international journal of andrology, 22:292–299 (1999)

Laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions

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Introduction

In addition to the 'testis determining factor' (SRY), the Y chromosome harbours genes important for spermatogenesis. The 'azoospermia factor' (*AZF*) on the long arm of the Y chromosome, originally defined by Tiepolo & Zuffardi (1976), is now progressively being dissected into discrete genes (Lahn & Page, 1997; Vogt *et al.*, 1997). The deletion of the *AZF* is thought to be pathogenetically involved in some cases of male infertility associated with azoospermia or severe oligozoospermia (Vogt, 1998). While until few years ago analysis of the *AZF* region was performed only for experimental purposes, the discovery of the *DAZ* gene (Reijo *et al.*, 1995), the description of three discrete *AZF* regions (Vogt *et al.*, 1996) and the diffusion of the PCR technology has rendered the molecular diagnosis of Y-chromosomal microdeletions popular and affordable (for review see Kostiner *et al.*, 1998 and Vogt, 1998). As a result, PCR analysis of the Y chromosome is now routinely performed in the work-up of the infertile male. However, there has been no consensus on how the diagnosis should be carried out and which DNA loci on the Y chromosome should be analysed.

The heterogeneity of the protocols employed and of the results published, together with the increasing diffusion of this diagnostic procedure, suggested the necessity of a quality control programme (Simoni *et al.*, 1998). Such an international programme started in 1997 as an official activity of the European Academy of Andrology. After completion of three trials the participants in the quality control programme and some experts in diagnostics of genetic diseases and in genetics of the Y chromosome were invited to evaluate the results and to contribute to the generation of the present *Guidelines for the molecular diagnosis of y-chromosomal microdeletions*.

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Frequency and clinical relevance of microdeletions of the Y chromosome

Existing evidence suggests that genes located on the euchromatic region of the long arm of the Y chromosome play an essential role in spermatogenesis (Edwards & Bishop, 1997; Vogt, 1998). In fact, microdeletions are found in infertile men with azoospermia or severe oligozoospermia, while they are not detected in control groups of proven fathers. Moreover, deletions in infertile men are usually de novo events, probably originating in the germ line of the patient's father (Edwards & Bishop, 1997). For practical purposes, the regions of the Y chromosome that contain genes probably relevant to male infertility are indicated as *AZFa*, *AZFb* and *AZFc*, respectively (Vogt *et al.*, 1996).

At the present time, the literature published after the description of the *DAZ* gene (Reijo *et al.*, 1995) reports data from more than 3000 infertile men and 1300 proven fathers (Nakahori *et al.*, 1996; Qureshi *et al.*, 1996; Stuppia *et al.*, 1996; Reijo *et al.*, 1996; Vogt *et al.*, 1996; Najmabadi *et al.*, 1996; Kent-First *et al.*, 1996; Pryor *et al.*, 1997; Foresta *et al.*, 1997; Vereb *et al.*, 1997; Kremer *et al.*, 1997; Mulhall *et al.*, 1997; Simoni *et al.*, 1997; van der Ven *et al.*, 1997; Stuppia *et al.*, 1996; Girardi *et al.*, 1997; Foresta *et al.*, 1998; Liow *et al.*, 1998; Stuppia *et al.*, 1998; Brandell *et al.*, 1998; Silber *et al.*, 1998; Grimaldi *et al.*, 1998; Kleiman *et al.*, 1999; Selva *et al.*, 1997; Kent-First *et al.*, 1999; Seifer *et al.*, 1999). Summarizing these published papers, microdeletions were found in about 7.3% of the infertile men. The vast majority of deletions were found in azoospermic men (66%), less so in men with sperm concentrations $<5 \times 10^6$/mL (28%) and sporadically in

men with sperm concentrations $>5 \times 10^6$/mL (6%). These data suggest that the expected frequency of microdeletions in 'selected' infertile men should be around 7%. However, in the literature the frequency varies between 1% (van der Ven *et al.*, 1997) and 55.5% (Foresta *et al.*, 1998). This variation is probably mainly due to the selection criteria of the patients. However, other factors should be considered, such as accuracy of the PCR results and relevance of the deletion detected, especially in case of discontinuous deletions or deletions of isolated STS loci. Reviews of the literature have been published recently (Simoni *et al.*, 1998; Vogt, 1998).

