EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. State of the art.

international journal of andrology, 27:240–249 (2004)

EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. State of the art 2004

M. SIMONI,* E. BAKKER† and C. KRAUSZ‡

*Institute of Reproductive Medicine, University of Münster, Münster, Germany, †Department of Human and Clinical Genetics, University of Leiden, The Netherlands, and ‡Andrology Unit, Department of Clinical Pathophysiology, University of Florence, Florence, Italy

Summary

Microdeletions of the Y chromosome are the second most frequent genetic cause of spermatogenetic failure in infertile men after the Klinefelter syndrome. The molecular diagnosis of Y-chromosomal microdeletions is routinely performed in the workup of male infertility in men with azoospermia or severe oligozoospermia. Since 1999, the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) support the improvement of the quality of the diagnostic assays by publication of the laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions and by offering external quality assessment trials. The present revision of the 1999 laboratory guidelines summarizes the results of a 'Best Practice Meeting' held in Florence (Italy) in October 2003. The basic protocol for microdeletion screening suggested in the 1999 guidelines proved to be very accurate, sensitive and robust. In the light of the recent advance in the knowledge of the Y chromosome sequence and of the mechanism of microdeletion it was agreed that the basic 1999 protocol, based on two multiplex polymerase chain reactions each covering the three *AZF* regions, is still fully valid and appropriate for accurate diagnosis.

Keywords: azoospermia, genetic testing, male infertility, microdeletion, oligozoospermia, quality control, Y chromosome

Introduction

Microdeletions of the Y chromosome are a recently discovered cause of spermatogenetic failure resulting in male infertility. After the Klinefelter syndrome, Y-chromosomal microdeletions are the second most frequent genetic cause of male infertility. In the last decade many investigators have described the occurrence of microdeletions in infertile patients around the world and the molecular diagnosis of deletions has become an important diagnostic test in the workup of male infertility (Vogt *et al.*, 1996; Vogt, 1998; Krausz & McElreavey, 1999; Maurer & Simoni, 2000).

Correspondence: Prof. Dr Manuela Simoni, Institute of Reproductive Medicine, Domagkstr.11, D – 40129 Münster, Germany. E-mail: simoni@uni-muenster.de

The portion of the male-specific region of the Y chromosome (MSY), comprising 95% of the Y chromosome and flanked by pseudoautosomal regions (Skaletsky *et al.*, 2003), which is affected by deletions has been classically subdivided into three regions called *AZF*a, *AZF*b and *AZF*c, respectively (Vogt *et al.*, 1996). Microdeletions are relatively frequent among infertile men, although the incidence can vary considerably depending on the selection criteria of the patients. Azoospermic men have a higher incidence of microdeletions than oligozoospermic men and consequently deletion frequency found in different laboratories may vary from 2 to 10% (or even higher) reflecting the composition of the study population (Krausz & McElreavey, 1999; Krausz *et al.*, 2001). Typically, routine laboratories receiving referrals

from outside institutions, without controlled patient selection, have a much lower incidence, <2%. Deletions of the three *AZF* regions occur with different frequency. According to the experience of the Institute of Reproductive Medicine in Münster, based on 34 patients with deletions of the Y chromosome, deletions of the *AZF*c region are the leading group (79% of all deletions), followed by *AZF*b (9%), *AZF*bc (6%), *AZF*a (3%) and *AZF*abc ('XX male': 3%).

The published data and the experience of the last years showed that diagnostic protocols can be quite different and that inaccurate or wrong diagnoses occur as well, suggesting the necessity of both standardization and quality control (Simoni, 2001). Therefore, the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) jointly supported the publication of the 'Laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions' (Simoni *et al.*, 1999) and started offering external quality control.

Four years after publication of the guidelines with the now complete knowledge of the sequence of the Y chromosome (Skaletsky *et al.*, 2003) and of the mechanism of microdeletions (Kamp *et al.*, 2000; Sun *et al.*, 2000; Kuroda-Kawaguchi *et al.*, 2001; Repping *et al.*, 2002), it was felt that the time for a revision was ripe and the experts in the field convened together with users in a 'Best Practice Meeting' held in Florence (Italy) in October 2003. The results of this meeting and the revised guidelines are now summarized in this paper.

Structure of the male-specific region of the Y chromosome (MSY)

The complete physical map and sequence of MSY are now available (Skaletsky *et al.*, 2003). This information was obtained by sequencing and mapping 220 BAC clones containing portions of the MSY from one man. The use of only one individual was necessary because, because of the presence of repetitive sequences with only minute differences characterizing the individual copies of each sequence (sequence family variants, SFV), inter-individual allelic variation or polymorphisms would have prevented the accurate mapping of SFV necessary to allocate the BAC clones. Three classes of sequences were found in MSY: X-transposed (with 99% identity to the X chromosome), X-degenerate (single-copy genes or pseudogene homologues of X-linked genes) and ampliconic. Ampliconic sequences are characterized by sequence pairs showing nearly complete (>99.9%) identity, organized in massive palindromes. According to current knowledge, the reference MSY contains 156 transcription units including 78 protein-coding genes encoding 27 proteins. Ampliconic sequences comprise 60 coding genes and 74 non-coding transcription units mostly grouped in families and expressed mainly or only in the testis. Ampliconic sequences recombine through gene conversion, i.e. non-reciprocal transfer of sequence information occurring between duplicated sequences within the chromosome, a process which maintains the >99.9% identity between repeated sequences organized in pairs in inverted orientation within palindromes.

Besides maintaining the gene content, this peculiar organization provides the structural basis for deletions and rearrangements. Today it is believed that deletions almost invariably arise through homologous recombination between identical repeated sequences with loss of the genetic material between them. Considering the architecture of the MSY, several different deletions are hypothetically possible (Yen, 2001), some of which have indeed been identified in infertile but also in fertile men (Repping *et al.*, 2003; Fernandes *et al.*, 2004). Those deletions clinically relevant for male infertility, as of today's knowledge, are briefly described below.

Mechanism and type of deletions

Three discrete *AZF*a, *AZF*b and *AZF*c regions were originally characterized by careful mapping of the MSY of a large number of men with microdeletions when the sequence of the Y chromosome was not completely known (Vogt *et al.*, 1996). Today the sequence of the MSY and the molecular mechanism resulting in microdeletions have been clarified. This resulted in a new model of deletions in which the *AZF*b and *AZF*c regions are overlapping. In addition, the *AZF*b and *AZFbc* deletions have been suggested to be the consequence of at least three different deletions patterns (Repping *et al.*, 2002) (Fig. 1). No consensus about the model and, consequently, nomenclature of deletions has been reached so far. From the practical, clinical point of view this is, however, uninfluential for the prognosis of the patient and, being aware of the current knowledge, either nomenclature can be adopted.

