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EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: State of the art 2023

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Abstract

Testing for AZoospermia Factor (AZF) deletions of the Y chromosome is a key component of the diagnostic workup of azoospermic and severely oligozoospermic men. This revision of the 2013 European Academy of Andrology (EAA) and EMQN CIC (previously known as the European Molecular Genetics Quality Network) laboratory guidelines summarizes recent clinically relevant advances and provides an update on the results of the external quality assessment program jointly offered by both organizations. A basic multiplex PCR reaction followed by a deletion extension analysis remains the gold-standard methodology to detect and correctly interpret AZF deletions. Recent data have led to an update of the sY84 primer sequences, as well as to a refinement of what were previously considered as interchangeable border markers for AZFa and AZFb deletion breakpoints. More specifically, sY83 and sY143 are no longer recommended for the deletion extension analysis, leaving sY1064 and sY1192, respectively, as first-choice markers. Despite the transition, currently underway in several countries, toward a diagnosis based on certified kits, it should be noted that many of these commercial products are not recommended due to an unnecessarily high number of tested markers, and none of those currently available are, to the best of our knowledge, in accordance with the new first-choice markers for the deletion extension analysis. The gr/gr partial AZFc deletion remains a population-specific risk factor for impaired sperm production and a predisposing factor for testicular germ cell tumors. Testing for this deletion type is, as before, left at the discretion of the diagnostic labs and referring clinicians. Annual participation in an external quality control program is strongly encouraged, as the 22-year experience of the EMQN/EAA scheme clearly demonstrates a steep decline in diagnostic errors and an improvement in reporting practice.

KEYWORDS
AZF, azoospermia, gr/gr deletion, human genetics, male infertility, oligozoospermia, quality control, spermatogenesis, Y chromosome microdeletion

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1 | INTRODUCTION

After Klinefelter syndrome, AZoospermia Factor (AZF) deletions of the Y chromosome are the second most recurrent genetic cause of male infertility. Over the years, many researchers have described the occurrence of these microdeletions in different populations across the globe, with their molecular diagnosis being an important test in the diagnostic workup of male infertility.[1–6]

Y microdeletions occur in about one in 4000 men in the general population, but their frequency is significantly increased in infertile men. Azoospermic men have a higher incidence of microdeletions than oligozoospermic men and, consequently, deletion frequencies may vary from 2% to 10% (or even higher) across different laboratories, reflecting the composition of the study population.[7–9] Typically, routine diagnostic laboratories receiving referrals from outside institutions without controlled patient selection have a much lower incidence, usually below 2%.

Both published data and the quality control program experience confirm that diagnostic protocols vary across labs, and that inaccurate or altogether wrong diagnoses do occur, suggesting the need for both standardization and quality control.[10] Therefore, the European Academy of Andrology (EAA) and EMQN CIC jointly started offering external quality assessment (EQA) and a periodic publication of laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions.[4,11,12]

During the last 10 years, novel data have surfaced for some of these deletion patterns at the level of breakpoint variability and on the prognostic value of the test in the context of testicular sperm extraction (TESE). These, together with some methodological issues of significant importance, led to this update on the EAA/EMQN guidelines on AZF microdeletion testing.

1.1 | What is new?

A) The AZFa proximal border markers sY83 and sY1064 are not interchangeable, as their result depends on observed variation in the proximal breakpoint of complete AZFa deletions. Importantly, complete AZFa deletions can be compatible with the presence of sY83 and the absence of sY1064. Accordingly, only sY1064, and not sY83, is now recommended in the mandatory deletion extension analysis.

B) The AZFb distal border markers sY143 and sY1192 are not interchangeable, with only sY1192 being able to distinguish between complete and partial AZFb deletions (and thus provide a reliable prognosis of TESE success). Accordingly, only sY1192, and not sY143, is now recommended in the mandatory deletion extension analysis.

C) The sequence of both the forward and the reverse primers of sY84 have been updated to avoid a mismatch in the forward primer, and to account for a SNP in Asian populations (in the region of the reverse primer). The use of these updated primers, or other suitable alternatives, is strongly recommended, particularly in Asian populations.

D) Variability in the breakpoint of AZFb deletions can lead to the retention of the sY1224 proximal border marker in a significant number of complete AZFb and AZFbc deletions. Although the phenotypical consequences of this variation are unclear, the use of sY121 followed by sY1224 (in case sY121 is absent) is recommended.

E) To the best of our knowledge, the changes in first-choice markers for the deletion extension analysis are not fully compatible with any of the currently available molecular diagnostic kits for AZF deletions.

2 | INDICATIONS FOR THE GENETIC TESTING OF THE Y CHROMOSOME

Diagnosis of a complete AZF deletion establishes the cause of the patient's azoo-/oligozoospermia phenotype, offers important information at the level of genetic counseling, and provides prognostic value when considering TESE and medically assisted reproduction (MAR). Based on an extensive body of literature, clinically relevant deletions are found in patients with azoospermia or severe oligozoospermia with sperm concentrations $< 2 \times 10^6$/mL. Very rarely, deletions can be found in infertile patients with sperm concentrations between 2 and 5×10^6/mL.[9,13] Only from its 2010 edition onward, the "WHO laboratory manual for the examination and processing of human semen"—the gold standard for performing semen analyses—moved from sperm concentration to the biologically more meaningful parameter of total sperm count in the ejaculate as a proxy for spermatogenic output.[14,15] Consequently, data on total sperm count in AZF deletion carriers are missing from the majority of the published papers. However, both for sperm concentration and total sperm count, a consensus remains to be reached regarding the appropriate threshold below which AZF testing should be offered. The current European clinical guidelines still indicate a threshold of < 5 million spermatozoa/mL[16]; however, the cost-effectiveness of the test has been questioned in men with sperm concentrations $> 1 \times 10^6$/mL.[17] It should nevertheless be emphasized that screening azoospermic men for Y-chromosomal deletions is unanimously advised, as it can provide not only a diagnosis but also a robust estimate of the success rate of TESE.

We provide a flow chart with these indications and the recommended analytic steps in Figure 1. Routine clinical parameters, such as hormone levels, testicular volume, varicocele, maldescended testis, infections, and so on, do not have any predictive value.[8,9,18–20] In general, molecular analysis of the Y chromosome is not indicated in patients with chromosomal abnormalities (except 46,XY/45,X karyotype), obstructive azoospermia (unless FSH is above the normal limit), or hypogonadotropic hypogonadism. However, a number of deletion carriers have been identified among non-idiopathic infertile men, namely, those with testicular tumors, epididymal occlusions, orchitis, varicocele, or after chemo-/radiotherapy.[21] Therefore, the presence of any diagnosis accompanied by azoospermia or severe oligozoospermia should be an indication for AZF testing. For instance, in men belonging to the above semen categories, AZF screening is important before varicocelectomy, as deletion carriers will most likely not benefit from