Although the role of Y-chromosomal deletions in male infertility appears obvious, formal proof of the involvement of discrete genes in male fertility is still lacking. This would require the discovery of cases of infertility due to point mutations and/or the demonstration in vitro of clear loss of function induced by site-directed mutagenesis. The function of these genes, however, is still unknown. Moreover, the nature of many Y-chromosomal genes, most of which are either multicopy or expressed in tissues other than the testis, renders the experimental analysis and the demonstration of a causal role in infertility extremely difficult. It is conceivable that several genes of the Y chromosome, which are specifically expressed in the testis and deleted in human male infertility, contribute to normal spermatogenesis (Vogt, 1998). What they exactly do, is basically unknown.

In summary, diagnosis of Y-chromosomal microdeletions as it is currently performed does not prove that the deletion caused the patient's infertility. However, such a causal relationship is highly probable, provided that the loci have been shown and confirmed to be deleted only in infertile males and not in proven fathers in well controlled studies.

Indications for diagnosis

Given these premises, there are no absolute criteria or guidelines indicating which patients are candidates for molecular analysis. Patients undergoing intra-cytoplasmatic sperm injection (ICSI) may represent an obvious population for deletion screening due to the fact that the microdeletions are transmitted to the male offspring if assisted reproduction is successfully performed (Kent-First *et al.*, 1996; Kamischke *et al.*, 1999; Page *et al.*, 1999). In this case, the diagnosis of a deletion can influence the therapeutic option. Genetic counselling is then mandatory in order to provide information about the risk of producing an infertile son, although there is no proof yet that the son will indeed be infertile (Meschede *et al.*, 1995; Meschede *et al.*, 1997). Therefore, for the time being indications for molecular diagnosis of Y-chromosomal microdeletions remain open and clinicians should decide in which patients they regard this diagnosis as worthwhile and meaningful. It should be kept in mind that this is still a very experimental area of reproductive medicine and genetics.

Results from the first three trials of the External Quality Control Assessment Programme

Six DNA samples were sent to the participating laboratories subdivided into three trials. Participants (see Appendix) were requested to perform analysis according to their usual protocol. Twenty, 25 and 29 laboratories participated in the consecutive trials, respectively. In summary, the results have shown that:

- both simplex and multiplex PCR are used;
- there is a wide inter-lab heterogeneity in the choice of primers used. Remarkably, none of the primers was common to all participants. Overall, 90 different primers were employed in trial 1 (9 of which amplified STSs in the *AZFa* region, 23 in *AZFb*, 36 in *AZFc* and 22 other STSs, respectively), 77 primers in trial 2 (8 in *AZFa*, 24 in *AZFb*, 30 in *AZFc* and 15 in other regions, respectively) and 80 primers in trail 3 (9 in *AZFa*, 24 in *AZFb*, 36 in *AZFc* and 11 in other regions, respectively). The reason for the choice of primers was not indicated;
- the rate of misdiagnosis was 5% (2/40 diagnoses) for trial 1, 4% for trial 2 (2/50 diagnoses) and 5.2% (3/58 diagnoses) for trial 3. Both false positive (i.e. failure to detect a deletion) and false negative (failure to amplify normal DNA) results were obtained. In general, the diagnosis of deletions involving the *DAZ* gene was accurate (no false positives), but false negative results leading to the wrong diagnosis of *AZFc* deletions were observed. False positive results were obtained in a sample with an *AZFb* deletion. Remarkably, both false positive and false negative results were obtained even when using more than one STS primer for each *AZF* interval. Finally, some laboratories produced 'uncertain' results for some STSs, suggesting that the quality control samples might have been handled differently from the patient samples;
- no data concerning internal quality control were given.

