Multiplex PCR with Multichannel Microchip Electrophoresis: An Ultrafast Analysis for Genetic Diseases

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Abstract

A Y chromosomal polymorphic markers screening strategy using a multiplex polymerase chain reaction (PCR) and DNA microchip electrophoresis technology has recently been developed. It is a part of the human Y chromosome haplotyping system for studying Japanese population genetics and its relationship with male spermatogenic failure. This strategy is based on optimizing and modifying the primer set concentrations while keeping all other components of the PCR mixtures and conditions similar to those of a singleplex PCR. Well-balanced PCR products are obtained without changing even the DNA oligomer melting temperatures. Here, a panel of primer sets are used to amplify two groups of Y chromosome markers. The first consists of five markers and the second consists of seven markers. Both are possibly deleted in infertile men. The microchip electrophoresis technology is fast and sensitive, enables direct molecular typing of several Y chromosomal markers, and is separated by a difference of as many as six base pairs.

Introduction

The development of the polymerase chain reaction (PCR) has enabled rapid and efficient analysis of specific DNA sequences. The aim of performing PCR is the amplification of one or more target sequences of the genome (1). Sometimes the experimental approach requires the analysis of a variety of DNA sequences simultaneously, using several sets of oligonucleotide primers and one DNA template. This multiplex PCR is performed in a single multiplex reaction tube, and the mixed reagents and template share the same conditions for amplification (2).

Optimization and establishing conditions for a multiplex PCR require great efforts toward testing a variety of reaction conditions. Indeed, there is no theoretical limit to the number of sequences that can be amplified simultaneously, but the constraints on establishing conditions for specific and interpretable reaction generally limit the useful number of target sequences. Difficulties generally arise when more primers are added to a reaction. To have a successful PCR, careful attention should be paid to primer and enzyme concentration, buffer composition, cycling parameters, and melting temperature (T_m) of the primers (3). The publication of the human genome sequence, with its vast amount of genetic information, created a great demand for such multiplex PCRs. Currently, multiple PCR primer sets have been used for studying genetic disorders, forensic disputes, and population origins and migrations (4).

The inheritance of the nonrecombining portion of the Y chromosome and its passing unchanged from fathers to sons has served so much in the forensic and population genetic studies (5). The Y chromosome is a popular and important part of genome study because of human spermatogenesis (6). Human spermatogenesis is regulated by a network of genes located on autosomes and sex chromosomes, especially on the Y chromosome (7,8). One approach for studying the genetic background of the spermatogenesis process is obtained by genomic breakpoint mapping studies of the Y chromosome of infertile patients, focusing on those Y regions that most likely contain the Y genes of functional importance. Some of these genes are polymorphic among the fertile men. In sterile patients, cytogenetic analyses mapped microscopically visible Y deletions and rearrangements in the same polymorphic Y regions (9–12). The presence of a Y chromosomal spermatogenesis locus was thought to be located in Yq11.23 and designated as AZoospermia Factor (AZF) (13). More recently, molecular deletion mapping in Yq11 has revealed a series of microdeletions that could be mapped to one of three different AZF loci: AZFa, AZFb, and AZFc (14).

On the other hand, microchip electrophoresis has proven its strong reliability for genomics separation with its high resolution and speed analysis and less sample consumption, which led to significant progress in genomics research (15).

This report focuses on the analysis methods for studying such Y deletions and polymorphisms that are related to male spermatogenic ability. Twelve short tandem repeats were selected that are related to male infertility. They were amplified

on two multiplex reactions and then electrophoresed on a DNA microchip.

Presented are the preparation and optimization of the two multiplex PCRs. The hope is that such an approach will help to examine a large number of markers and samples while saving time, effort, reagents, and sample amounts.

Experimental

Instrumentation

Microchip electrophoresis was performed on two-microchip electrophoresis systems. An Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) with epifluorescent detection and semiconductor laser (emits at 630 nm) was used. The chips are made from soda lime glass and have 12-sample wells with three gel-dye mix wells and one for the ladder, with a depth of 10 µm, width of 50 µm, and effective separation length of 15 mm.

A Hitachi SV 1210 microchip CE system (Hitachi, Tokyo, Japan) was used. The detector is light-emitting diode confocal semiconductor fluorescence with an excitation wavelength at 470 nm and emission wavelength at 530 nm. The chips are polymethyl methacrylate micro *i*-chip with 12 lanes that can be detected simultaneously.