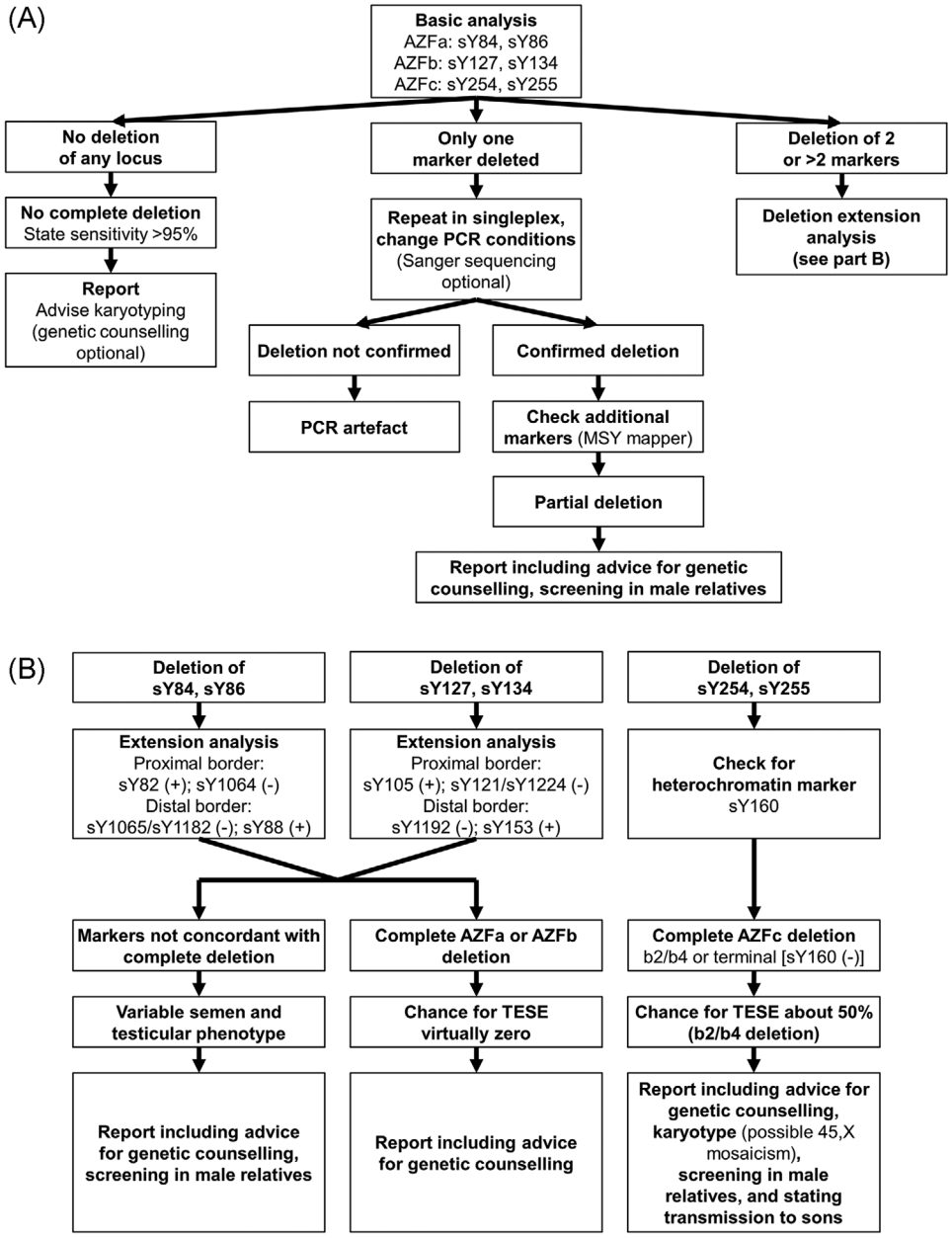

FIGURE 1 Flow chart of common analytical steps and consequences: (A) basic marker analyses and (B) deletion extension analyses.

the surgical procedure. Despite initial indications suggesting otherwise, more adequately powered studies have failed to establish the clinical utility of testing for Y-chromosomal deletions in Klinefelter patients.[8,22,23]

3 | STRUCTURE OF THE MALE-SPECIFIC REGION OF THE Y CHROMOSOME

The origin of Y-chromosomal microdeletions can be traced back to the uniqueness of the male-specific region of the Y chromosome (MSY).[24] This sequence was obtained by mapping 220 DNA clones containing portions of the MSY from just one man. Using DNA from only one individual was necessary as the presence of extensive repetitive sequences prone to inter-individual variation would severely complicate the assembly of sequences from multiple Y chromosomes. Tellingly, the first complete telomere-to-telomere assembly of the human Y was only made available in late 2022, thanks to critical advances in next-generation sequencing techniques and genome assembly algorithms.[25]

The reference MSY contains 156 transcription units (78 protein-coding) for a total of 27 different protein types. Three classes of sequences can be found in the MSY: X-degenerate (single-copy genes or pseudogenes derived from the ancestral state of the chromosome), X-transposed (more recently acquired from the X chromosome, and still 99% identical to the corresponding X regions), and ampliconic.[24] The latter correspond to repeat regions, divided across multiple family types, each sharing nearly complete (> 99.9%) identity between

intra-family repeats. Ampliconic sequences contain nine protein-coding gene families with testis-specific or enriched expression. Initially, these families were estimated to harbor at least 60 coding genes, a number that has been increased to more than 100 in the latest assemblies.[25] Due to their highly repetitive nature, ampliconic sequences can undergo both gene conversion[26] and non-allelic (intra-chromosomal) homologous recombination (NAHR).[27] Amplicons can be arranged in an ordered manner along the MSY, thus forming palindromes: mirror-image blocks of different ampliconic repeats that extend over considerable stretches of the MSY. Not surprisingly, the ampliconic (and palindromic) organization potentiates the generation of structural variants, such as deletions, duplications, and inversions. It is widely accepted that NAHR is the main driving force for the generation of complete AZF deletions. These exchanges occur between highly homologous repeat sequences with the same orientation, leading to loss (and the reciprocal duplication in the sister chromatid) of the genetic material between them. Considering the highly repetitive architecture of the MSY, many different MSY rearrangements have been identified.[28–34] Only those that have been clearly established as clinically relevant for male infertility are the focus of these guidelines.

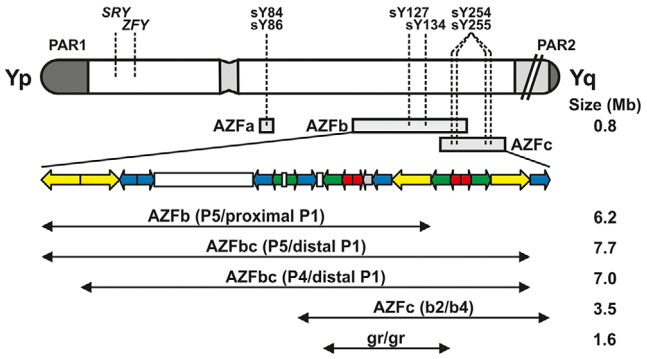

FIGURE 2 Schematic representation of the Y chromosome and of clinically relevant microdeletion patterns (based on the diagram of Repping and colleagues[30]). Repetitive sequences (color-coded palindromes) explain the origin of deletions in the AZFbc region by homologous recombination between identical sequences. The location of the STS primers suggested for the basic analysis step in these guidelines is indicated by dashed lines. Since four copies of the *DAZ* gene are present in the Y chromosome reference sequence, the STS primers sY254 and sY255 amplify four loci in AZFc. The AZFc (b2/b4) deletion is by far the most frequent type (~ 80%) of Y-chromosomal microdeletions found in men with severe oligo/azoospermia.

4 | DELETION TYPES AND THEIR MOLECULAR MECHANISM

Three different regions required for spermatogenesis were mapped to the long arm of the Y before the sequence of the MSY was actually known.[1] The three regions (AZFa, AZFb, and AZFc) were primarily defined based on phenotypical criteria, as each was associated with a specific type of spermatogenic impairment (see below). When these regions were finally sequenced, it became clear that the sequence of the AZFb and AZFc regions partially overlapped.[28] The fact that complete AZFb and AZFc deletions vary in their extension and affect different gene copies ultimately explains why AZFb deletions are associated with a more severe phenotype (azoospermia) than their AZFc counterparts (that can also be compatible with severe oligozoospermia).

The AZFa region is 792 kb long and contains the single-copy genes *USP9Y* (formerly *DFFRY*) and *DDX3Y* (formerly *DBY*). A third gene, UTY, with largely unknown functions, maps distally to this region.[35] The origin of complete AZFa deletions has been traced to NAHR between similarly oriented identical sequence blocks within the HERVyq1 and HERVyq2 retroviral sequences.[36–38] Recombination can occur in one of two identical blocks in these retroviral sequences (ID1 and ID2), giving rise to at least two slightly different deletion patterns.[37–39] In any case, these correspond to *de facto* complete AZFa deletions, removing both *USP9Y* and *DDX3Y*.