Guidelines for diagnostic testing

The diagnostic testing of deletions is performed by PCR amplification of selected regions of the Y chromosome. Most of the STS primers used so far amplify anonymous sequences of the chromosome, which have been more or less precisely mapped but are not yet known to belong to specific genes. Although discrete genes are progressively being identified, there is no proof of their causal role in infertility, not even in the case of *DAZ* and *RBM* (Ruggiu & Cooke, 1999). Moreover, using STS primers specific for discrete genes does not increase the detection rate of microdeletions in DNA samples from ICSI candidates (Krausz *et al.*, 1999). Therefore, for the time being, it is basically unimportant whether the STS primers used amplify anonymous regions or *AZF* candidate genes. What is

important for the diagnosis is that the panel of STS primers are derived from regions of the Y chromosome which are unique and not polymorph in the Caucasian population and are well known to be deleted specifically in infertile men. These regions correspond to the *AZFa*, *AZFb* and *AZFc* regions according to Vogt *et al.* (1996). A putative fourth *AZF* region has been recently postulated by Kent-First *et al.* (1999). According to the authors, a subgroup of patients with idiopathic infertility and variable sperm count would display a deletion of a new Yq11 subinterval. However, all the STS loci analysed for defining *AZF*d (i.e. sY145, sY153, sY152, sY220, sY150, sY232, sY262, sY221) had been earlier mapped to the *AZFc* region (interval 6C-6E, Reijo *et al.*, 1995; Vogt *et al.*, 1997). Therefore, there is no need to define a fourth *AZF* region. Rather, it would be important to establish the causal relationship of microdeletions in *AZFc* to the male infertility phenotype, since the pathological phenotypes of these patients range from azoospermia to normozoospermia (Kent-First *et al.*, 1999). Hence, these data await confirmation.

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. A very useful publication with recommendations for good laboratory practice and internal quality control as well as troubleshooting in diagnostic amplification techniques has appeared recently (Neumaier *et al.*, 1998) and should be carefully followed when implementing the diagnostics of Y-chromosomal microdeletions. Each set of PCRs 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. It was agreed that an appropriate *internal PCR control* in *AZF* diagnostics is the *ZFX/ZFY* gene. The *ZFX/ZFY* gene was chosen 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 fertile man and from a women, respectively. The DNA sample from a fertile man controls for the sensitivity and specificity of the assay. The female DNA sample controls for the 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.

For a more detailed description of internal quality control please consult Neumaier *et al.* (1998).

First choice (minimal 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 *AZFa*, *AZFb* or *AZFc*. However, analysing two STS loci in each region reinforces diagnostic accuracy, since deletions usually involve more than one STS locus. Therefore, it was agreed that at least two STS loci in each *AZF* region should be analysed. Based on the experience of the first three trials of external quality control and considering the multiplex PCR format, the STS primers included in the first choice set are:

For *AZFa*: sY84, sY86
For *AZFb*: sY127, sY134
For *AZFc*: sY254, sY255 (both in the *DAZ* gene)

These STS primers have been shown to give reproducible results in multiplex PCR reactions by several laboratories and are those most frequently used among the participants in the quality control assessment scheme. 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 first choice for the diagnosis of microdeletion of the AZFa, AZFb and AZFc region includes: sY14 (SRY), ZFX/ZFY, sY84, sY86, sY127, sY134, sY254, sY255.

The sequence of the primers and the suggested PCR conditions are reported in the Appendix. The use of this primer set will enable the detection of over 90% of the deletions in the three *AZF* regions. This is the first choice set for diagnosis and it should be kept in mind that the extent of the deletion is not assessed by this method and genotype/phenotype correlations are not possible. Furthermore, deletions outside the *AZFa*, *AZFb* and *AZFc* regions will not be detected. Nevertheless, the adoption of this minimal set of primers by all laboratories is judged to be sufficient, at the moment, for routine diagnostics. It will also allow a minimal standardization and a better comparison of laboratory performance and inter-laboratory variability.