The *AZF*a region is about 1100 kb long and contains the single copy genes *DFFRY* (or *USP9Y*) and *DBY*. Recent data obtained simultaneously by different groups identify the origin of complete *AZF*a deletions in the homologous recombination between identical sequence blocks within the retroviral sequences in the same orientation HERVyq1 and HERVyq2 (Blanco *et al.*, 2000; Kamp *et al.*, 2000; Sun *et al.*, 2000). Within these retroviruses, recombination can occur in either one of two identical sequence blocks (ID1 and ID2), giving rise to two major pattern of deletions slightly different in their precise breakpoints (Kamp *et al.*, 2000, 2001; Sun *et al.*, 2000). In any case the complete deletion of the *AZF*a region removes about 792 kb including both *USP9Y* and *DBY* genes, the only two genes in *AZF*a. Deletions involving only the *USPY9* gene or the *DBY* gene have been reported only by one group (Ferlin *et al.*, 1999; Foresta *et al.*, 2000) and not found in >1600 patients tested elsewhere (Silber *et al.*, 1998; Krausz *et al.*, 2003 and references therein). Moreover, the mechanism responsible for such selective loss has not been elucidated.

The type and mechanism of deletions of the *AZF*b and *AZF*c region have been recently clarified as well (Kuroda-

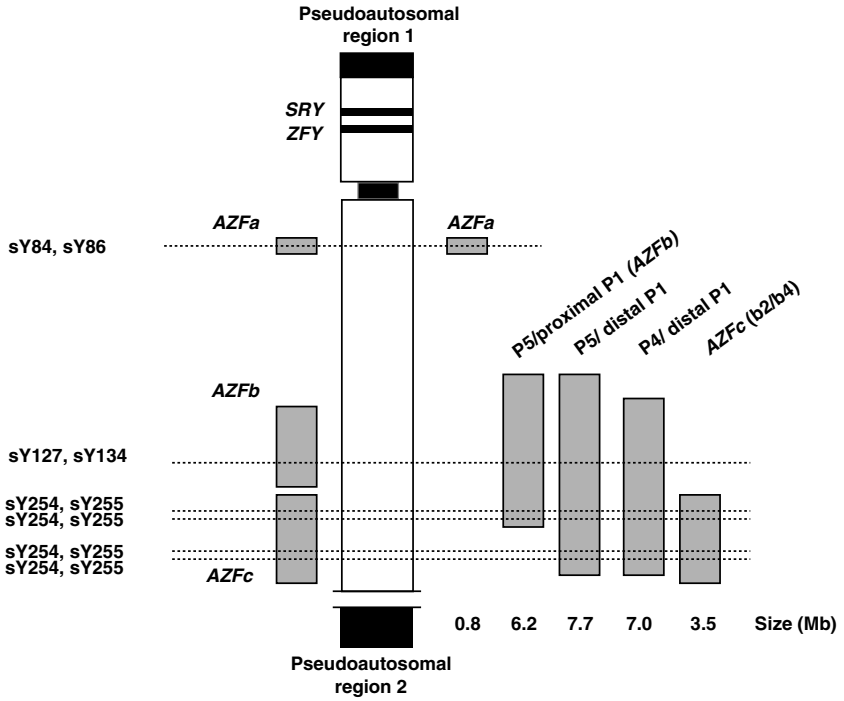


Figure 1. Schematic representation of the Y chromosome and of the current deletion models. The model proposed by Vogt *et al.* (1996) with the subdivision into the three *AZF*a, *AZF*b and *AZF*c regions is depicted on the left-hand side of the chromosome. The model of Repping *et al.* (2002) is represented on the right-hand side of the chromosome. The location of the STS primers suggested by the present guidelines is indicated by dotted lines. As four copies of the *DAZ* gene are normally present on the Y chromosome, the STS primers sY254, sY255 amplify four loci in *AZF*c. The *AZF*c (b2/b4) deletion is by far the most frequent type (approximately 80%) of Y-chromosomal microdeletion found in men with oligo/azoospermia.

Kawaguchi *et al.*, 2001). Both regions together comprise 24 genes, most of which present in multiple copies for a total of 46 copies. The complete deletion of *AZF*b removes 6.2 Mb (including 32 copies of genes and transcription units) and results from homologous recombination between the palindromes P5/proximal P1 (Repping *et al.*, 2002). The *AZF*c region includes 12 genes and transcription units, each present in a variable number of copies making a total of 32 copies (Repping *et al.*, 2003). The classical complete deletion of *AZF*c, the most frequent pattern among men with deletions of the Y chromosome, removes 3.5 Mb, originates from the homologous recombination between amplicons b2 and b4 in palindromes P3 and P1, respectively and removes 21 copies of genes and transcription units (Kuroda-Kawaguchi *et al.*, 2001). Deletions of both *AZF*b and *AZF*c together occur by to two major mechanisms involving homologous recombination between P5/distal P1 (7.7 Mb and 42 copies removed) or between P4/distal P1 (7.0 Mb, 38 copies removed) (Repping *et al.*, 2002).

Therefore, according to the present knowledge, the following recurrent microdeletions of the Y chromosome are clinically relevant and are found in men with oligo- or azoospermia (Fig. 1):

- *AZF*a,
- *AZF*b (P5/proximal P1),
- *AZF*bc (P5/distal P1 or P4/distal P1),
- *AZF*c (b2/b4).

Given the palindromic structure of the MSY and the presence of several amplicons which could undergo recombination, other types of deletions are possible (Yen, 2001). Some of them have been identified, although their clinical significance has not yet been fully explored (Fernandes *et al.*, 2002; Repping *et al.*, 2003).

Genotype/phenotype correlation

Y microdeletions are specific for spermatogenic failure as no deletions have been reported in a large number of normospermic men (Krausz *et al.*, 2003). Although 'fertility' can be compatible with Y deletions, it simply reflects the fact that natural fertilization may occur even with relatively low sperm counts depending on the female partner's fertility status. For this reason, it is more appropriate to consider Y deletions as a cause of oligo/azoospermia rather than cause of 'infertililty'.