The type and mechanisms of deletion in the AZFb and AZFc regions have been clarified by Kuroda-Kawaguchi and colleagues.[28] Together, the two regions contain 24 different genes, most of which are present in multiple copies. The complete AZFb deletion removes 6.2 Mb (including 32 transcription units) and results from NAHR between the P5/proximal P1 palindromes.[30] Thus, complete AZFb deletions can also be referred to as P5/proximal P1 deletions.

The AZFc region includes 12 genes, each present in a variable number of copies for a total of 32 transcription units.[24,32] Complete AZFc deletions remove 3.5 Mb (for a total of 21 transcription units, of which 10 are considered protein-coding) and originate from NAHR between the b2 and b4 amplicons in palindromes P3 and P1, respectively.[28] Similar to AZFb deletions, complete AZFc deletions can also be referred to based on the intervening targets of the NAHR event (i.e., as b2/b4 deletions).

The extraordinarily repetitive organization of the AZFb and AZFc regions potentiates the occurrence of additional rearrangements that may also be of relevance in terms of their effects on spermatogenesis. Some correspond to deletion patterns removing both the AZFb and AZFc regions almost in their entirety. These AZFbc deletions occur by two major mechanisms involving NAHR between P5/distal P1 (7.7 Mb, for a loss of 42 transcription units) or between P4/distal P1 (7.0 Mb, with 38 transcription units lost).[30] In addition, less extensive deletions within the AZFc region (partial deletions) can nevertheless also impose constrains to spermatogenesis, with these being the focus of a specific section of the guidelines.

In summary, the following recurrent complete microdeletions of the Y chromosome are clinically relevant, being the cause of spermatogenic impairment in azoospermic or severely oligozoospermic men (Figure 2):

- AZFa
- AZFb (P5/proximal P1)
- AZFc (b2/b4)
- AZFbc (P5/distal P1 or P4/distal P1)

The most frequent complete deletion type is the AZFc deletion (70%–80%) followed by AZFa (0.5%–9%), AZFb (1%-7%), and AZFbc

(1%–20%).[8,9,33,40] Deletions that are detected as AZFabc are most likely associated with an abnormal karyotype such as 46,XX male or iso(Y).[41]

5 | GENOTYPE-PHENOTYPE CORRELATIONS IN COMPLETE AZF DELETIONS

AZF deletions are specifically associated with spermatogenic failure since they have never been reported in normozoospermic men.[7,8] Although some rare cases of natural transmission of complete AZFc deletions have been described,[42] they mainly reflect the fact that natural fertilization can occur, under extraordinary conditions, in men with very low sperm counts. For this reason, it is more appropriate to consider Y deletions as a cause of azoo/oligozoospermia rather than a cause of "infertility."

Deletions of the entire AZFa region invariably result in a Sertoli cell-only (SCO) testicular phenotype, hence in azoospermia.[1,39,43–45] Complete deletions of the AZFb and AZFbc regions (P5/proximal P1, P5/distal P1, P4/distal P1) are associated with azoospermia, with approximately half of the men having an SCO testicular phenotype and the other half a spermatogenic arrest (often with normal FSH and normal testis volume) that prevents the successful production of male gametes. However, severe oligozoospermia has been reported in association with rare cases of atypical AZFb deletions[46] (see below). Complete AZFc deletions (b2/b4) have a strong deleterious impact on spermatogenesis, as they associate either with azoospermia or severe oligozoospermia.[9,19,47,48] Contrary to their AZFa and AZFb counterparts, complete AZFc deletions have been convincingly shown to be compatible with residual spermatogenesis in a significant proportion of cases. A number of reports indicate that the spermatogenic impairment phenotype associated with this deletion type can aggravate with time,[49–52] yet a study that looked for significant differences over a time span of 14 months to 7 years in four patients failed to identify such an effect.[19]

5.1 | Prognostic value of AZF deletion testing

The diagnosis of AZF deletions has prognostic value and can influence therapeutic options. Deletion screening should be offered to all patients with azoospermia who are eligible for TESE coupled to intracytoplasmic sperm injection (TESE-ICSI). A complete deletion of the AZFa region indicates the virtual impossibility of retrieving testicular sperm for TESE-ICSI. Accordingly, there have been no recorded instances of successful TESE in men with complete AZFa deletions. Similarly to complete AZFa deletions, it is virtually impossible to obtain sperm via TESE in men with complete AZFb deletions.[43,44,53] However, TESE may be attempted in azoospermic carriers of atypical AZFb or AZFbc deletions characterized by a proximal breakpoint in the P4 palindrome (instead of in P5). Indeed, a smaller deletion with a proximal breakpoint at P4 may be associated with the retention of additional AZFb gene copies, such as *XKRY*, *CDY2*, and *HSFY*, and consequently with a less severe, TESE-positive outcome.[53–55] Similarly, partial AZFb deletions as identified by the retention of the sY1192 distal marker (part of the deletion extension analysis) can also be compatible with residual spermatogenesis. Indeed, positive sperm retrieval has been reported in a patient showing discrepant results between the two distal markers (sY143 and sY1192) that are typically used to define the extension of AZFb deletions.[46] The presence of sY1192 (a more distal marker than sY143) in this patient's deletion pattern strongly suggests that additional copies of *RMBY1* remain intact, likely leading to a less severe testis phenotype. Another recent study reached a similar conclusion,[33] emphasizing the importance of distinguishing between complete (P5/proximal P1) and partial AZFb deletions. Considering the above, the guidelines have now been updated to include sY1192 instead of sY143 as the recommended marker when mapping the distal breakpoint of AZFb deletions in the mandatory deletion extension step. It is important to emphasize that not only the distal but also the proximal breakpoint can differentiate between TESE-negative and TESE-positive patients. Therefore, it is of utmost importance that the deletion extension analysis is performed, as this information will ultimately determine if TESE should be attempted or not. Only complete test results (including basic and deletion extension markers) can provide the necessary insight on the type of reproductive options available to men with AZFb deletions. TESE is not recommended in cases of sY1192 negative P5/proximal P1 deletions due to a very low/virtually zero chance of retrieving spermatozoa. It should nevertheless be noted that there are exceptionally rare cases in the literature of likely sY1192 negative AZFb and AZFbc deletions being compatible with complete spermatogenesis, possibly due to genetic background effects.[56,57] In azoospermic men with complete AZFc deletions, there is an approximately 50% chance of retrieving sperm by TESE based on the average of 32 studies.[58] However, the TESE success rate varied greatly across these studies (from 15% to 71%), illustrating not only biological variation but also the crucial importance of technical aspects for the success of the TESE procedure. According to available data, the presence of 45,X cell lines above 5% in peripheral blood can be considered as a negative predictive factor for spermatogenesis.[59]

6 | IMPLICATIONS FOR GENETIC COUNSELING

Genetic counseling is mandatory in order to provide information about the risk of conceiving a son with impaired spermatogenesis. In the case of AZFc deletions (and also in partial AZFa and partial AZFb deletions), counseling may also be relevant for other male family members since the transmission of these deletion types has been reported.[60–62] Complete AZFa, AZFb, and AZFabc deletions are generally incompatible with sperm production, thus family screening is not indicated and should not be mentioned in the report. Non-terminal AZFbc deletions are often associated with non-reference Y chromosomes, and complete, albeit severely impaired, spermatogenesis may be possible in some atypical deletion cases. Such patients should, therefore, be informed of the risk of conceiving a son with impaired

spermatogenesis, as the deletion will be obligatory transmitted to their male offspring.