Second choice of STS primers

Once a deletion is detected, if one wishes to determine whether the *extent* of the deletion is similar to that of the regions designated as *AZFa*, *AZFb* and *AZFc*, the analysis can be extended to STS loci known to cross the proximal and the distal borderlines of each *AZF* region. This analysis could include:

AZFa: sY82(+), sY83(–) (proximal border),//(distal border) sY87(–), sY88(+)

AZFb: sY135(+), sY114(–) (proximal border),//(distal border) sY143(–), sY152(+)

AZFc: sY143(+), sY152(–) (proximal border),//(distal border) sY157(–), sY158(+)

Yq12 (distal heterochromatin, positive control): sY160/DYZ1

(+) indicates that the locus should be present in the corresponding patient group

(–) indicates that the locus should be absent in the corresponding patient group

This choice might be useful to establish genotype/phenotype correlation. Even in this case, deletions outside the *AZFa*, *AZFb* and *AZFc* regions will not be detected.

In summary: *in any case* the routine diagnostics of deletions of the Y chromosome should include the set of primers indicated under 5b. If a more extensive analysis is desired, the set of primers indicated under 5c *might* be used. However, the choice of the primers for the extended analysis is left to the individual laboratories. The extended analysis should be performed in multiplex PCR reactions using the internal and external controls indicated above.

STSs primers which should not be used

A number of STS primers currently used by some participants in the quality control assessment scheme should *no longer be used* for the following reasons:

- sY16: is redundant when using sY14
- sY55: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 3C, 6A)
- sY75: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 4A, 6)
- sY109: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals D11, D23)
- sY112: this STS locus is repetitive and dispersed throughout the Y chromosome (D12, D14, D22). sY135 is recommended instead of sY112 for the analysis of interval D12
- sY132: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 3 and 6)
- sY164: this STS locus is repetitive
- sY138: this STS locus is repetitive
- sY153: this STS locus is polymorphic
- sY155: this STS locus is repetitive
- sY272: this STS locus is polymorphic

Moreover: sY156 and Y6HP52 analyse the same locus in interval D20. Therefore only one of them should be used.

In summary: the use of the following primers should be abandoned: sY16, sY55, sY75, sY109, sY112, sY132, sY164, sY138, sY153, sY155, sY272.

Control and repetition of the test

If the results of a multiplex PCR indicate a deletion, the whole set of primers should be repeated in simplex PCR, since there is no reason to repeat the test in the same manner. It is known that simplex PCR is less subject to amplification failure. 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. 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 internet site:

http://www.leeds.ac.uk/cmgs/guidelines/report-writing

According to those guidelines, reports must be clear, concise, accurate, fully interpretable, credible and authoritative. Reports should be typed, word-processed or created by computer. Hand-written 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.

Participation in External Quality Control Assessment Programme

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.

The assessment of the performance of the laboratories participating in the international quality control assessment scheme organized by the European Academy of Andrology will include both a general report and individual reports, and recommendations to the participants. The results will be assessed by independent reviewers.

The present guidelines represent a first effort towards standardization and improvement of the quality of the molecular diagnosis of Y chromosomal microdeletions. They have been compiled with the active participation of many colleagues working in this field (see Appendix) who have expressed consensus on the issues described here. These guidelines derive from the state-of-the-art knowledge on this rapidly evolving, still experimental field and will be updated as soon as new data from the published literature suggest its necessity. In any case, a formal review of these guidelines will be made one year from the date of issue.

References

Brandell, R. A., Mielnik, A., Liotta, D., Ye, Z., Veeck, L. L., Palermo, G. D. & Schlegel, P. N. (1998) *AZFb* deletions predict the absence of spermatozoa with testicular sperm extraction: preliminary report of a prognostic genetic test. *Human Reproduction* **13**, 2812–2815.