Deletions of the entire *AZF*a region as described above invariably result in complete sertoli cell only (SCO) syndrome and azoospermia (Vogt *et al.*, 1996; Krausz *et al.*, 2000; Kamp *et al.*, 2001; Kleiman *et al.*, 2001; Hopps *et al.*, 2003). Deletions of isolated genes of the *AZF*a region, involving only the *USPY9* gene or the *DBY* gene were related to a variable testicular phenotype (Ferlin *et al.*, 1999; Foresta *et al.*, 2000). Such deletions, however, have been described only sporadically and need further confirmation by other groups. The diagnosis of a complete deletion of the *AZF*a region implies the virtual impossibility to retrieve testicular sperm for intracytoplasmic sperm injection (ICSI).

Complete deletions of *AZF*b and *AZF*b+c (P5/proximal P1, P5/distal P1, P4/distal P1) are characterized by a histological picture of SCO or spermatogenetic arrest resulting in azoospermia. Several reports have shown that,

similarly to the complete deletions of the *AZF*a region, no sperm are found upon attempts of testicular sperm extraction (TESE) in these patients (Krausz *et al.*, 2000; Hopps *et al.*, 2003). As in the case of complete deletions of *AZF*a, a diagnosis of complete deletions of *AZF*b or *AZF*b+c (P5/proximal P1, P5/distal P1, P4/distal P1) is incompatible with sperm retrieval and ICSI should not be recommended to these patients.

Deletions of the *AZF*c region (b2/b4) are associated with a variable clinical and histological phenotype (Reijo *et al.*, 1996; Luetjens *et al.*, 2002; Oates *et al.*, 2002). In general, *AZF*c deletions are compatible with residual spermatogenesis. *AZF*c deletions can be found in men with azoospermia or severe oligozoospermia and, in rare cases, can even be transmitted naturally to the male offspring (Kühnert *et al.*, 2004 and references therein). In men with azoospermia and *AZF*c deletion there is a fairly good chance of retrieving sperm from TESE and children can be conceived by ICSI (Kent-First *et al.*, 1996; Mulhall *et al.*, 1997; Jiang *et al.*, 1999; Kamischke *et al.*, 1999; Kleiman *et al.*, 1999; Page *et al.*, 1999; Cram *et al.*, 2000; van Golde *et al.*, 2001; Oates *et al.*, 2002; Peterlin *et al.*, 2002). The sons of these patients will be *AZF*c-deleted.

Indications for molecular screening of the Y chromosome

Diagnosis of a microdeletion of the Y chromosome permits the cause of the patient's azoospermia/oligozoospermia to be established and to formulate a prognosis. In which patients should molecular screening of the Y chromosome be performed? The world literature, now based on several thousands of patients screened, indicates that, as a rule, clinically relevant deletions are found in patients with azoospermia or sperm concentration $<1 \times 10^6$/mL. Very rarely, deletions can be found in infertile patients with sperm concentration between 1 and 5×10^6/mL (Maurer & Simoni, 2000; Foresta *et al.*, 2001). Although the incidence of microdeletions is higher when patients are selected by testicular histology, e.g. SCO (Foresta *et al.*, 2001; Kamp *et al.*, 2001), no absolute selection criteria can be given according to which patients are candidates for molecular analysis. In general, in patients with chromosomal abnormalities, obstructive azoospermia or hypogonadotropic hypogonadism molecular analysis of the Y chromosome is not indicated but, the usual clinical parameters such as hormone levels, testicular volume, varicocele, maldescended testis, infections, etc. do not have any predictive value (Maurer *et al.*, 2001; Frydelund-Larsen *et al.*, 2002; Oates *et al.*, 2002; Tomasi *et al.*, 2003). Patients with azoospermia or severe oligozoospermia who may be candidate for ICSI or TESE/ICSI should be offered deletion screening because TESE should not be recommended in cases of complete deletion of the *AZF*a region, or complete deletion of *AZF*b or deletions of the *AZF*b+c regions. Moreover, microdeletions of the *AZF*c region are transmitted to the male offspring if assisted reproduction is performed (Kent-First *et al.*, 1996; Mulhall *et al.*, 1997; Jiang *et al.*, 1999; Kamischke *et al.*, 1999; Kleiman *et al.*, 1999; Page *et al.*, 1999; Cram *et al.*, 2000; van Golde *et al.*, 2001; Oates *et al.*, 2002; Peterlin *et al.*, 2002). Therefore, the diagnosis of a deletion has prognostic value and can influence therapeutic options.

Genetic counselling is mandatory in order to provide information about the risk of producing a son with impaired spermatogenesis. However, the exact phenotype cannot be predicted entirely because of the different genetic background and the impact of environmental factors on reproductive functions and on the fertility potential of father and son. Concerns have been raised about the potential risk for the offspring of developing 45,X0 Turner's syndrome and other phenotypic anomalies associated with sex chromosome mosaicism, including ambiguous genitalia. Data on men with Y microdeletions (Siffroi *et al.*, 2000) and in patients bearing a mosaic 46XY/45X0 karyotype with sexual ambiguity and/or Turner stigmata (Patsalis *et al.*, 2002) suggest that some Yq microdeletions are associated with an overall Y chromosomal instability which might result in the formation of 45,X0 cell lines. Until now only 17 male and 18 female ICSI babies born from fathers affected by Yq microdeletions have been reported (Kent-First *et al.*, 1996; Mulhall *et al.*, 1997; Jiang *et al.*, 1999; Kamischke *et al.*, 1999; Kleiman *et al.*, 1999; Cram *et al.*, 2000; van Golde *et al.*, 2001; Oates *et al.*, 2002; Peterlin *et al.*, 2002). It appears that the children are phenotypically normal, except for one son born with pulmonary atresia and a hypoplastic right ventricle (Page *et al.*, 1999) and no ambiguous genitalia or Turner syndrome have been observed among them. Considering that embryos bearing a 45,XO karyotype have a higher risk of spontaneous abortion, it would be important to know whether there is a higher incidence of spontaneous abortion among the partners of Y deleted men. Until this information is available, it should be mentioned to the patients with caution.

In general, the indication for molecular diagnosis of Y-chromosomal microdeletions remains an important clinical decision which should be taken case by case and clinicians should decide in which patients they regard this diagnosis as worthwhile and meaningful.