If spermatozoa are present in the ejaculate or in the testis, AZF-deleted patients can conceive their own biological children either naturally (in some extremely rare cases of complete AZFc deletions) or through MAR. Several studies have been published on ICSI outcome in couples with male partners carrying complete AZFc deletions.[8,9,19,63–74] While several studies have reported a negative impact of the deletion on key MAR parameters, such as fertilization rate, embryo quality, and live birth rate, others have failed to identify any significant differences in fertilization and pregnancy rates between men with or without complete AZFc deletions.[8,70,74–77] Thus, whether this deletion has any noticeable impact on MAR outcome is still a matter of debate. However, a recent meta-analysis based on 12 studies, indicated that apart from a significant decrease in fertilization rate, all the other reproductive outcomes (e.g., embryo quality, clinical pregnancy rate, miscarriage rate, and live birth rate) in these patients are equivalent to patients with no genetic anomalies (both using ejaculated or testicular spermatozoa).[78]

The potential consequences for offspring health associated with the transmission of Y microdeletions is a matter of intense debate. While the deletion of the father will be obligatory transmitted to the son—who will consequently have impaired sperm production—the exact testicular phenotype cannot be predicted due to the effects of a different genetic background and the impact of varying environmental factors on reproductive functions. The number of reported ICSI babies born from fathers affected by Yq microdeletions is still relatively low, with just 268 cases being well-documented[8,9,19,63–67,69–76,79–95] (Table 1). It appears that these children are phenotypically normal, except for one son born with pulmonary atresia and a hypoplastic right ventricle[68] and one child with a non-specified "major malformation."[76] In both cases, the father carried an AZFc deletion. Concerns have been raised about the potential risk for Turner's syndrome (45,X) in the offspring and other phenotypic anomalies associated with sex chromosome mosaicism, including ambiguous genitalia. Data on men with Y microdeletions[96] and in patients bearing a mosaic 46,XY/45,X karyotype with sexual ambiguity and/or Turner stigmata[97] suggest that some Yq microdeletions may be associated with an overall Y chromosomal instability that might result in the formation of 45,X cell lines. Yet, no ambiguous genitalia or Turner syndrome have been observed in the 268 reported ICSI babies born from Yq-deleted fathers. Two papers provide data on the aneuploidy rate in embryos by performing preimplantation genetic diagnosis in embryos derived from Y chromosome deletion carriers. In the first study, no sex chromosome anomalies were found,[72] whereas in the other, a higher percentage of monosomy X embryos were detected.[74] This recommends caution when the risk of chromosomal abnormalities is discussed with patients. In addition, as embryos bearing a 45,X karyotype have a very high risk of spontaneous abortion, miscarriage rates may be higher in partners of Yq-deleted men. Yet, this possibility remains to be formally tested.

Reassuringly, there is no evidence for an association of complete AZFc deletions with intellectual, psychological, or motor development disorders in the progeny. A proposed association with haploinsufficiency of the *SHOX* (Short-stature HOmeoboX-containing) gene located in the pseudoautosomal region (PAR)[98] was not replicated in a much larger multicenter study,[99] nor in an independent study in a Chilean population.[100] The latter identified PAR abnormalities exclusively in patients with terminal AZFbc deletions associated with isochromosome Yp and/or Y nullisomy. This study also reported neuropsychiatric disorders in 5 out of the 7 patients with a terminal AZFbc deletion and abnormal karyotype. It should be noted that the validation of this finding in a larger cohort is still pending.

In conclusion, the indication for the molecular diagnosis of Y-chromosomal microdeletions is based on critically reduced spermatogenic output and it is strongly advised in patients affected by azoospermia and severe oligozoospermia. However, the decision limit for severe oligozoospermia warranting AZF deletion analysis remains under debate in the range of 1×10^6/mL up to 5×10^6/mL sperm concentration (see above). AZF testing has prognostic value for sperm retrieval and in case spermatozoa can be found in the ejaculate or by testicular biopsy, the deletion will be obligatory transmitted to the male offspring. While the data are heterogeneous, the latest meta-analysis indicates that MAR outcomes, with the exception of fertilization rate, are not significantly affected by these Y chromosome rearrangements. Although the number of babies born to fathers bearing Y deletions has further increased in the last 10 years, we still do not have conclusive information about the definitive risk for Turner syndrome, ambiguous genitalia, or other chromosomal anomalies. Clearly, there is a need to establish robust follow-up programs directed at Yq-deleted men and their potential offspring. In cases of complete AZFc deletions and of partial AZFb or AZFa deletions, analysis of the male members of the family should be requested in the laboratory report. Moreover, extensive karyotype analysis (counting 100 metaphases) is recommended in the presence of complete AZFc or Yq terminal deletions in order to rule out 46,XY/45,X mosaicism associated with structurally aberrant Y chromosomes.

7 | PARTIAL AZF DELETIONS

7.1 | Gene-specific partial AZFa deletions

Although some authors have previously found an extraordinarily high frequency of single AZF gene deletions,[101,102] these data are in stark contrast with the collective experience resulting from the analysis of more than 2000 patients.[7,8,21,103–105] All confirmed AZF gene-specific deletions (with defined breakpoints) map to the AZFa region. Five of them specifically affect the *USP9Y* gene, either partial or completely.[61,106] None of the deletions were due to NAHR and are thus likely unique, supporting the extreme rarity of such events. The associated phenotype of these partial AZFa deletions is largely variable: from azoospermia to normozoospermia. This indicates that *USP9Y* acts as a fine tuner rather than an essential regulator of spermatogenesis.[60] Indeed, loss-of-function variants in *DDX3Y* have very recently been described as a cause of azoospermia, thus formally validating this gene as the key spermatogenic factor in the AZFa region.[107]

TABLE 1 Summary of the health status of babies born from fathers affected by AZF microdeletions.

No. of babies born to fathers carrying AZF deletions	No. of healthy babies	No. of babies with malformations	Type of deletion	Reference
27	26	1 major malformation (not specified)	1 Partial AZFa[b] + 26 AZFc	Goncalves et al., 2017
1	1	0	AZFb[c]	Zhang et al., 2017
6	6	0	1 AZFb[c] + 5 AZFc	Liu et al., 2017
20	20[a]		2 AZFbc[d] + 18 AZFc	Choi et al., 2013
1	1	0	AZFc	Hu et al., 2019
3	3	0	AZFc	Kleiman et al., 1999
5	5	0	AZFc	van Golde et al., 2001
11	11	0	AZFc	Oates et al., 2002
2	2	0	AZFc	Peterlin et al., 2002
4	4	0	AZFc	Choi et al., 2004
3	3[a]		AZFc	Stouffs et al., 2005
3	3	0	AZFc	Gambera et al., 2010
13	13[a]		AZFc	Kim et al., 2012
35	35	0	AZFc	Liu et al., 2013
39	39[a]		AZFc	Zhu et al., 2015
1	1	0	AZFc	Schwarzer et al., 2016
16	16[a]		AZFc	Sabbaghian et al., 2018
14	14	0	AZFc	Patrat et al., 2010
6	6[a]		AZFc	Choi et al., 2007
15	15[a]		AZFc	Wu et al., 2011
2	2[a]		AZFc	Abur et al., 2019
2	2	0	AZFc	Mulhall et al., 1997
4	3	1 case of pulmonary atresia + hypoplastic right ventricle	AZFc	Page et al., 1999
33	33[a]		Deletion type not specified	Zhu et al., 2014
2	2	0	AZFc	Lo Giacco et al., 2014
Total: **268**	**266**	**2**		

Note: All deletions are complete, unless specified otherwise.
[a]Assumed to be healthy (not specified in the article).
[b]Isolated deletion of *DDX3Y*.
[c]Only sY143 was tested at the distal border (possible partial AZFb deletion).
[d]Genotype cannot be verified (no data on STS markers were reported).