Standard PCR conditions

Primers

Two groups were designed for this study. The first consisted

Table I. Primer Sequence and Yield PCR Products Size of the Multiplex PCR in Which Five Primer Sets Were Used to Simultaneously Amplify Corresponding Y Chromosome Markers

Name	Marker size (bp)	Average size (bp)	RSD% of sizing	RE% of sizing	Locus	Primer sequencing	T_m	GC (%)
Sy592	65	68	0.97	4.61	UTY	F:GCATCATAATATGGATCTAGTAGG	52	38
						R:GGAGATACTGAATAGCATAGC	50	43
Sy594	82	82	0.98	0	TTY1	F:CTCTGGGAATCAAATTCGAGG	52	48
						R:GTCTTTCAGCCAATCCAAGG	52	50
Sy600	88	91	1.01	3.41	TTY2	F:GACAACTCTGACAGCCAGG	53	58
						R:GTCAGAACTCCCAAACAGG	51	53
Sy591	94	98.5	0.73	4.79	XKRY	F:CACTCATGGAGAAGGGTAGG	54	55
						R:GTCACACTCAGCCTCTTTAC	52	50
Sy593	102	106	0.80	3.92	TB4Y	F:CAAAGACCTGCTGACAATGG	52	50
						R:CTCCGCTAAGTCTTTCACC	51	53

Table II. Primer Sequence and Yield PCR Products Size of Multiplex PCR in Which All Seven Primer Sets of Corresponding Y Chromosome Markers Were Simultaneously Amplified

Name	Marker size (bp)	Average size (bp)	RSD% of sizing	RE% of sizing	Locus	Primer sequencing	T_m	GC (%)
Sy610	61	60.5	0.84	0.82	DBY	F:AGTTCCGCTATTCGGTCTCA	52	50
						R:CCCTGAAGAGAAGCGAAAAA	50	45
Sy202	121	118	0.95	2.48		F:ACAGTTTGAAATGAAATTTTAAATGTGTT	50	21
						R:TGACAAAGTGAGACCCTACTACTA	54	42
Sy90	176	176	0.39	0	KALY	F:CAGTGCCCCATAACACTTTC	52	50
						R:ATGGTAATACAGCAGCTCGC	52	50
Sy276	216	212	0.54	1.85	AMELY	F:CCTACCGCATCAGTGAATTTC	52	48
						R:TCTGTATGTGGAGTACACATGG	53	45
Sy624	256	243	0.33	5	DAZ	F:TTTAAATCTGTTGGATCCTCTCA	50	35
						R:TTCTTTCAGTCTCGATTATTTGTTA	49	28
Sy57	288	286	0.24	0.69	DYS257	F:GAACTTGTCGGGAGGCAAT	51	53
						R:CCACATTTAAACTGAGTACAGT	49	36
Sy274	361	352	0.32	2.5	RPS 4Y	F:TTAAGGGGACAGTATTTCAACTTC	52	38
						R:CCACATTTAAACTGAGTACAGT	49	36

of five Y chromosomal polymorphic markers that are distributed along the human Y chromosome and were suspected to play a role in the spermatogenesis process. Those markers are UTY, TTY1, TTY2, XKRY, and TB4Y, with PCR products fragment lengths of 65, 82, 88, and 94, and 102 base pairs, respectively. Table I shows the names of these markers, amplified fragment size (bp), locus name, primer sequence, melting temperature of each primer, and the gas chromatographic percentage for each primer. The second group consists of seven Y chromosomal polymorphic markers that play a role in the spermatogenesis process. Those markers are DBY, Sy202, KALY, AMELY, DAZ, DYS257, and RPS4Y (Table II).

Optimization of the multiplex PCR

Multiplex PCR was performed using a 20-µL reaction containing 2 µL of template DNA with a concentration of 1.5 ng/µL, 1µM primers, 250µM deoxyribonucleoside 5-prime-triphosates, 10X PCR buffer (10mM Tris-HCL, pH 8.3, 50mM KCl), 2.5mM $MgCl_2$, and 2.5 units AmpliTaq Gold DNA polymerase enzyme. Primer sequences were obtained from published sources (NCBI, Gene Bank STS accession numbers, http://www.ncbi.nlm.nih.gov/), and their T_m were calculated using the public software (http://www.micro.nwfsc.noaa.gov) for prediction of T_m values.