Guidelines for diagnostic testing

Diagnostic testing of deletions is performed by polymerase chain reaction (PCR) amplification of selected regions of the Y chromosome. MSY-specific STS primers amplify both anonymous sequences of the chromosome or genes and can be now mapped precisely (Skaletsky *et al.*, 2003). Although the map of the MSY is now known, virtually nothing is known about the role of the individual genes and transcription units in spermatogenesis and their causal role in infertility. It has been shown that using STS primers specific

for discrete genes does not increase the detection rate of clinically relevant microdeletions in DNA samples from ICSI candidates (Silber *et al.*, 1998; Krausz *et al.*, 1999, 2001; Peterlin *et al.*, 2002). In addition, the palindromic/ampliconic structure of MSY makes this region particularly prone to rearrangements and loss of genetic material, which should not necessarily be regarded as pathological microdeletions and might represent relatively common polymorphisms (Repping *et al.*, 2003; Fernandes *et al.*, 2004). Therefore it remains basically unimportant whether the STS primers used amplify anonymous regions or specific MSY genes. What is important for the diagnosis is that the panel of STS primers are derived from regions of the Y chromosome, which are not polymorphic and are well known to be deleted specifically in men affected by oligo/azoospermia according to the known, clinically relevant microdeletion pattern. The sequence of MSY and the mechanism underlying the microdeletions have shown definitely that a putative fourth *AZF*d region postulated by Kent-First *et al.* (1999) and considered in a popular commercial kit does not exist.

PCR format and internal quality control

The PCR amplification of genomic DNA for clinical diagnosis requires strict compliance with good laboratory practice and basic principles of quality control. Guidelines for internal quality control should be carefully followed when implementing the diagnostics of Y-chromosomal microdeletions and can be downloaded at http://www.emqn.org/bpguidelines.php (25.5.04).

In parallel to the patient's DNA sample, a female sample has to be processed as a control for DNA contamination during the whole procedure. Each set of PCR reactions should be carried out at least in duplex or, even better, multiplex PCR. The multiplex format is helpful to distinguish a negative result from a technical failure through the use of an internal control. An appropriate internal PCR control in *AZF* diagnostics is the *ZFX/ZFY* gene because the primers amplify a unique fragment both in male and female DNA, respectively. External positive and negative controls must be run in parallel with each multiplex, i.e. with each set of primers. Appropriate positive and negative controls are a DNA sample from a man with normal spermatogenesis and from a woman, respectively. The DNA sample from a normal man controls for sensitivity and specificity of the assay. The female DNA sample controls for specificity and for contamination. In addition, a water sample, which contains all reaction components but water instead of DNA, must be run with each set of primers. The water sample controls for reagent contamination.

In summary, the diagnostics of Y-chromosomal microdeletions should be performed by multiplex (at least duplex) PCR amplification of genomic DNA, using the *ZFX/ZFY* as internal PCR control. A DNA sample from a fertile male and from a women and a blank (water) control should be run in parallel with each multiplex.

Basic set of STS primers

In principle, the analysis of only one non-polymorphic STS locus in each *AZF* region is sufficient to determine whether *any* STS deletion is present in *AZF*a, *AZF*b or *AZF*c. However, analysing two STS loci in each region reinforces diagnostic accuracy, as deletions involve well-defined regions including many STS loci. Therefore, the concept that at least two STS loci in each *AZF* region should be analysed remains valid. Based on the experience of many laboratories and the results of external quality control and considering the multiplex PCR format, the first choice of STS primers recommended in the first version of the guidelines remains basically valid. These primers include:

For *AZF*a: sY84, sY86
For *AZF*b: sY127, sY134
For *AZF*c: sY254, sY255 (both in the *DAZ* gene)

These STS primers have been shown to give robust and reproducible results in multiplex PCR reactions by several laboratories and in external quality control trials. Moreover, the *SRY* gene should be included in the analysis as a control for the testis-determining factor on the short arm of the Y chromosome and for the presence of Y-specific sequences when the *ZFY* gene is absent (e.g. in XX males).

In summary, the set of PCR primers which should be used in multiplex PCR reactions as best choice for the diagnosis of microdeletion of the *AZF*a, *AZF*b and *AZF*c region includes: sY14 (*SRY*), *ZFX/ZFY*, sY84, sY86, sY127, sY134, sY254, sY255. The location of these primers on the Y chromosome is indicated in Fig. 1.

The sequence of the primers and an example of a PCR protocol are reported in the Appendix A and B. The use of this primer set will enable the detection of almost all the clinically relevant deletions and of over 95% of the deletions reported in the literature in the three *AZF* regions and is sufficient for routine diagnostics. Adoption of this favourite set of primers by all laboratories is strongly recommended as it allows a minimal standardization and good comparison of laboratory performance and inter-laboratory variability.

Significance of the basic primer set and extension analysis

AZFa The molecular analysis of the *AZF*a region involves the use of the two STS markers sY84 and sY86. Both markers are located upstream of the USPY9 and DBY genes and are anonymous. According to the pathogenic mechanism of the deletion and current experience, once a deletion of both sY84 and sY86 is detected, the probability of dealing with a complete deletion approaches 100%. However, as *AZF*a deletions are rather rare and it cannot be excluded that even more rare, still unknown deletion patterns exist, when such a deletion if found it is advisable to complete diagnosis by determining whether the deletion is complete or not.

As indicated in the previous guidelines (Simoni *et al.*, 1999), determination of the extension of the deletion (complete/not complete) can be performed by using the

STS primers sY82(present), sY83(absent) for the proximal border and sY87(absent), sY88(present) for the distal border. A more sophisticated determination of the breakpoints can be obtained with the protocol suggested by Kamp *et al.* (2001).

If only one of the two *AZF*a STS loci (only sY84 or only sY86) is deleted and amplification failures can be excluded, the *AZF*a region should be studied in more detail testing for the presence/absence of the two *AZF*a genes (DBY and USP9Y) and the borders according to the map provided by Kamp *et al.* (2001). This event, however, is presently considered to be extraordinarily rare.

AZF*b *(P5/proximal P1) The two anonymous markers sY127 and sY134 are located in the median and distal part of *AZF*b. According to the present knowledge, in the vast majority of cases the deletion of both markers indicates a complete deletion of the *AZF*b region. This might be confirmed by using the following second choice markers: sY105 (present) and sY114 (absent) for the proximal border and sY143 (absent) and sY152 (present) for the distal border. If one wishes to determine the breakpoints of the deletion more precisely, in particular to check if the *AZF*b deletion corresponds to the P5/proximal P1 pattern, which also removes two copies of the *DAZ* gene (Fig. 1), the primers described by Repping *et al.* (2002) should be used. However, it should be pointed out that this is not required for routine clinical diagnosis of a complete deletion of the *AZF*b region.

