the multiplex PCR sets. However, with the primer concentration of 0.8 μM all 25 STSs in the five multiplex PCR sets gave rise to strong and specific PCR bands.

DNA Polymerase in the Multiplex PCR

The use of AmpliTaq Gold DNA polymerase can reduce or eliminate the generation of nonspecific

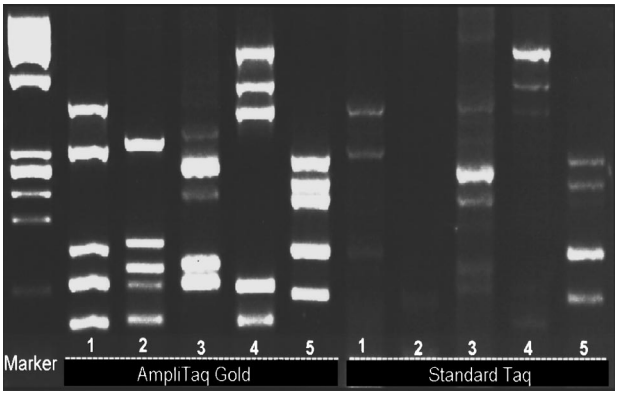

Fig. 3. AmpliTaq Gold DNA polymerase was compared with the standard Taq DNA polymerase. The same DNA sample was analyzed using the AmpliTaq Gold and standard Taq DNA polymerase, respectively, in the multiplex PCR. A significant enhancement in the yield of PCR products was obtained and nonspecific PCR products were eliminated in all multiplex sets with the use of AmpliTaq Gold. The DNA marker, which shows the nine upper bands with sizes of 1353, 1078, 872, 603, 310, 282–271, 234, 194, and 118 bp, was used.

PCR products that can result from mispriming and primer oligomerization when using several primers, suboptimal reaction conditions, and/or reactions set up at ambient temperature, particularly in multiplex PCR. The undesired PCR products compete with target sequence for dNTPs and primers and, as a result, reduce the yield of some of the true amplification products. Thus, in the present study the usefulness of AmpliTaq Gold DNA polymerase was assessed in our multiplex protocol.

The specificity and efficiency of the multiplex PCR using AmpliTaq Gold DNA polymerase are compared with those using the standard Taq DNA polymerase in Fig. 3. A significant enhancement in the yield of PCR products was obtained with the use of AmpliTaq Gold. Furthermore, nonspecific PCR products were eliminated in all multiplex sets.

Fresh dNTP Used in the Multiplex PCR

The dNTP concentration has been reported to be important in multiplex PCR. The best results in our multiplex PCR were obtained at the concentration of 0.4 m*M* dNTP. We found that dNTP stocks are sensitive to freezing/thawing cycles. After four such cycles, using the same dNTP stock, the multiplex PCR did not work very well; some products became very weak and others became invisible as shown in Fig. 4A. Therefore, small aliquots of fresh dNTP was prepared before each experiment and used immediately after preparation. Figure 4 B shows the multiplex PCR prepared with fresh dNTP of the same DNA as presented in Fig. 4A.

Use of Adjuvant: DMSO and Formamide

Various authors have used dimethyl sulfoxide (DMSO) or formamide to improve the amplification efficiency and specificity of PCR (18,22,28). The application of denaturing agents such as DMSO and formamide in our multiplex PCR protocol did not improve either the amplification efficiency or the specificity.

DISCUSSION

By simultaneously amplifying several loci in one reaction, multiplex PCR is a rapid and convenient method for screening of many loci in a short time. Thus, multiplex PCR has been suggested to be the choice for routine screening of microdeletions on the Y chromosome in infertile men, since analysis of many STSs is impractical by single PCR. In the present study, we propose a multiplex PCR protocol that can be used for screening of Y microdeletions, since it has been optimized to work under identical conditions for all 25 STSs used.

Establishment of multiplex PCR often poses difficulties in making different primer pairs work together. Numerous optimization steps are involved in developing a robust and efficient multiplex PCR method, since multiplexing is frequently complicated by an extra band(s). To overcome this potential problem and to achieve a higher template specificity, the use of AmpliTaq Gold DNA polymerase is often recommended (32). In the present study, we confirmed this by demonstrating that AmpliTaq Gold DNA polymerase achieved a higher specificity and efficiency in the multiplex PCR compared to standard Taq DNA polymerase. Furthermore, we found that in multiplex PCR it is important to use a fresh dNTP mixture, since freezing/thawing cycles significantly lower the efficiency of PCR.

Though there have been numerous studies in which infertile men have been screened for Y chromosome microdeletions, the number and map site of STSs employed have varied broadly, making meaningful comparison of data sets difficult. Likewise, most protocols focus only on specific regions. Consequently, significant microdeletions may have been missed.