Thermal cycling for the first group was performed using the following conditions: 95°C for 4 min as an initial denaturation step, followed by 30 cycles of 94°C for 1 min, 60°C for 45 s, and 72°C for 45 s. after completion of the 30 cycles, there was a final extension step at 72°C for 10 min before holding the PCR products at 4°C until the time of electrophoresis. The conditions for the second group were the same as the first group, except the T_m was 58°C.

Chemicals

Four polyethylene oxides (M_n: 8 × 10^6Da, 1 × 10^6 Da, 4 × 10^5 Da, and 1 × 10^5 Da) were purchased from Aldrich (Milwaukee, WI).

Results and Discussion

Microdeletions of the Y chromosome long arm are the most common mutations in infertile males, which involves one or more AZF (AZFa, b, and c). The understanding of the AZF structure and gene content and mapping of the deletion breakpoints in infertile men is still incomplete (16). Thus, the human Y-linked polymorphisms are considered useful tools for investigating the the spermatogenic ability of males (17,18). Many studies have yet to be performed to uncover the real problems underlying the spermatogenic failure that makes a proportion of males infertile. Multiplex PCR technology is one important tool required for determining the genotypes of infertile men faster with higher sensitivity. The genotype is directly determined by simultaneously amplifying several polymorphic markers in the same reaction with a multiplex PCR and a base extension reaction (3).

For that purpose, a Y chromosomal polymorphic markers screening strategy using multiplex PCR and DNA microchip electrophoresis technology was recently developed. It is a part of the human Y chromosome haplotyping system for studying Japanese population genetics and its relationship with male spermatogenic failure that is being carried out at the School of Medicine of Tokushima University (Tokushima, Japan).

This strategy was based on optimizing and modifying the primer set concentrations while keeping all other components of the PCR mixtures and conditions similar to those of a singleplex PCR. Well-balanced PCR products were obtained without changing even the DNA oligomer T_m or cycling parameters.

Two panels of primer sets to amplify two groups of Y chromosome markers were selected. The first group consists of five markers and the second group consists of seven markers. Both groups of markers are possibly deleted in infertile men and their typing will be helpful in investigating the underlying causes of spermatogenic failure in those infertile men. Products of the multiplex PCRs were resolved on a DNA microchip, using the microchip electrophoresis technology

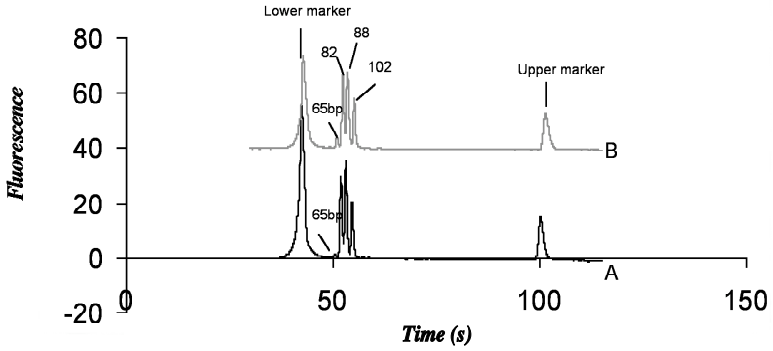

Figure 1. Chromatograms of the first group for four multiplex PCR products showing a poorly amplified fragment (65 bp) (A). This was improved after doubling the primer concentration of that marker when preparing the PCR (B).

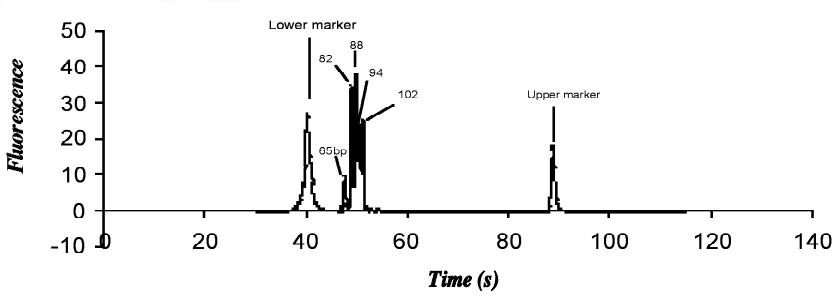

Figure 2. Chromatograms of the five multiplex PCR products showing a satisfactory separation on Agilent 2100 microchip machine.

that helped in fast separation and clear detection of all the amplicons with a diff e rence of as many as 6 bp in the amplified fragments' lengths.