AZF*c *(b2/b4) The two markers sY254 and sY255 are specific for the *DAZ* gene, which is present in four copies arranged in two complexes of two genes each in head-to-head orientation located in the palindromes P2 and P1 respectively in the reference MSY sequence (Saxena *et al.*, 2000). The absence of both markers indicates deletion of the entire *AZF*c region, which removes all copies of *DAZ*. According to current knowledge, the deletion of only one of these two markers is impossible and should be always regarded as a methodological error. Deletion of individual copies of the *DAZ* gene is possible but cannot be explored with this protocol. Deletions of only two copies of the *DAZ* gene have been reported both in infertile and in fertile men (Fernandes *et al.*, 2002; de Vries *et al.*, 2002; Repping *et al.*, 2003). As the pathophysiological meaning of partial deletions of the *AZF*c region remains to be determined, this analysis should be considered experimental and is currently not recommended for routine purposes.

The experience accumulated until now has shown that when both markers sY254 and sY255 are deleted, a diagnosis of complete deletion of the *AZF*c region can be made. As the histological picture of the testis is variable in such cases (Vogt *et al.*, 1996; Luetjens *et al.*, 2002) and sperm can be found in the testis and/or in the ejaculate, TESE/ICSI can be attempted in these patients. Some studies have shown that the *AZF*c deletion pattern is rather constant, although not always identical (Kuroda-Kawaguchi *et al.*, 2001; Luetjens *et al.*, 2002). The primers indicated by Kuroda-Kawaguchi *et al.* (2001) permit the user to determine if the deletion corresponds to the b2/b4 pattern. This analysis, however, will not change the prognosis for the patient and is not necessary for routine purposes.

AZF*bc *(P5/distal P1 or P4/distal P1) The complete deletion of both *AZF*b and *AZF*c regions is indicated by the lack of amplification of all four markers sY127, sY134, sY254 and sY255. The use of more specific markers as indicated by Repping *et al.* (2002) determine whether the deletion corresponds to the P5/distal P1 or P4/distal P1 pattern but is not necessary for clinical routine, as in view of current knowledge this will not change the prognosis for these patients.

Interpretation of the results, control and repetition of the test

The protocol suggested by these guidelines (see appendix A) has been conceived and optimized so that each of the two multiplex reactions contains a marker for each *AZF* region. Thus, when a complete deletion occurs in a sample both PCR reactions should show the lack of amplification for the marker specific for that region. While partial deletions of the *AZF*a and *AZF*b region, as indicated by the lack of amplification of only one marker for the relevant region, are possible, the elective deletion of only sY254 or sY255 should always be regarded as a methodological error. If only one marker for *AZF*a or *AZF*b is deleted, the deletion must first be carefully confirmed (see below) and then the entire region should be studied in more detail. This event, however, is presently regarded as exceptional.

The PCR conditions should be carefully optimized in each laboratory according to the equipment available (e.g. type of Thermocycler) and DNA quality. If the result is ambiguous and/or a technical failure is suspected, the multiplex reaction should be repeated. If the multiplex does not work for a specific DNA sample, the primer set may be run in simplex reactions. If the results of both multiplex PCR consistently speak in favour of a deletion, the deletion is confirmed. If the results of the two multiplexes are not in agreement, the whole set of primers should be repeated in simplex PCR, as there is no reason to repeat the test in the same manner. It is known that simplex PCR is less subject to amplification failure and it is strongly advised to repeat the amplification at a lower annealing temperature. There is no general advice as to the number of repetitions. The test should be repeated until the results are clear and reproducible (good laboratory practice).

Reporting

Reports should be written in a standardized format and should be clear to the non-specialist. Guidelines on how to write reports on the outcome of molecular genetics investigations on a patient can be found at the web site: http://www.emqn.org/bpguidelines.php

In general, reports must be clear, concise, accurate, fully interpretative, credible and authoritative. Reports should be typed, word-processed or produced by computer. Handwritten reports are not acceptable. Reports must include the following information:

- Clear identification of the laboratory.
- Date of referral and reporting.
- Patient identification: full name, date of birth and unique laboratory accession/identification number.
- Restatement in some form of the clinical question being asked (e.g. diagnosis of microdeletion of the Y chromosome), and the indication (e.g. azoospermia, ICSI, etc.).
- Tissue studied (e.g. blood, buccal smear, etc).
- Method used (e.g. multiplex PCR amplification).
- Outcome of the analysis: a tabular form of the various STS loci analysed is preferred. Avoid the use of + and −, which can be misinterpreted. Use words instead (e.g. present/absent, or similar).
- A written interpretation understandable by the non-specialist.
- Signature of at least one person responsible for the results. Preferably, all laboratory data are reviewed and all outgoing reports are signed by two independent assessors.

External quality assessment

The laboratories performing *AZF* diagnostics should join an external quality assessment (EQA) scheme. It is fundamental that the DNA samples received from the organizers of the EQA programme are processed exactly in the same way as patients' samples are handled, including reporting. Results should be returned by mail to the organizers as patient (i.e. DNA sample) reports written in English. E-mail or fax can be used for quick communication, but the final evaluation of the results will be performed exclusively on reports received by mail.

Assessment of the performance of the laboratories participating in the international quality control assessment scheme organized by the EAA and EMQN includes both a general report and individual reports and recommendations to the participants. The results are assessed by independent reviewers. Laboratories, which correctly diagnose and report about the EQA samples receive a certificate.

Clinical relevance of partial deletions and rearrangements of AZFc

Very recent data indicate that partial deletions of the *AZFc* region (Repping *et al.*, 2003; Vogt & Fernandes, 2003) and possibly other types of rearrangements (Fernandes *et al.*, 2004; Repping *et al.*, 2004) occur relatively frequently. While some of these deletions and rearrangements might be of clinical significance, the exact role of such findings in male infertility has not yet been comprehensively analysed. Therefore, this should still be regarded as a very experimental area of infertility genetics and more data are necessary before clear-cut indications can be given for clinical routine.