7.2 | gr/gr partial AZFc deletions

The AZFc region is particularly susceptible to NAHR events which may lead to both partial deletions and duplications.[27–29,31,108,109] Although several different partial AZFc deletion patterns have been described, many of them occur at such a low frequency that it is quite challenging to determine their clinical significance. Of these, the gr/gr deletion is the one that has been more extensively studied. This deletion is named after the ampliconic targets (two "green" and "red" amplicon blocks) that are involved in its NAHR-mediated generation.[32] Although it removes half of the AZFc gene content (genes with germ cell-exclusive or predominant expression), the clinical significance of gr/gr deletions is still a matter of debate due to the striking phenotypical variability observed in deletion carriers (from azoospermia to normal sperm counts). A large body of data supports that gr/gr deletions represent a risk factor of spermatogenic impairment in a population-dependent manner. In a large multi-ethnic study, this deletion type was found in one of every 41 men and it almost doubled the risk of severe spermatogenic failure, accounting for ~2% of the latter condition.[110] Studies conducted in European populations tend to report such a risk, especially when normozoospermic men are used as controls,[5] whereas the association is less evident, or altogether absent, in other geographic regions.[27,111,112] Although methodological issues may account for some of these differences (lack of ethnic/geographic matching of

cases and controls, inappropriate selection criteria, and unsuitable validation of deletions), a population effect is clearly present as gr/gr deletions are fixed without any phenotypical consequences in specific evolutionary lineages of the Y chromosome. This is particularly obvious in Y haplogroups D2b, Q3, and Q1, common in Japan and certain areas of China.[113,114] Despite multiple efforts aimed at clarifying the molecular basis for the highly variable phenotypic presentation of gr/gr deletions, it has been up to now difficult to define specific deletion subtypes associated with either neutral or pathogenic effects. Indeed, the genetic factors that can modulate the phenotypical penetrance of this variant remain unclear: in addition to contradictory data on the effect of secondary duplications, the number and copy identity of the retained AZFc genes do not seem to influence the severity of the phenotype.[115] Furthermore, the sequence diversity of the retained genes across different gr/gr deleted men has been shown to be quite low, at least in the Estonian population.[34]

7.3 | Clinical implications of gr/gr deletions

As stated above, the heterogeneity of the study populations available in the literature exerts a major confounding effect on the analysis of the clinical significance of gr/gr deletions. However, five meta-analyses have been attempted, all achieving significant odds ratios (OR) of a 1.8–2.5 fold increased risk of reduced sperm output/infertility.[27,111,112,116,117] As stated above, an equivalent risk estimation was reported in a large multi-ethnic study analyzing 20000 Y chromosomes.[110] Therefore, the gr/gr deletion represents a unique example in Andrology of a confirmed genetic risk factor for impaired sperm production. The combined data of Spanish and Italian cohorts (in total 944 patients vs. 1044 normozoospermic controls) indicate a four-fold increased risk of spermatogenic impairment associated with this variant.[5] Infertile gr/gr deletion carriers were mainly oligozoospermic, further supporting the relatively milder effect of this deletion type in respect to the complete AZFc deletion. gr/gr deletions have also been proposed as a predisposing factor for testicular germ cell tumors (TGCTs).[118,119] This effect has been recently confirmed in a large multicenter Mediterranean cohort (497 TGCT patients and 2030 controls), with a three-fold increased risk of TGCT being associated with the variant (OR = 3.1, 95% CI = 1.4–7.0).[120]

As in the case of other AZFc rearrangements, gr/gr deletions will be obligatorily transmitted to the male offspring. This issue is particularly relevant as some evidence suggests that partial AZFc deletions may expand into a complete AZFc deletion (i.e., into a clear-cut cause of spermatogenic impairment) in the next generation.[121,122] Yet, more data are still required to establish a firm conclusion on this matter.

Overall, since gr/gr deletions cannot be considered a *de facto* cause of spermatogenic impairment, testing for this deletion type is left at the discretion of the diagnostic labs and the referral clinician. The population-dependent effects of this variant and the recent confirmation of its role as a predisposing factor for TGCT are additional aspects to take into consideration during the decision-making process.

7.4 | Guidelines for diagnostic testing

The preferred method for diagnostic testing of AZF deletions is PCR amplification of selected regions of the Y chromosome. The study of DNA samples from ICSI candidates has revealed that using gene-specific sequence tagged-sites (STS) markers does not increase the detection rate of clinically relevant microdeletions when compared with well-validated markers mapping to non-coding regions.[7,71,103,105] Therefore, from a clinical perspective, it remains basically unimportant whether the selected markers amplify anonymous regions or specific MSY genes. What is indeed crucial is that the selected markers map to Y regions consistently shown to be deleted specifically in azo/oligozoospermic men affected by a given microdeletion pattern.

7.4.1 | Testing for AZFa deletions

The molecular analysis of the AZFa region involves the use of two STS markers: sY84 and sY86. Both are located upstream of the *USP9Y* and *DDX3Y* genes and are anonymous. According to the pathogenic mechanism of the deletion and based on available data, once a deletion of both sY84 and sY86 is detected, the probability of dealing with a complete deletion is very high. However, it is possible (although rare) that both markers are deleted without the two AZFa genes being affected,[123] or that just *USP9Y* is affected.[60,61] Therefore, testing for AZFa deletions requires a mandatory deletion extension analysis including the following markers: sY82 (present), sY1064 (absent) for the proximal border; and sY1065 or sY1182 (absent), sY88 (present) for the distal border (Figure 2B). Importantly, our recent data have shown that the proximal breakpoint marker sY83, recommended in previous versions of the guidelines as an equivalent option to sY1064, can still be retained in a sizeable fraction of complete AZFa deletions. Hence, to avoid the misidentification of a complete AZFa deletion as partial, the use of sY1064 instead of sY83 is strongly recommended in the deletion extension analysis (or at least sY1064 should be tested to corroborate that the deletion is indeed complete in sY83 positive cases). It is important to consider that the two AZFa genes map to the more distal part of the AZFa region, hence testing the recommended distal AZFa markers is extremely relevant for TESE prognosis, and the exact location of the proximal breakpoint (sY83 present or absent) does not actually influence the clinical interpretation of the deletion. If only sY84 or sY86 are found to be deleted (and amplification failures can be excluded), the AZFa region should be studied in more detail according to the map provided by Kamp and colleagues,[39] or by consulting publicly available databases.[124] This event, however, is presently considered to be extraordinarily rare.

7.4.2 | Testing for AZFb deletions

The two anonymous markers sY127 and sY134 are located in the median and distal part of the AZFb region. According to available data, in most cases, the deletion of both markers indicates a complete deletion of the AZFb region. As previously discussed, to clearly establish the

prognostic value of the deletion in terms of TESE outcome, a mandatory deletion extension analysis with additional markers is required. These include: sY105 (present) and sY121 and sY1224 (absent) for the proximal border, and sY1192 (absent) and sY153 (present) for the distal border (Figure 2B). It is important to note that in previous versions of the guidelines the use of sY143 or sY1192 was considered equivalent. This is no longer the case as more recent data have established that sY1192, but not sY143, has prognostic value in terms of TESE outcome.[33,46] Our recent data also have shown that sY1224—recommended in previous versions of the guidelines as equivalent to sY121—can be retained in a still significant number of complete AZFb and AZFbc deletions. Although the phenotypical consequences of this variation remain to be clarified, the use of both sY121 and sY1224 (when sY121 is absent) is strongly encouraged. We renew our previous recommendation against testing for markers sY114 and sY152 (still included in some commercial kits) as they map to more than one genomic region. In particular, sY152 maps to the *DAZ* genes, similarly to sY255 and sY254. In this regard, we again emphasize that the so-called "AZFd" region, defined on the putative absence of sY152, does not exist. Although some commercial kits still refer to this supposed region, we strongly advise against the use of such products.