Four amplicons (sY592, sY593, sY594, and sY600) were first amplified in a multiplex PCR without changing any parameters of the singleplex PCR. Three of them were clearly amplified, but the sY592 (65bp) was poorly amplified.

To overcome this problem, the concentration of the relevant primer set (sY592, 65bp) was doubled. The amplification in the multiplex PCR and the separation on a DNA microchip was then repeated; the results were clear and satisfactory (Figure 1).

Interestingly, more successful results were achieved when amplifying the mentioned four markers and adding a fifth marker (sY591, 94bp) that is also related to male infertility (Figure 2).

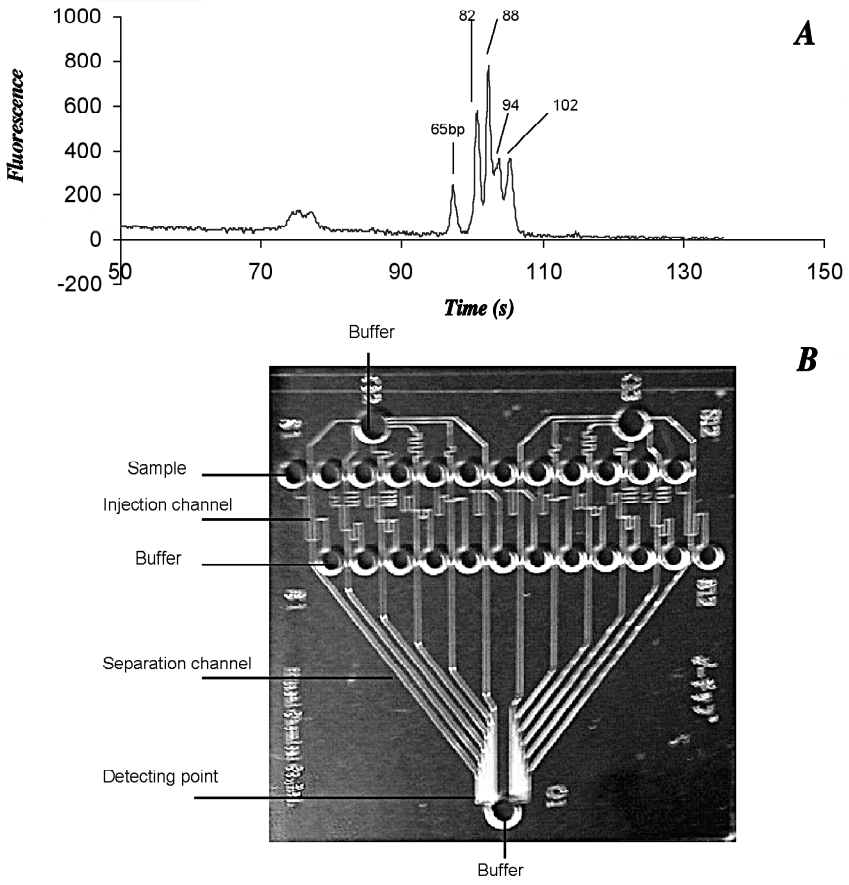

Figure 3. Chromatograms of the five multiplex PCR products showing a good resolution after using a newly developed gel matrix on Hitachi 1210 (A). A picture of the used multichannel microchip device showing its 12 lanes is also presented (B).