Acknowledgements

The Best Practice Meeting was held in Florence, Italy, on October 5, 2003, as a satellite symposium of the EAA International Symposium on 'Genetics of male infertility: from research to clinic'. 61 persons from over 20 countries participated in the meeting and contributed actively to the discussion. The Best Practice Meeting and the External Quality Assessment Scheme for the molecular diagnosis of Y-chromosomal microdeletions are supported by the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN).

References

Blanco, P., Shlumukova, M., Sargent, C. A., Jobling, M. A., Affara, N & Hurles, M. E. (2000) Divergent outcomes of intrachromosomal recombination on the human Y chromosome: male infertility and recurrent polymorphism. *Journal of Medical Genetics* **37**, 752–758.

Cram, D. S., Ma, K., Bhasin, S., Arias, J., Pandjaitan, M., Chu, B. *et al.* (2000) Y chromosome analysis of infertile men and their sons conceived through intracytoplasmic sperm injection: vertical transmission of deletions and rarity of de novo deletions. *Fertility and Sterility* **74**, 909–915.

Ferlin, A., Moro, E., Garolla, A. & Foresta, C. (1999) Human male infertility and Y chromosome deletions: role of the *AZF*-candidate genes *DAZ*, RBM and DFFRY. *Human Reproduction* **14**, 1710–1716.

Fernandes, S., Huellen, K., Goncalves, J., Dukal, H., Zeisler, J., Rajpert De Meyts, E. *et al.* (2002) High frequency of *DAZ*1/*DAZ*2 gene deletions in patients with severe oligozoospermia. *Molecular Human Reproduction* **8**, 286–298.

Fernandes, S., Paracchini, S., Meyer, L. H., Floridia, G., Tyler-Smith, C. & Vogt, P. H. (2004) A Large *AZFc* deletion removes *DAZ3*/*DAZ4* and nearby genes from men in Y haplogroup N. *American Journal of Human Genetics* **74**, 180–187.

Foresta, C., Ferlin, A. & Moro, E. (2000) Deletion and expression analysis of *AZFa* genes on the human Y chromosome revealed a major role for DBY in male infertility. *Human Molecular Genetics* **9**, 1161–1169.

Foresta, C., Moro, E. & Ferlin, A. (2001) Y chromosome microdeletions and alterations of spermatogenesis. *Endocrine Reviews* **22**, 226–239.

Frydelund-Larsen, L., Krausz, C., Leffers, H., Andersson, A. M., Carlsen, E., Bangsboell, S., McElreavey, K., Skakkebaek, N. E. & Rajpert-De Meyts, E. (2002) Inhibin B: a marker for the functional state of the seminiferous epithelium in patients with azoospermia factor c microdeletions. *Journal of Clinical Endocrinology and Metabolism* **87**, 5618–5624.

van Golde, R.J,, Wetzels, A. M., de Graaf, R., Tuerlings, J. H., Braat, D. D. & Kremer, J. A. (2001) Decreased fertilization rate and embryo quality after ICSI in oligozoospermic men with microdeletions in the azoospermia factor c region of the Y chromosome. *Human Reproduction* **16**, 289–292.

Hopps, C. V., Mielnik, A., Goldstein, M., Palermo, G. D., Rosenwaks, Z. & Schlegel, P. N. (2003) Detection of sperm in men with Y chromosome microdeletions of the *AZF*a, *AZF*b and *AZF*c regions. *Human Reproduction* **18**, 1660–1665.

Jiang, M. C., Lien, Y. R., Chen, S. U., Ko, T. M., Ho, H. N. & Yang, Y. S. (1999) Transmission of de novo mutations of the deleted in azoospermia genes from a severely oligozoospermic male to a son via intracytoplasmic sperm injection. *Fertility & Sterility* **71**, 1029–1032.

Kamischke, A., Gromoll, J., Simoni, M., Behre, H. M. & Nieschlag, E. (1999) Transmission of a Y chromosomal deletion involving the deleted in azoospermia (*DAZ*) and chromodomain (CDY1) genes from father to son through intracytoplasmic sperm injection: case report. *Human Reproduction* **14**, 2320–2322.

Kamp, C., Hirschmann, P., Voss, H., Huellen, K. & Vogt, P. H. (2000) Two long homologous retroviral sequence blocks in proximal Yq11 cause *AZF*a microdeletions as a result of intrachromosomal recombination events. *Human Molecular Genetics* **9**, 2563–2572.

Kamp, C., Huellen, K., Fernandes, S., Sousa, M., Schlegel, P. N., Mielnik, A. *et al.* (2001) High deletion frequency of the complete *AZF*a sequence in men with Sertoli-cell-only syndrome. *Molecular Human Reproduction* **7**, 987–994.

Kent-First, M. G., Kol, S., Muallem, A., Ofir, R., Manor, D., Blazer, S., First, N. & Itskovitz-Eldor, J. (1996) The incidence and possible relevance of Y-linked microdeletions in babies born after intracytoplasmic sperm injection and their infertile fathers. *Molecular Human Reproduction* **2**, 943–950.

Kent-First, M., Muallem, A., Shultz, J., Pryor, J., Roberts, K., Nolten, W. *et al.* (1999) Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth *AZF* region (*AZF*d) by Y-chromosome microdeletion detection. *Molecular Reproduction and Development* **53**, 27–41.

Kleiman, S. E., Yogev, L., Gamzu, R., Hauser, R., Botchan, A., Paz, G., Lessing, J. B., Yaron, Y. & Yavetz, H. (1999) Three-generation evaluation of Y-chromosome microdeletion. *Journal of Andrology* **20**, 394–398.

Kleiman, S. E., Bar-Shira Maymon, B., Yogev, L., Paz, G. & Yavetz, H. (2001) The prognostic role of the extent of Y microdeletion on spermatogenesis and maturity of Sertoli cells. *Human Reproduction* **16**, 399–402.

Krausz, C. & McElreavey, K. (1999) Y chromosome and male infertility. *Frontiers in Bioscience* **4**, E1–E8.

Krausz, C., Bussani-Mastellone, C., Granchi, S., McElreavey, K., Scarselli, G. & Forti, G. (1999) Screening for microdeletions of Y chromosome genes in patients undergoing intracytoplasmic sperm injection. *Human Reproduction* **14**, 1717–1721.

Krausz, C., Quintana-Murci, L. & McElreavey, K. (2000) Prognostic value of Y deletion analysis: what is the clinical prognostic value of Y chromosome microdeletion analysis? *Human Reproduction* **15**, 1431–1434.