7.4.3 | Testing for AZFc deletions

The two markers sY254 and sY255 are specific for the *DAZ* gene, which is present in the reference Y chromosome sequence in four copies arranged in two clusters.[125] A vast body of knowledge accumulated over the years has shown that when sY254 and sY255 are deleted, a diagnosis of complete deletion of the AZFc region can be made.[28] Based on the available data, the deletion of only one of these two markers is extremely unlikely and should be regarded as a technical error. The mandatory deletion extension analysis using the sY160 heterochromatin marker allows the distinction between complete AZFc deletions (b2/b4, sY160 present) and terminal deletions (sY160 absent; Figure 2B). Terminal deletions, as well as b2/b4 deletions, can be associated with a mosaic karyotype (46,XY/45,X)[41,59,97] and, thus, karyotype analysis should be requested also for TESE prognostic reasons.

If the lab decides to also test for the gr/gr partial AZFc deletion, this can be achieved by using two markers: sY1291 and sY1191.[32] The diagnosis is based on the absence of marker sY1291 and the presence of sY1191. It is worth noting that a 5% false deletion rate has been detected in a multicenter study,[115] underlining the importance of the optimization of the PCR conditions and of additional confirmatory steps. Y haplogroup analysis is recommended in Asian patients in order to exclude Y lineage-fixed deletions, which are unlikely to affect spermatogenesis (see above).

7.4.4 | Testing for AZFbc deletions

The complete deletion of both the AZFb and AZFc 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 and colleagues[30] can determine whether the deletion corresponds to the P5/distal P1 or P4/distal P1 pattern (sY116 is present in case of P4/distal P1, and absent in case of P5/distal P1). This definition has clinical prognostic value as stated above.[53] Testing for the sY160 heterochromatin marker to identify terminal deletions in these patients is highly recommended.

7.5 | Setting up the PCR reaction: Internal quality control and recommended markers

The PCR amplification of genomic DNA for clinical diagnosis requires strict compliance with good laboratory practice and basic principles of quality control. Positive and negative controls must be run in parallel with each reaction. In the case of AZF deletion testing, these correspond to a DNA sample from a man with normal spermatogenesis and that from a woman. In addition, a water sample, which contains all reaction components but water instead of DNA, must be run with each set of primers as control for contamination. 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. A marker for the *SRY* gene (sY14) 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 in XX males. Another essential internal PCR control in AZF diagnostics is the *ZFX/ZFY* gene as it will amplify a unique fragment both in male and female DNA. Testing for *ZFX/ZFY* is relevant not only for the female control DNA but also in *SRY* negative 46,XX males since it will be the only positive marker.

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, analyzing two STS loci in each region reinforces diagnostic accuracy, since deletions involve well-defined regions including many STS loci. Therefore, at least two STS loci in each AZF region should be analyzed in the first step of the test (basic marker analysis). Based on the experience of many laboratories, the results of external quality control and considering the multiplex PCR format, the first choice of STS primers recommended in the previous versions of the guidelines remains valid. These primers include:

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

*updated sequences for both sY84-F and sY84-R, see below.

These STS primers provide robust and reproducible results in multiplex PCR reactions, as demonstrated by many laboratories and in external quality control trials. However, it must be noted that, according to the latest sequencing data, there is a mismatch in the middle of the sequences of primer sY84-F (which does not preclude the efficacy of amplification) and thus the sequence has been changed in

the table accordingly. Regarding sY84-R, an SNP (rs72609647) in the fifth nucleotide of the primer sequence has been detected in Asian populations.[126] Hence, in case of amplification failure in samples of this origin, an alternative primer for sY84 and/or an alternative neighboring marker to sY84 should be tested.[124]

For the mandatory deletion extension analysis, the recommended primers are:

For AZFa: proximal breakpoint: sY82 and sY1064; distal breakpoint: sY1065 (or sY1182) and sY88
For AZFb: proximal breakpoint: sY105 and sY121/sY1224; distal breakpoint: sY1192 and sY153
For AZFc: sY160

In conclusion, the use of these two primer sets is sufficient for routine diagnostics as it enables the detection of virtually all clinically relevant AZF deletions (and of over 95% of the deletions reported in the literature) while offering adequate prognostic value for TESE. Adoption of these markers by all laboratories is strongly encouraged since it allows for minimal standardization and comparison of laboratory performance and variability. The location of these primers on the Y chromosome is indicated in Figure 2. Their sequence and an example of a PCR protocol are reported in Appendices A and B.

7.6 | Interpretation of the results and repetition of the test

The protocol suggested by these guidelines (Appendix A) has been conceived and optimized so that each of the two first-round 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 AZFa and AZFb 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 (AZFc/DAZ) should always be regarded as a methodological error. If only one marker for AZFa or AZFb 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. In case of an AZFabc deletion (all the eight Yq markers are absent), the interpretation of the control markers (SRY and ZFX/ZFY) is of outstanding importance in order to rule out technical problems.

PCR conditions should be carefully optimized in each laboratory according to the available equipment and reagents (e.g., type of thermocycler and of DNA polymerase), as well as DNA quality/quantity/source. 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 PCRs consistently favor 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, since there is no reason to repeat the test in the same manner. It is known that simplex PCR is less prone to amplification failure, and it is strongly advised to repeat the reaction at a lower annealing temperature. Repeated freeze/thaw cycles of aliquoted primers should be avoided as this may lead to amplification failure. 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).

8 | REPORTING GENETIC TEST RESULTS

Reports should be written in a standardized format and should be clear to the non-specialist. Details of recommendations for reports of diagnostic genetic testing are described by Deans and colleagues.[127] In general, reports must be clear, concise, accurate, and easily interpretable. Hand-written reports are not acceptable. Reports must include the following general information:

- identification of the physician referring the patient
- clear and complete identification of the laboratory performing the analyses and issuing the report (including unique laboratory accession/identification number)
- title
- date of referral and reporting
- patient identification: full given name(s), surname, date of birth, and biological sex
- tissue studied (e.g., blood, buccal smear, etc.)
- a written interpretation and conclusion understandable by a non-specialist
- signatures of (at least) two independent assessors (including their role)
- page numbers
- if the report includes more than one page, the pagination should be clear (e.g., page X of Y) and patient identification should be on each page

The following specific information must also be included:

- restatement in some form of the reason for referral (e.g., diagnosis of Y chromosome microdeletions) and the indication (e.g., azoospermia, preparation for ICSI, pre-TESE, etc.)
- method used (e.g., multiplex PCR amplification), including limitations of the assay; if a commercially available kit was used, its name, manufacturer, and version number must be disclosed
- if the test/analysis was outsourced to an outside laboratory or company, this information must be clearly stated
- outcome of the analysis: the result on the tested markers must be reported. The preferred option is a table listing the result of all STS loci that support the interpretation (note: if a kit containing an extensive number of markers was used, it is recommended to only list in the report the marker results relevant for the interpretation). Avoid the use of + and −, which can be misinterpreted. Use words instead (e.g., present/absent, or similar)

Examples of reports concerning the most frequent deletions are provided in the Supplementary Materials.

8.1 | Alternative methods for Y microdeletion testing

Since the publications of the first guidelines in 1999, several alternative methods to assess Y-chromosomal deletions have been published. In addition, various commercial kits have become available. These, however, tend to contain an unnecessarily high number of markers that can confound the analysis (Appendix C). Ultimately, they may lead to the detection of "false" deletions, especially if DNA quality and/or the PCR conditions are suboptimal.[128,129] Moreover, most kits do not allow the validation of suspected deletions by single PCR (Appendix C). Multiplex PCRs based on gene-specific markers have also been proposed.[130] Although these approaches allow the detection of isolated gene-specific deletions, the extreme rarity and unclear clinical significance of these deletions precludes its use in the routine diagnostics.[106] Also, the fact that some of these kits contain gene-specific markers brings forward the need of a particularly careful interpretation of the results, as well as of validating suspected single gene deletions, if at all possible.