Edwards, R. G. & Bishop, C. E. (1997) On the origin and frequency of Y chromosome deletions responsible for severe male infertility. *Molecular Human Reproduction* **3**, 549–554.

Foresta, C., Ferlin, A., Garolla, A., Moro, E., Pistorello, M., Barbaux, S. & Rossato, M. (1998) High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell–only syndrome. *Human Reproduction* **13**, 302–307.

Foresta, C., Ferlin, A., Garolla, A., Rossato, M., Barbaux, S. & De Bortoli, A. (1997) Y-chromosome deletions in idiopathic severe testiculopathies. *Journal of Clinical Endocrinology and Metabolism* **82,** 1975–1080.

Girardi, S. K., Mielnik, A. & Schlegel, P. (1997) Submicroscopic deletions in the Y chromosome of infertile men. *Human Reproduction* **12**, 1635–1641.

Grimaldi, P., Scarponi, C., Rossi, P., Rocchietti March, M., Fabbri, A., Isidori, A., Spera, G., Krausz, C. & Geremia, R. (1998) Analysis of Yq microdeletions in infertile males by PCR and DNA hybridization techniques. *Molecular Human Reproduction* **4**, 1116–1121.

Kamischke, A., Gromoll, J., Simoni, M., Behre, H. M. & Nieschlag, E. (1999) Transmission of a Y-chromosomal deletion involving the *DAZ* and CDY genes from father to son though ICSI. *Human Reproduction* **14**, in press.

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**, 943–959.

Kent-First, M., Muallem, A., Schultz, J., Pryor, J., Roberts, K., Nolten, W., Meisner, L., Chandley, A., Gouchy, G., Jorgensen, L., Havighurst, T. & Grosch, J. (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., Lessing, J. B., Paz, G. & Yavetz, H. (1999) Genetic evaluation of infertile men. *Human Reproduction* **14**, 33–38.

Kostiner, D. R., Turek, P. J. & Reijo, R. A. (1998) Male infertility: analysis of the markers and genes on the human Y chromosome. *Human Reproduction* **13**, 3032–3038.

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

Kremer, J. A. M., Tuerlings, J. H. A. M., Meuleman, E. J. H., Schoute, F., Mariman, E., Smeets, D. F. C. M., Hoefsloot, L. H., Braat, D. D. M. & Merkus, H. M. W. M. (1997) Microdeletions of the Y-chromosome and intracytoplasmic sperm injection: from gene to clinic. *Human Reproduction* **12**, 687–691.

Lahn, B. T. & Page, D. C. (1997) Functional coherence of the human Y chromosome. *Science* **278**, 675–680.

Liow, S. L., Ghadessy, F. J., Ng, S. C. & Yong, E. L. (1998) Y chromosome microdeletions, in azoospermic or near-azoospermic subjects, are located in the *AZFc* (*DAZ*) subregion. *Molecular Human Reproduction* **4**, 763–768.

Meschede, D., DeGeyter, C., Nieschlag, E. & Horst, J. (1995) Genetic risk in micromanipulative assisted reproduction. *Human Reproduction* **10**, 2880–2886.

Meschede, D., Nieschlag, E. & Horst, J. (1997) Assisted reproduction for infertile couples at high genetic risk: an ethical consideration. *Biomedical Ethics* **2**, 4–6.

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 intracytoplasmatic sperm injection. *Human Reproduction* **12**, 503–508.

Najmabadi, H., Huang, V., Yen, P., Subbaru, M. N., Bhasin, D., Banaag, L., Naseeruddin, S., De Kretser, D. M., Baker, H. W. G., McLachlan, R. I., Loveland, K. A. & Bhasin, S. (1996) Substantial prevalence of microdeletions of the Y-chromosome in infertile men with idiopathic azoospermia and oligozoospermia detected using a sequence-tagged site-based mapping strategy. *Journal of Clinical Endocrinology and Metabolism* **81**, 1347–1352.