Krausz, C., Rajpert-De Meyts, E., Larsen, L. F., Quintana-Murci, L., McElreavey, K. & Skakkebaek, N. (2001) Double blind screening for microdeletions of Y chromosome genes in infertile and fertile/normospermic Danish men. *Journal of Clinical Endocrinology and Metabolism* **86**, 2638–2642.

Krausz, C., Forti, G. & McElreavey, K. (2003) The Y chromosome and male fertility and infertility. *International Journal of Andrology* **26**, 70–75.

Kühnert, B., Gromoll, J., Kostova, E., Tschanter, P., Luetjens, C. M., Simoni, M. & Nieschlag, E. (2004) Natural transmission of an *AZF*c Y-chromosomal microdeletion from father to his sons. *Human Reproduction* **19**, 886–888.

Kuroda-Kawaguchi, T., Skaletsky, H., Brown, L. G., Minx, P. J., Cordum, H. S., Waterston, R. H. *et al.* (2001) The *AZF*c region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nature Genetics* **29**, 279–286.

Luetjens, C. M., Gromoll, J., Engelhardt, M., Von Eckardstein, S., Bergmann, M., Nieschlag, E. & Simoni, M. (2002) Manifestation of Y-chromosomal deletions in the human testis: a morphometrical and immunohistochemical evaluation. *Human Reproduction* **17**, 2258–2266.

Maurer, B. & Simoni, M. (2000) Y chromosome microdeletion screening in infertile men. *Journal of Endocrinological Investigation* **23**, 664–670.

Maurer, B., Gromoll, J., Simoni, M. & Nieschlag, E. (2001) Prevalence of Y chromosome microdeletions in infertile men who consulted a tertiary care medical centre: the Münster experience. *Andrologia* **33**, 27–33.

Mulhall, J. P., Reijo, R., Alagappan, R., Brown, L., Page, D., Carson, R. & Oates, R. D. (1997) Azoospermic men with deletion of the DAZ gene cluster are capable of completing spermatogenesis: fertilization, normal embryonic development and pregnancy occur when retrieved testicular spermatozoa are used for intracytoplasmic sperm injection. *Human Reproduction* **12**, 503–508.

Oates, R. D., Silber, S., Brown, L. G. & Page, D. C. (2002) Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the *AZF*c region of the Y chromosome, and of 18 children conceived via ICSI. *Human Reproduction* **17**, 2813–2824.

Page, D. C., Silber, S. & Brown, L. G. (1999) Men with infertility caused by *AZF*c deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. *Human Reproduction* **14**, 1722–1726.

Patsalis, P. C., Sismani, C., Quintana-Murci, L., Taleb-Bekkouche, F., Krausz, C. & McElreavey, K. (2002) Effects of transmission of Y chromosome *AZF*c deletions. *Lancet* **360**, 1222–1224.

Peterlin, B., Kunej, T., Sinkovec, J., Gligorievska, N. & Zorn, B. (2002) Screening for Y chromosome microdeletions in 226 Slovenian subfertile men. *Human Reproduction* **17**, 17–24.

Reijo, R., Alagappan, R. K., Patrizio, P. & Page, D. C. (1996) Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. *Lancet* **347**, 1290–1293.

Repping, S., Skaletsky, H., Lange, J., Silber, S., Van Der Veen, F., Oates, R. D., Page, D. C. & Rozen, S. (2002) Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *American Journal of Human Genetics* **71**, 906–922.

Repping, S., Skaletsky, H., Brown, L., van Daalen, S. K., Korver, C. M., Pyntikova, T. *et al.* (2003) Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nature Genetics* **35**, 247–251.

Repping, S., van Daalen, S. K. M., Korver, C. M., Brown, L. G., Marszalek, J. D., Gianotten, J. *et al.* (2004) A family of human Y chromosomes has dispersed throughout northern Eurasia despite

a 1.8-Mb deletion in the azoospermia factor c region. *Genomics*, in press.

Saxena, R., de Vries, J. W., Repping, S., Alagappan, R. K., Skaletsky, H., Brown, L. G., Ma, P., Chen, E., Hoovers, J. M. & Page, D. C. (2000) Four *DAZ* genes in two clusters found in the *AZF*c region of the human Y chromosome. *Genomics* **67**, 256–267.

Siffroi, J. P., Le Bourhis, C., Krausz, C., Barbaux, S., Quintana-Murci, L., Kanafani, S. *et al.* (2000) Sex chromosome mosaicism in males carrying Y chromosome long arm deletions. *Human Reproduction* **15**, 2559–2562.

Silber, S. J., Alagappan, R., Brown, L. G. & Page, D. C. (1998) Y chromosome deletions in azoospermic and severely oligo-zoospermic men undergoing intracytoplasmic sperm injection after testicular sperm extraction. *Human Reproduction* **13**, 3332–3337.

Simoni, M. (2001) Molecular diagnosis of Y chromosome micro-deletions in Europe: state-of-the-art and quality control. *Human Reproduction* **16**, 402–409.

Simoni, M., Bakker, E., Eurlings, M. C., Matthijs, G., Moro, E., Müller, C. R. & Vogt, P. H. (1999) Laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions. *International Journal of Andrology* **22**, 292–299.

Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P. J., Cordum, H. S., Hillier, L., Brown, L. G. *et al.* (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* **423**, 825–837.

Sun, C., Skaletsky, H., Rozen, S., Gromoll, J., Nieschlag, E., Oates, R. & Page, D. C. (2000) Deletion of azoospermia factor a (*AZF*a) region of human Y chromosome caused by recombination between HERV15 proviruses. *Human Molecular Genetics* **9**, 2291–2296.

Tomasi, P. A., Oates, R., Brown, L., Delitala, G. & Page, D. C. (2003) The pituitary-testicular axis in Klinefelter's syndrome and in oligo-azoospermic patients with and without deletions of the Y chromosome long arm. *Clinical Endocrinology (Oxford)* **59**:214–222.

Vogt, P. H. (1998) Human chromosome deletions in Yq11, *AZF* candidate genes and male infertility: history and update. *Molecular Human Reproduction* **4**, 739–744.

Vogt, P. H. & Fernandes, S. (2003) Polymorphic *DAZ* gene family in polymorphic structure of *AZF*c locus: artwork or functional for human spermatogenesis? *APMIS* **111**, 115–126.