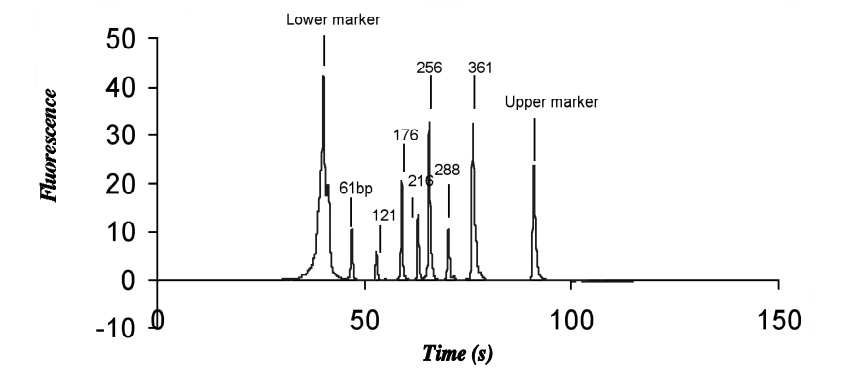

Figure 4. Chromatograms of the second group of multiplex PCR products in which a different group of seven Y chromosome markers were amplified and simultaneously separated with good resolution on an Agilent 2100 microchip machine.

To evaluate the reproducibility and accuracy of the instruments in measuring the size of the markers of the study, the precent relative standard deviation (RSD%) and the percent relative error (RE%) of the sizes of the markers were calculated. Statistically, RSD indicates the reproducibility, and the lower the value of the RSD, the better reproducibility obtained. Although RE indicates the accuracy, the lower the value of RE, the better the accuracy.

The results showed quite a good reproducibility (RSD%) and accuracy (RE%). The RSD% has ranged from 0.73% to 1.01%, and the RE% has ranged from 0% to 4.79% (Table I).

To achieve a better resolution, a new gel matrix was developed and applied that consisted of 0.30% polyethylene oxide (PEO) (8×10^6 Da)–0.60% PEO (1×10^6 Da)–0.90% PEO (4×10^5 Da)–0.80% PEO (1×10^5 Da/1 × TBE), EtBr, 1 µg/mL. Because Agilent bioanalyzer 2100 cannot accept any changes in the separation parameters, a Hitachi 1210 was used, using the newly developed gel matrix. On this Hitachi 1210, a much better separation was obtained for the same products of the mentioned multiplex PCR of the five markers (Figure 3A).

Another advantage of the Hitachi 1210 is that the chip used for separation consists of 12 lanes, which enabled 12 separations to be performed simultaneously, within 110 s (Figure 3B). This is considered to be a very important advantage because, by using multiplex PCR of the five markers with Hitachi 1210 of 12 lanes chips, 60 markers could be detected simultaneously, within 110 s. This confirms the great potential properties of microchip electrophoresis technology by its ultrafast separation and detection with high resolution within a very short time for a mixture of multiple fragments.

Using the same concept, the amplification of another group of seven markers related to the same disease was successful. Table II presents the names of the seven

markers, primers used, and different yield fragments sizes. In a multiplex PCR, all seven of these markers were amplified under the same conditions without changing any parameter of the singleplex PCR. Neither the T_m point nor the primer concentration was modified more than the basic conditions mentioned previously (Experimental section). Both the amplification and the separation were successful and with good resolution (Figure 4).

Similar to the first group, the reproducibility and accuracy were shown to be quite good. The RSD% was less than 0.95%, though the RE% was less than 5% (Table II).

By separating this set of seven markers on the 12-lane Hitachi microchip, the opportunity will be presented to separate 84 genomic markers simultaneously, within only 120 s.

Conclusion

Merging the newly optimized multiplex PCRs with the multichannel microchip electrophoresis system can lead to simple amplification and ultrafast separation of the genomic markers. In the future, such improvements in genetic investigations will be of great importance for the routine analysis for diagnosis of the genetic diseases. Significant innovations are still expected because of widespread applications of micromachining in chemistry and biotechnology.