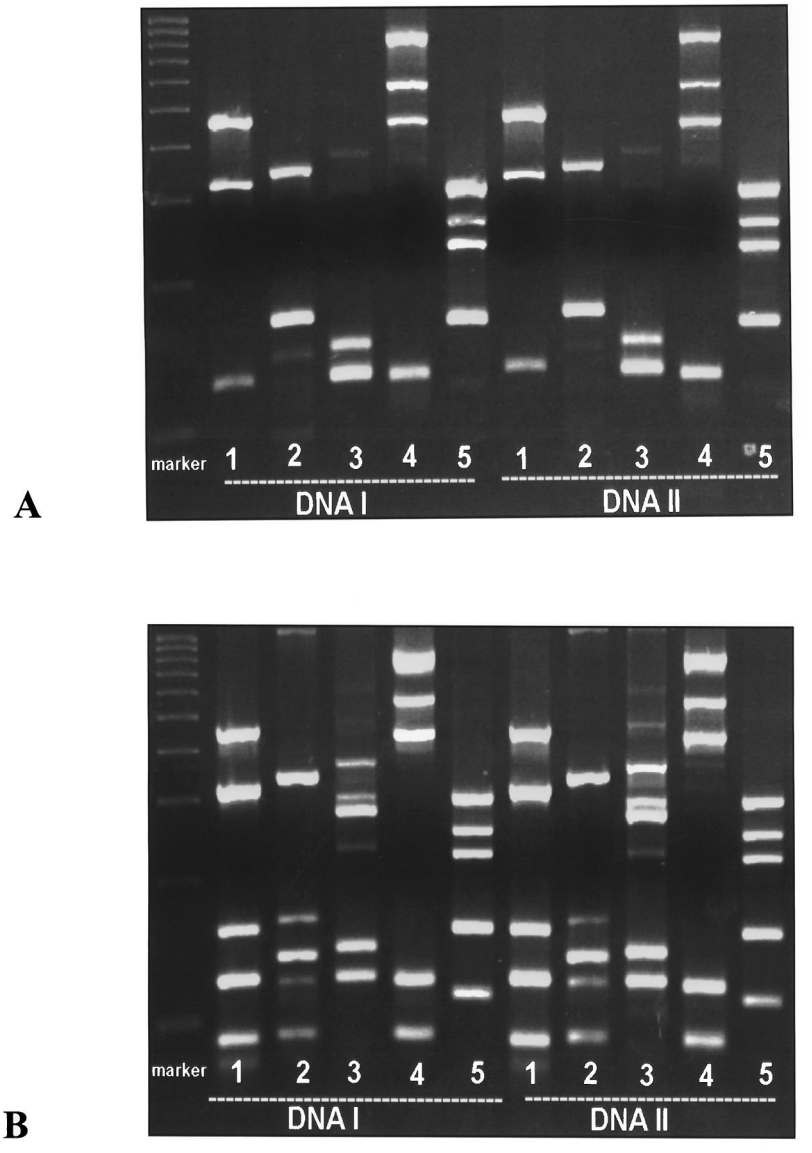

Fig. 4. Multiplex PCR amplification of two DNA templates using a dNTP exposed to more than four cycles of freezing/thawing (A) and fresh dNTP (B). Some PCR products became very weak or invisible using a dNTP exposed to freezing/thawing cycles in the five sets of multiplex PCR. The DNA ruler, which shows 10 bands ranging from 100 to 1000 bp, with 100-bp increments, was used as a marker.

Screening protocols have ranged from the use of a single STS in the DAZ region to the use of 131 STSs that cover the Y chromosome (7,8,14–26). The microdeletion detection rates also vary widely, from 1 to 58.3%, in these studies. It is still a question how many and which loci should be included in the screening of Y chromosome microdeletions. It has been suggested that the use of a larger number of STSs in a screening protocol does not increase the frequency of microdeletions detected (33). However, it is also true that a larger number of STSs can protect against inaccuracy and also lead to the detection of clinically irrelevant polymorphic variants (26). Furthermore, using a larger number of STSs may provide additional information to evaluate the relationship between the type of Y microdeletions and the clinical phenotype in infertile men, since the genetic regulation of spermatogenesis is not fully elucidated. Thus,

in our study, we decided to use 25 STSs, which can detect most of the microdeletions reported in previous studies (7,8,14–26).

There has been no consensus on which STSs are polymorphic or repetitive in the literature (22,26,33). For example, sY109, sY132, sY138, sY153, sY155, and sY164 were suspected be either polymorphic or repetitive (33). On the other hand, Liow *et al.* and Kent-First *et al.* suggested that these STSs were neither polymorphic nor repetitive in their recent studies (22,26), which is also supported by the fact that microdeletions have been found by using these STSs, which are claimed to be repetitive (17,21,22,26,34).

The STS sY272 has been reported to have a low frequency of polymorphism, since in a population of 920 fertile men, 2 were found to have a deletion in this locus (26). However, of 92 deletions found in a population of 514 infertile men, the STS sY272 was involved in 34 of the deletions (26). Therefore, we decided to include the STS sY272 in our multiplex PCR, since it can be used to determine the size of a larger deletion.

In our protocol, the PCR result is confirmed by Southern blot analysis when fewer than three consecutive STSs are missing since, in principle, all the STSs might have a low frequency of polymorphism that has just not been discovered yet. When more than three STSs are found to be deleted, it is likely that a true microdeletion is present.

In the present paper, we conclude that our multiplex PCR approach together with appropriate quality control procedures provides an easy, efficient, suitable, and accurate method for screening of microdeletions on the Y chromosome since this multiplex design can amplify 25 Y-linked STSs simultaneously using the same amplification conditions.

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