Vogt, P. H., Edelmann, A., Kirsch, S., Henegariu, O., Hirschmann, P., Kiesewetter, F. *et al.* (1996) Human Y chromosome azoospermia factors (*AZF*) mapped to different subregions in Yq11. *Human Molecular Genetics* **5**, 933–943.

de Vries, J. W., Repping, S., van Daalen, S. K., Korver, C. M., Leschot, N. J. & van der Veen, F. (2002) Clinical relevance of partial *AZF*c deletions. *Fertility and Sterility* **74**, 1209–1214.

Yen, P. (2001) The fragility of fertility. *Nature Genetics* **29**, 243–244.

Received 2 April 2004; revised 6 May 2004; accepted 10 May 2004

Appendix A

Example of a PCR protocol

Two multiplex reactions were designed for the analysis of the three *AZF* deletion regions on the Y chromosome. Both multiplexes contain five fragments, i.e. the three *AZF* loci and the two control fragments *SRY* and *ZFY*. Each laboratory should set up and validate its own protocol. Here we give an example of the protocol validated and currently in use at the Institute of Reproductive Medicine in Münster.

PCR kit: Quiagen Multiplex PCR Kit (Cat.No. 206143, Quiagen, Hilden, Germany).

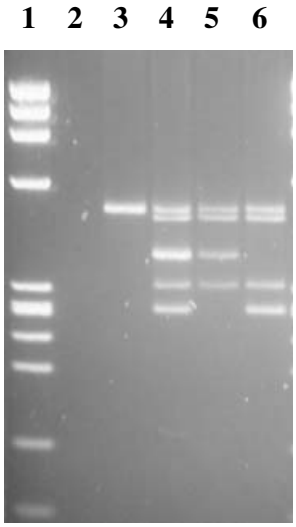
Preparation of 10x primer mix A and B (containing 2 μM each primer). Primer mixes are prepared in batches sufficient for about 100 reactions, and packaged in smaller size aliquots (sufficient for 10 or 20 reactions) for storage at −20 °C.

The 50 μL PCR reaction mix contains:

25 μL 2x Quiagen Multiplex PCR MasterMix [containing HotStarTaq DNA Polymerase, Qiagen Multiplex PCR Buffer (containing 6 mM $MgCl_2$) and dNTP Mix], 5 μL 10x Primer mix (2 μM each primer), approximately 1 μg template DNA, sterile distilled water to 50 μL.

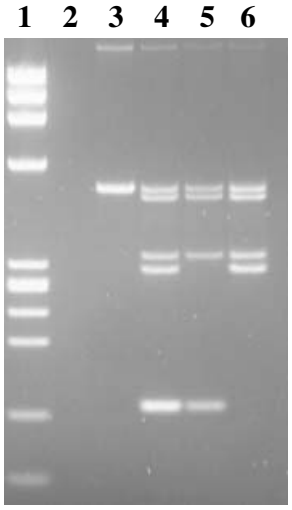
Amplification conditions (as established using a Hybaid Touch Down Thermocycler, Dynex Hybald Lab System, Frankfurt, Germany) start with an initial activation step of 15 min at 95 °C, followed by 35 cycles of 30 sec denaturation (94 °C), 90 sec annealing (57 °C) and 60 sec elongation (72 °C), ended by a last elongation step of 10 min and cooling to 4 °C.

Reaction products (30 μL) are separated on a 2% Agarose (Peqbold Universal Agarose, Peqlab, Erlangen, Germany) plus 0.5% DNA Agar (Serva, Heidelberg, Germany) gels in 1 × TBE for 25 V overnight. An example of both multiplexes is given in Fig. 2.



Multiplex A:

ZFY	: 495 bp
SRY	: 472 bp
sY254	: 400 bp (*AZFc*)
sY86	: 320 bp (*AZFa*)
sY127	: 274 bp (*AZFb*)



Multiplex B:

ZFY	: 495 bp
SRY	: 472 bp
sY84	: 326 bp (*AZFa*)
sY134	: 301 bp (*AZFb*)
sY255	: 126 bp (*AZFc*)

Figure 2. Examples of both Multiplex PCR. Multiplex A: lane 1, phi X-HeaIII size marker; lane 2, water; lane 3, female DNA; lane 4, DNA of normal male; lane 5, DNA of *AZF*b (P5/proximal P1)-deleted patient; lane 6, DNA of *AZF*c (b2/b4)-deleted patient.

Appendix B

Sequence of the PCR primers

Multiplex Primers	
A and B	ZFY-F: 5′ – ACC RCT GTA CTG ACT GTG ATT ACA C – 3′
	ZFY-R: 5′ – GCA CYT CTT TGG TAT CYG AGA AAG T – 3′
A and B	*SRY*-F: 5′ – GAA TAT TCC CGC TCT CCG GA – 3′
	SRY-R: 5′ – GCT GGT GCT CCA TTC TTG AG – 3′
A.	sY86-F: 5′ – GTG ACA CAC AGA CTA TGC TTC – 3′
	sY86-R: 5′ – ACA CAC AGA GGG ACA ACC CT – 3′
A.	sY127-F: 5′ – GGC TCA CAA ACG AAA AGA AA – 3′
	sY127-R: 5′ – CTG CAG GCA GTA ATA AGG GA – 3′
A.	sY254-F: 5′ – GGG TGT TAC CAG AAG GCA AA – 3′
	sY254-R: 5′ – GAA CCG TAT CTA CCA AAG CAG C – 3′
B.	sY84-F: 5′ – AGA AGG GTC TGA AAG CAG GT – 3′
	sY84-R: 5′ – GCC TAC TAC CTG GAG GCT TC – 3′
B.	sY134-F: 5′ – GTC TGC CTC ACC ATA AAA CG – 3′
	sY134-R: 5′ – ACC ACT GCC AAA ACT TTC AA – 3′
B.	sY255-F: 5′ – GTT ACA GGA TTC GGC GTG AT – 3′
	sY255-R: 5′ – CTC GTC ATG TGC AGC CAC– 3′

Appendix C

Useful web sites:
European Academy of Andrology (EAA):
http://www.uni-leipzig.de/~eaa/index.html
European Molecular Genetics Quality Network (EMQN):
http://www.emqn.org/
E-mail addresses of the authors:
M. Simoni: simoni@uni-muenster.de
E. Bakker: e.bakker@lumc.nl
C. Krausz: c.krausz@dfc.unifi.it