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References

1. K. Hayashi. "Manipulation of DNA by PCR". In *The Polymerase Chain Reaction*. K.B. Mullis, F. Ferre, and R.A. Gibbs, Eds. Birkhauser, Boston, MA, 1994, pp. 3–12.
2. J.S. Chamberlain and J.R. Chamberlain. "Optimization of multiplex PCRs". In *The Polymerase Chain Reaction*. K.B. Mullis, F. Ferre, and R.A. Gibbs, Eds. Birkhauser, Boston, MA, 1994, pp. 39–45.
3. E.M. Elnefro, A.M. Ashishi, R.J. Cooper, and P.E. Klapper. Multiplex PCR: optimization and appliction in diagnostics virology. *Clin. Microbiol. Rev.* **13:** 559–70 (2000).
4. E. Bosch, A.C. Lee, F. Calafell, E. Arroyo, P. Henneman, P. de Knijff, and M.A. Jobling. High resolution Y chromosome typing: 19 STRs amplified in three multiplex reactions. *Forensic Sci. Int.* **125:** 42–51 (2002).
5. M. Jobling and C. Tyler-Smith. Father and sons – the Y chromosome and human evolution. *Trends Genet.* **11:** 449–56 (1995).
6. M.A. Jobling and C. Tyler-Smith. New uses for new haplotypes the human Y chromosome, disease and selection. *Trends Genet.* **16:** 356–62 (2000).
7. B.T. Lahn and D.C. Page. Functional coherence of the human Y chromosome. *Science* **278:** 675–80 (1997).
8. Y.F. Lau. Gonadoblastoma, testicular and prostate cancers, and the TSPY gene. *Am. J. Hum. Genet.* **64:** 921–27 (1999).
9. L.S. Whitfield, J.E. Sulston, and P.N. Goodfellow. Sequence variation of the human Y chromosome. *Nature* **378:** 379–80 (1995).
10. R. Reijo, T.Y. Lee, P. Salo, R. Alagappan, L.G. Brown, M. Rosenberg, S. Rozen, T. Jaffe, D. Straus, O. Hovatta, A. de la Chapelle, S. Silber, and D.C. Page. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat. Genet.* **10:** 383–93 (1995).
11. C.A. Sargent, C.A. Boucher, S. Kirsch, G. Brown, B. Weiss, A. Trundley, P. Burgoyne, N. Saut, C. Durand, N. Levy, P. Terrriou, T. Hargreave, H. Cooke, M. Mitchell, G.A. Rappold, and N.A. Affara. The critical region of overlap defining the AZFa male infertility interval of proximal Yq contains three transcribed sequences. *J. Med. Genet.* **36:** 670–77 (1999).
12. R. Saxena, J.W.A. de Vries, S. Repping, R.K. Alagappan, H. Skaletsky, L.G. Brown, P. Ma, E.S. Chen, J.M.N. Hoovers, and C.D. Page. Four DAZ genes in two clusters found in the AZFc region of the human Y chromosome. *Genomics* **67:** 256–67 (2000).
13. P.H. Vogt, A. Edelmann, S. Kirsch, O. Henegariu, P. Hirschmann, F. Kiesewetter, F.M. Köhn, W.B. Schill, S. Farah, C. Ramos, M. Hartmann, W. Hartschuh, D. Meschede, H.M. Behre, A. Castel, E. Nieschlag, W. Weidner, H.J. Cröne, A. Jung, W. Engel, and G. Haidl. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum. Mol. Genet.* **5:** 933–43 (1996).
14. P.H. Vogt and S. Fernandes. Polymorphic DAZ gene family in polymorphic structure of AZFc locus: artwork or functional for human spermatogenesis? *APMIS* **111:** 115–26 (2003).
15. M. Jabasini, L. Zhang, F. Dang, F. Xu, M.R. Almoti, A.A. Ewis, J. Lee, Y. Nakahori, and Y. Baba. Analysis of DNA polymorphisms on the human Y chromosome by microchip electrophoresis. *Electrophoresis* **23:** 1537–42 (2002).
16. A. Ferlin, E. Moro, A. Rossi, B. Dallapiccola, and C. Foresta. The human Y chromosome's azoospermia factor b (AZFb) region: sequence, structure, and deletion analysis in infertile men. *J. Med. Genet.* **40:** 18–24 (2003).
17. Y. Kuroki, T. Iwamoto, J.W. Lee, M. Yoshike, S. Nozawa, T. Nishida, A.A. Ewis, H. Nakamura, T. Toda, K. Tokunaga, S. Kotliarova, N. Kondoh, E. Koh, M. Namiki, T. Shinka, and Y. Nakahori. Spermatogenic ability is different among males in different Y chromosome lineage. *J. Hum. Genet.* **44:** 289–92 (1999).
18. C. Krausz, L. Quintana-Murci, E. Rajpert-De Meyts, N. Jorgensen, M.A. Jobling, Z.H. Rosser, N.E. Skakkebaek, and K. McElreavey. Identification of a Y chromosome haplogroup associated with reduced sperm counts. *Hum. Mol. Genet.* **10:** 1873–77 (2001).

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