Nakahori, Y., Kuroki, Y., Komaki, R., Kondoh, N., Namiki, M., Iwamoto, T., Toda, T. & Kobayashi, K. (1996) The Y chromosome region essential for spermatogenesis. *Hormone Research* **46** (Suppl. 1), 20–23.

Neumaier, M., Braun, A. & Wagner, C. (1998) Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. *Clinical Chemistry* **44**, 12–36.

Page, D. C., Silber, S. & Brown, L. G. (1999) Men with infertility caused by *AZFc* deletion can produce sons by intracytoplasmic

sperm injection, but are likely to transmit the deletion and infertility. *Human Reproduction* **14**, 1722–1726.

Pryor, J. L., Kent-First, M., Muallem, A., Van Bergen, A. H., Nolten, W. E., Meisner, L. & Roberts, K. P. (1997) Microdeletion of the Y chromosome of infertile men. *New England Journal of Medicine* **336**, 534–539.

Qureshi, S. J., Ross, A. R., Ma, K., Cooke, H. J., McIntyre, M. A., Chandley, A. C. & Hargreave, T. B. (1996) Polymerase chain reaction screening for Y chromosome microdeletions: a first step towards the diagnosis of genetically-determined spermatogenic failure in men. *Molecular Human Reproduction* **2**, 775–779.

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.

Reijo, R., Lee, T.-Y., Salo, P., Alagappan, R., Brown, L. G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., de la Chapelle, A., Silber, S. & Page, D. C. (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nature Genetics* **10**, 383–393.

Ruggiu, M. & Cooke, H. (1999) Y bind RNA for spermatogenesis? *International Journal of Andrology* **22**, 19–27.

Seifer, I., Amat, S., Delgado-Viscoglioci, P., Boucher, D. & Bignon, Y. J. (1999) Screening for microdeletions on the long arm of chromosome Y in 53 infertile men. *International Journal of Andrology* **22**, 148–154.

Selva, J., Kanafani, S., Prigent, Y., Poncet, V. & Bergere, M. (1997) Incidence of *AZF* (azoospermia factor) deletions and familial forms of infertility among patients requiring intracytoplasmic spermatozoa injection (ICSI). *Journal of Assisted Reproduction and Genetics* **14**, 593–595.

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

Simoni, M., Gromoll, J., Dworniczak, B., Rolf, C., Abshagen, K., Kamischke, A., Carani, C., Meschede, D., Behre, H. M., Horst, J. & Nieschlag, E. (1997) Screening for deletions of the Y chromosome involving the *DAZ* (Deleted in Azoospermia) gene in azoospermia and severe oligozoospermia. *Fertility and Sterility* **67**, 542–547.

Simoni, M., Kamischke, A. & Nieschlag, E. (1998) Current status of the molecular diagnosis of Y-chromosomal microdeletions in the work-up of male infertility. *Human Reproduction* **13**, 1764–1768.

Stuppia, L., Gatta, V., Calabrese, G., Guanciali Franchi, P., Morizio, E., Bombieri, C., Mingarelli, R., Sforza, V., Frajese, G., Tenaglia, R. & Palka, G. (1998) A quarter of men with idiopathic oligo-azoospermia display chromosomal abnormalities and microdeletions of different types in interval 6 of Yq11. *Human Genetics* **102**, 566–570.

Stuppia, L., Mastroprimiano, G., Calabrese, G., Peila, R., Tenaglia, R. & Palka, G. (1996) Microdeletions in interval 6 of the Y chromosome detected by STS-PCR in 6 of 33 patients with idiopathic oligo- or azoospermia. *Cytogenetics and Cell Genetics* **72**, 155–158.