The list of published alternative methods, some partially based on the guideline protocols, others not, mainly includes capillary electrophoresis, real-time PCR, MLPA, array-CGH, or next-generation sequencing (NGS).[131–135] Some laboratories have adapted the EAA/EMQN multiplexes, adding a fluorescent label to the primers to allow detection with capillary electrophoresis. These are found in the form of either in-house-validated methods or commercially available kits (Appendix C). Real-time PCR has the advantage of being relatively fast, as it bypasses the need for gel electrophoresis, but the required equipment is less readily available than that of the standard methodology. The particular organization of the AZF regions and their significant structural variation across different populations poses significant challenges to the adequate implementation of NGS-based methods. These challenges can lead to diagnostic errors and strongly advise for caution when considering the use of NGS-based approaches in a diagnostic setting without proper validation and extensive expertise in Y chromosome analysis. As the availability of this technology and the know-how to properly implement it become more commonplace, it may lead to new, more encompassing tests that can further improve diagnostic yield in azoospermic men.[136]

In conclusion, the two-step multiplex PCR established in previous versions of the guidelines remains the most cost-effective, reproducible, and easily available methodology to perform Y-chromosomal deletion testing. In case a laboratory decides to establish an alternative method, the new approach needs to be validated on a suitable number of samples, including positive and negative controls, as to estimate test specificity and sensitivity. Whenever an alternative method is employed, the report should clearly include the exact experimental design used instead of referring to it as "according to the guidelines." The latter only applies to the two-step multiplex PCR + mandatory deletion extension analysis (the latter in case of confirmed deletions in the first step) described herein.

9 | 22 YEARS OF EXPERIENCE OF THE EAA/EMQN EXTERNAL QUALITY ASSESSMENT

Laboratories performing AZF diagnostics are strongly encouraged to join a yearly EQA scheme. A suitable option is available from EMQN CIC, an ISO 17043:2010 accredited EQA provider, implemented in collaboration with the EAA. In this EAA/EMQN AZF scheme, every year three validated DNA samples with mock clinical case descriptions are distributed to participating laboratories. It is fundamental that the DNA samples from the EQA program are processed exactly in the same way as patients' samples are handled, including reporting. Laboratory results are assessed by at least two independent reviewers. Both a general report summarizing overall performance and common problems, as well as individual laboratory reports with lab-specific feedback and recommendations are issued. Laboratories receive a participation certificate evaluating their performance.

Between 2000 and 2012, the number of participating laboratories in the EAA/EMQN AZF scheme almost tripled from 57 to 148 (Figure 3A), and this number has stabilized over the last 10 years. The diagnostic error rate (an incorrect genotype that would lead to a misdiagnosis) decreased steeply during the first 5 years of the program, from almost 8% to the current 1%–2% (Figure 3B). While more variable, the quality of result interpretation and reporting has also significantly improved, with more than 70% of all analyses in the last 4 years having scored full marks in this category. Of note, the testing of an unnecessarily high number of markers—often part of commercially available kits (see *Alternative methods for Y microdeletion testing* section)—is still a recurrent source of interpretation errors. The two dips in interpretation scores in 2006 and 2012 (Figure 3B) are explained by the inclusion of an atypical (46,XX male) case in both years. This led to wide-spread problems in interpreting marker results, recommending further testing (karyotyping) and correctly estimating the prognostic value of the test (it is impossible to obtain testicular sperm in these patients).

Overall, this established EAA/EMQN AZF scheme has demonstrated that it is a valuable tool for raising the performance of participating labs, as illustrated by the significant improvements in genotyping accuracy and the quality of reporting practice. Online registration for this program is available at www.emqn.org.

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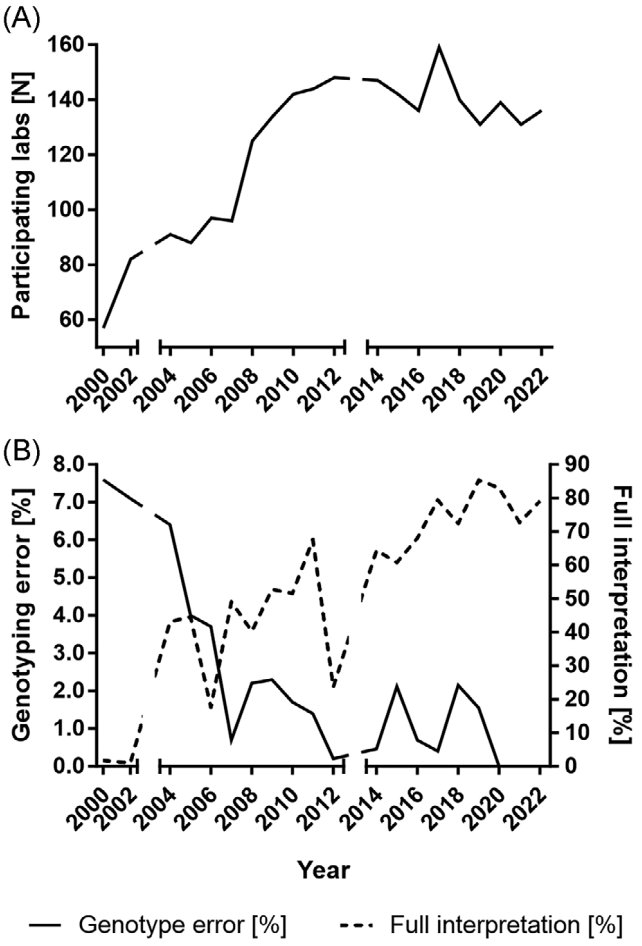

FIGURE 3 Main outcomes of the 22-year experience of the EAA/EMQN external quality control scheme in assessing laboratory performance. The number of participating labs has steadily increased until 2014 and then remained fairly stable (A). Genotyping error rates have steeply declined, while interpretation scores gradually increased (B).

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AUTHORITY STATEMENT

These guidelines were developed based on the experience of the authors as assessors of the EAA/EMQN quality control AZF scheme, as well as on their collective expertise on the genetic basis of spermatogenic impairment. The content of this document reflects the consensus of experts both at EAA and the EMQN CIC.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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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: the three AZF loci and the two control fragments (*SRY* and *ZFX/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 Human Genetics in Münster.

PCR kit: Qiagen Multiplex PCR Kit (Cat.No. 206143, Qiagen).

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 Qiagen 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), ~ 1 μg template DNA, sterile distilled water to 50 μL.

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

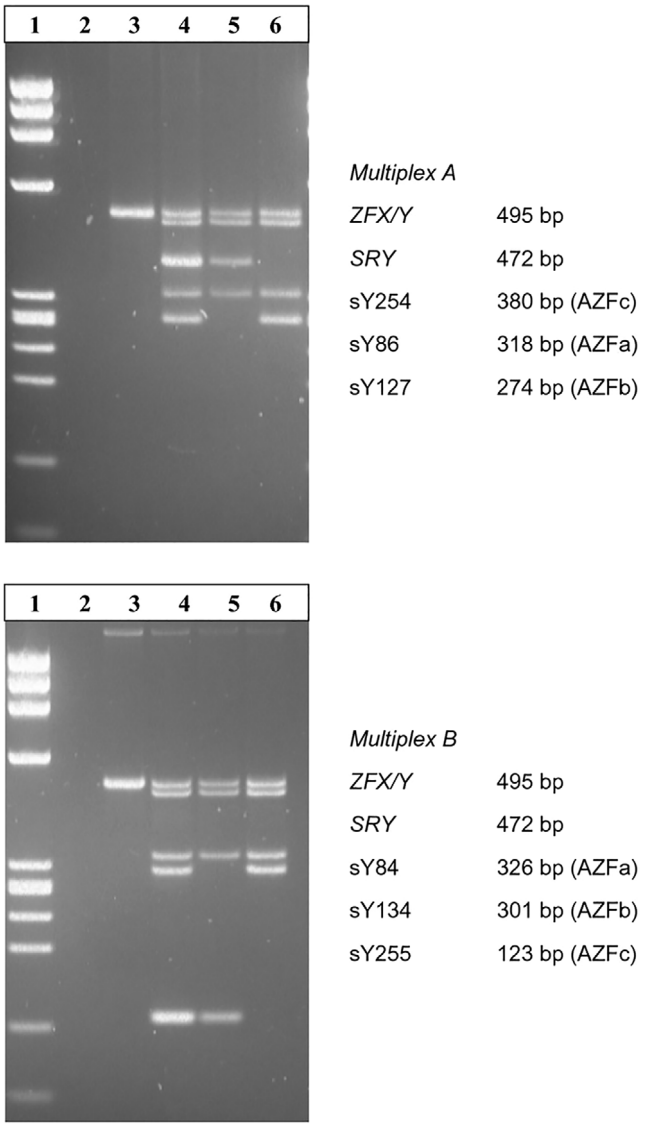

FIGURE A1 Examples of both Multiplex PCRs. Multiplex A: lane 1 phi X-HeaIII size marker, lane 2 water, lane 3 female DNA, lane 4 DNA of normal male, lane 5 DNA of AZFb (P5/proximal P1)-deleted patient, and lane 6 DNA of AZFc (b2/b4)-deleted patient.

Reaction products (30 μL) are separated on a 2% Agarose gel (Peqbold Universal Agarose, Peqlab) with 0.5% DNA Agar (Serva) in 1 x TBE for 25 V overnight. An example of both multiplexes is given in Figure A1.

APPENDIX B

LOCUS AND SEQUENCE OF THE PCR PRIMERS (FOR FURTHER INFORMATION, SEE ALSO "MSY BREAKPOINT MAPPER")

Locus	Primer	Sequence	Product size (bp)	Result in complete deletion
Reaction A and B				
ZFX/ZFY	*ZFX/Y*-F	5′ - ACC RCT GTA CTG ACT GTG ATT ACA C - 3′	495	Present
	ZFX/Y-R	5′ - GCA CYT CTT TGG TAT CYG AGA AAG T - 3′		
SRY	sY14-F	5′ - GAA TAT TCC CGC TCT CCG GA - 3′	472	Present
	sY14-R	5′ - GCT GGT GCT CCA TTC TTG AG - 3′		
Reaction A				
AZFa	sY86-F	5′ - GTG ACA CAC AGA CTA TGC TTC - 3′	318	Absent
	sY86-R	5′ - ACA CAC AGA GGG ACA ACC CT - 3′		
AZFb	sY127-F	5′ - GGC TCA CAA ACG AAA AGA AA - 3′	274	Absent
	sY127-R	5′ - CTG CAG GCA GTA ATA AGG GA - 3′		
AZFc	sY254-F	5′ - GGG TGT TAC CAG AAG GCA AA - 3′	380	Absent
	sY254-R	5′ - GAA CCG TAT CTA CCA AAG CAG C - 3′		
Reaction B				
AZFa	sY84-F	5′ - AGA AGG GTC CTG AAA GCA GGT - 3′	326	Absent
	sY84-R	5′ - GCC TAC TAC CTG GAG GCT TC - 3′		
AZFb	sY134-F	5′ - GTC TGC CTC ACC ATA AAA CG - 3′	301	Absent
	sY134-R	5′ - ACC ACT GCC AAA ACT TTC AA - 3′		
AZFc	sY255-F	5′ - GTT ACA GGA TTC GGC GTG AT - 3′	123	Absent
	sY255-R	5′ - CTC GTC ATG TGC AGC CAC - 3′		
Deletion extension analysis				
AZFa				
AZFa	sY82-F	5′ - ATC CTG CCC TTC TGA ATC TC - 3′	264	Present
	sY82-R	5′ - CAG TGT CCA CTG ATG GAT GA - 3′		
AZFa	sY1064-F	5′ - GGG TCG GTG CAC CTA AAT AA - 3′	110	Absent
	sY1064-R	5′ - TGC ACT AAA GAG TGA TAA TAA ATT CTG - 3′		
AZFa[a]	sY1065-F	5′ - TCA GGT ACT GTG ATG CCG TT - 3′	239	Absent
	sY1065-R	5′ - TGA AGA GGA CAC AAA GGG AAA - 3′		
AZFa[a]	sY1182-F	5′ - ATG GCT TCA TCC CAA CTG AG - 3′	247	Absent
	sY1182-R	5′ - CAT TGG CCT CTC CTG AGA CT - 3′		
AZFa	sY88-F	5′ - TTG TAA TCC AAA TAC ATG GGC - 3′	123	Present
	sY88-R	5′ - CAC CCA GCC ATT TGT TTT AC - 3′		
AZFb				
AZFb	sY105-F	5′ - AAG GGC TTC TTC TCT TGC TT - 3′	301	Present
	sY105-R	5′ - AGG GAG CTT AAA CTC ACC GT - 3′		
AZFb	sY1224-F	5′ - GGC TTA AAC TTG GGA GGG TG - 3′	640	Variable
	sY1224-R	5′ - CAA AGA GCC TCC CAG ACC A - 3′		
AZFb	sY121-F	5′ - AGT TCA CAG AAT GGA GCC TG - 3′	190	Absent
	sY121-R	5′ - CCT GTG ACT CCA GTT TGG TC - 3′		
AZFb	sY1192-F	5′ - ACT ACC ATT TCT GGA AGC CG - 3′	255	Absent
	sY1192-R	5′ - CTC CCT TGG TTC ATG CCA TT - 3′		
AZFb	sY153-F	5′ - GCA TCC TCA TTT TAT GTC CA - 3′	139	Present
	sY153-R	5′ - CAA CCC AAA AGC ACT GAG TA - 3′		
gr/gr	sY1291-F	5′ - TAA AAG GCA GAA CTG CCA GG - 3′	527	Absent
	sY1291-R	5′ - GGG AGA AAA GTT CTG CAA CG - 3′		
gr/gr	sY1191-F	5′ - CCA GAC GTT CTA CCC TTT CG - 3′	385	Present
	sY1191-R	5′ - GAG CCG AGA TCC AGT TAC CA - 3′		
Heterochromatin	sY160-F	5′ - TAC GGG TCT CGA ATG GAA TA - 3′	236	Present
	sY160-R	5′ - TCA TTG CAT TCC TTT CCA TT - 3′		

[a]Equivalent markers.

APPENDIX C

COMMERCIALLY AVAILABLE KITS AND THEIR CHARACTERISTICS WITH RESPECT TO THE EAA/EMQN GUIDELINES

Name of the kit (producer)	Fully respects the Guidelines (STSs)	Confirmation step by simplex or duplex PCR
AB Analitica	No[c,d]	No
ChromoQuantAZF	No[c,d]	No
Devyser	Yes[c]	No
Diachem	Yes[c]	Yes
DNA-Technology	No[b,d]	No
Elucigene	No[c,d]	No
Experteam	Yes[c]	No
Genetek-biopharma	No[d]	No
Molgen	No[b]	No
Promega 2.0	No[d]	No
Sacacae	No[b]	No
Qiagen	No[a]	No

Note: Information retrieved from the products' official webpages (as listed in July 2023). Kits which are based on the standard gel electrophoresis method are shaded.

[a]Different STS panel, only one marker for AZFa.

[b]No deletion extension markers.

[c]Offering deletion extension analysis, but not in accordance with the current guidelines.

[d]Excessive number of markers.