Tiepolo, L. & Zuffardi, O. (1976) Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Human Genetics* **34**, 119–124.

van der Ven, K., Montag, M., Peschka, B., Leygraaf, J., Schwanitz, G., Haidl, G., Krebs, D. & Van der Ven, H. (1997) Combined cytogenetic and Y chromosome microdeletion screening in males undergoing intracytoplasmic sperm injection. *Molecular Human Reproduction* **3**, 699–704.

Vereb, M., Agulnik, A. I., Houston, J. T., Lipschultz, L. I., Lamb, D. J. & Bishop, C. E. (1997) Absence of *DAZ* gene mutations in cases of non-obstructed azoospermia. *Molecular Human Reproduction* **3**, 55–59.

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., Edelmann, A., Kirsch, S., Henegariu, O., Hirschmann, P., Kiesewetter, F., Köhn, F. M., Schill, W. B., Farah, S., Ramos, C., Hartmann, M., Hartschuh, W., Meschede, D., Behre, H. M., Castel, A., Nieschlag, E., Weidner, W., Gröne, H.-J., Jung, A., Engel, W. & Haidl, G. (1996) Human Y chromosome azoospermia factors (*AZF*) mapped to different subregions in Yq11. *Human Molecular Genetics* **5**, 933–943.

Vogt, P. H., Affara, N., Davey, P., Hammer, M., Jobling, M. A., Lau, Y.-F. C., Mitchell, M., Schempp, W., Tyler-Smith, C., Williams, G., Yen, P. & Rappold, G. A. (1997) Report on the third international workshop on Y chromosome mapping 1997. *Cytogenetics and Cell Genetics* **79**, 1–20.

Appendix A. Participants in the 1st meeting

The first meeting to evaluate the results of the first three trials and generate guidelines for the diagnosis of Y-chromosomal microdeletions was held in Noordwijkerhout, The Netherlands, on 13 May 1999 during the first conference of the centres of the European Academy of Andrology. The following persons participated in the meeting and/or contributed to the guidelines:

Organizer

- Prof. Dr M. Simoni, Institute of Reproductive Medicine of the University, Domagkstr.11, D-48129 Münster, Germany.

Participants in the meeting

- Dr E. Baldi, Unità di Andrologia, Dipartimento di Fisiopatologia Clinica, Viale Pieraccini 6, I-50139 Firenze, Italy
- Dr E. Bakker, Department of Human and Clinical Genetics, Wassenaarseweg 72, NL-2333 Leiden, The Netherlands
- PD Dr J. Gromoll, Institute of Reproductive Medicine of the University, Domagkstr. 11, D-48129 Münster, Germany
- Dr B. Janssen, Institute of Human Genetics, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany
- Prof. Dr G. Matthijs, Center for Human Genetics, University of Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

- Dr E. Moro, Patologia Medica III, Via Ospedale Civile 105, I-35128 Padova, Italy
- Prof. Dr C. R. Müller-Reible, Institute of Human Genetics, Biozentrum, Am Hubland, D-97074 Würzburg, Germany
- Dr A. Tzschach, Institute of Human Genetics, University of Leipzig, Philipp-Rosenthal-Str. 55, D-4103 Leipzig, Germany
- L. Van Landuyt, Center for Medical Genetics, AZ-VUB, Laarbeeklaan 101, B-1090 Brussels, Belgium

Other contributors

- PD Dr P. H. Vogt, Institute of Human Genetics, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany
- Dr R. Reijo, Reproductive Genetics Division, Department of Obstetrics, Gynecology and Reproductive Sciences, UCSF, HSW 1480, 513 Parnassus Avenue, Box 0546, S. Francisco, CA 94143–0546

Appendix B. Suggested PCR protocol for the first choice testing

Two multiplex reactions were designed for the analysis of the three *AZF* deletion regions on the Y chromosome. Both multiplexes contain 5 fragments, i.e. the three *AZF* loci and the two control fragments *SRY* and *ZFY*. Reaction mixes (kits) are prepared in batches sufficient for 200 reactions, tested and filled out in smaller size aliquots (sufficient for 10 or 20 reactions) for storage at –70 °C.

For a deletion test 200 ng of genomic DNA and 2U of AmpliTaq are added to 20.6 μL of the prepared mix, giving a total reaction mix with an end volume of 25 μL. The mix then contains 0.75–6.1 pmol of each primer, multiplex buffer (16.6 mM $(NH_4)_2SO_4$, 66.7 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 6.8 μM EDTA and 5 mM Beta-mercaptoethanol) 10% DMSO, 1.5 mM dNTP's, 160 ng/μL BSA, 2U AmpliTaq (Perkin-Elmer), and 200 ng DNA.

Amplification conditions (as tested using a Perkin-Elmer 9700/9600 or 2400 PCR machine) start with an initial denaturation step of 5 min, followed by 25 cycles of 30 s denaturation (94 °C), 30 s annealing (55 °C) and 4 min elongation (65 °C), ended by a last elongation step of 5 min and cooling to 4 °C.

Reaction products (10 μL) are separated on a 2% NuSieve (FMC BioProducts, Rockland, ME) plus 0.5% MP (Boehringer) agarose gels in 1 × TBE for 480 Vhr. An example of both multiplexes is given in Fig. 1.

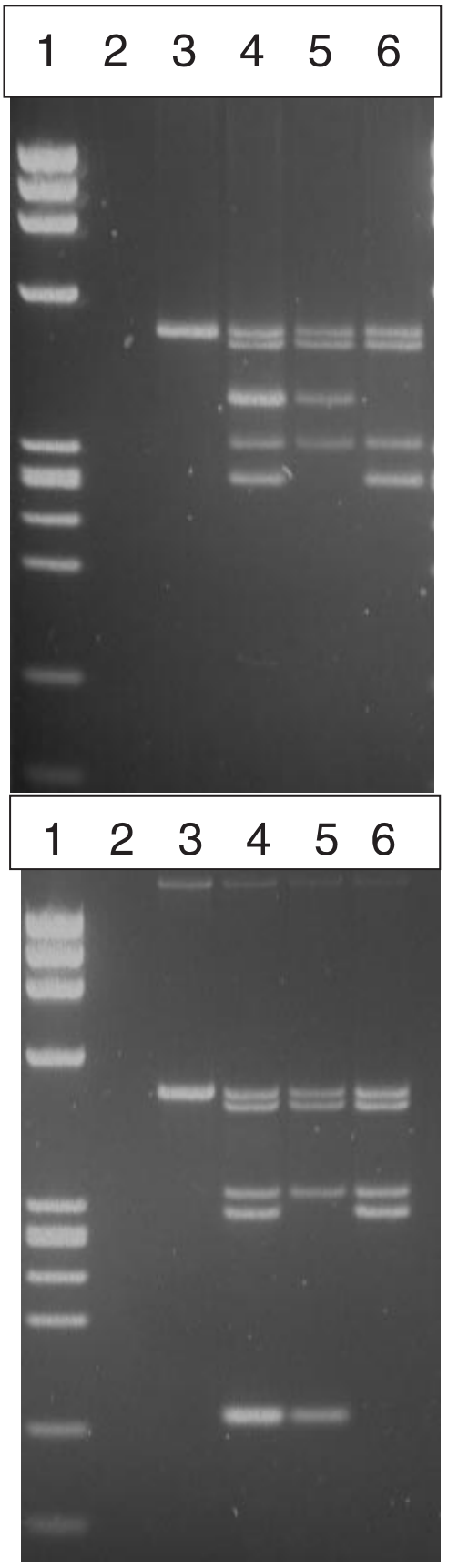

Figure 1. Examples of both Multiplex PCR. Multiplex A: lane 1 phi X-HealII size marker, lane 2 water, lane 3 female DNA, lane 4 DNA of normal male, lane 5 DNA of *AZFb* deleted patient, lane 6 DNA of *AZFc* deleted patient.

Appendix C. 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′

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*)