A One-Step Real-Time Multiplex PCR for Screening Y-Chromosomal Microdeletions without Downstream Amplicon Size Analysis

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Abstract

Background: Y-chromosomal microdeletions (YCMD) are one of the major genetic causes for non-obstructive azoospermia. Genetic testing for YCMD by multiplex polymerase chain reaction (PCR) is an established method for quick and robust screening of deletions in the AZF regions of the Y-chromosome. Multiplex PCRs have the advantage of including a control gene in every reaction and significantly reducing the number of reactions needed to screen the relevant genomic markers.

Principal Findings: The widely established “EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions (2004)” were used as a basis for designing a real-time multiplex PCR system, in which the YCMD can simply be identified by their melting points. For this reason, some AZF primers were substituted by primers for regions in their genomic proximity, and the ZFX/ZFY control primer was exchanged by the novel and high-performing DNA-binding dye EvaGreen™ and put into automated melting point detectors. By using an open-source system for real-time PCR analysis, we further demonstrate the applicability of automated melting point and YCMD detection.

Significance: With these changes, we were able to develop a platform-independent and robust real-time based multiplex PCR, which makes the need for amplicon identification by electrophoretic sizing expendable. By using an open-source system for real-time PCR analysis, we further demonstrate the applicability of automated melting point and YCMD detection.

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Introduction

In Western countries, infertility is a major health problem that affects about 15% of couples trying to conceive [1]. Y-chromosomal microdeletions (YCMD), along with Klinefelter syndrome, are the major genetic cause for primary spermatic failure on the male side, accounting for 5–10% of all non-obstructive azoospermic men [2].

Three regions, commonly referred to as “azoospermia factors” (AZFa, AZFb and AZFc) have been defined as spermaticogenic loci and are recurrently deleted in YCMD [3], distributing among 60% (AZFc), 16% (AZFb) and 14% (AZFa), albeit with significant bias in different populations [4,5]. The testicular phenotype in men bearing YCMD depends on the type of deletion, ranging from Sertoli cell-only syndrome (SCO) in AZFa [6,7], SCO to meiotic arrest in cases of complete AZFb/AZFc deletions [2], up to the very heterogeneous pattern from hypospermatogenesis to SCO in AZFc deletions [8,9].

To date, there are no defined criteria as to when patients should undergo YCMD genetic testing. An obvious target are patients subjected to intracytoplasmic sperm injection (ICSI) treatment which present with severe oligozoospermia (<1×10⁶/mL) [10]. Identification of YCMD prior to ICSI treatment can predict their transmission to offspring, as these are inevitably herited [11]. Furthermore, the extent of YCMD in a patient might be prognostic for finding spermatozoa in testicular biopsies [12]. There are also strong indications that an increased frequency of nullisomic sperm in the ejaculate of men with AZFc deletions might raise the potential risk of 45,X Turner’s syndrome [13]. Consequently, genetic testing of YCMD would optimally be a routine diagnostic for the andrological laboratory, with emphasis on being relatively affordable for the consulting couple and delivering fast results for initial decision making. These demands have been solved by the development of multiplex polymerase chain reactions (PCR) using combinations of primers to simultaneously amplify sequence-tagged sites (STS) within the three genomc AZF regions, usually in combination with a non-deleted Y-chromosomal control gene such as sex-determining region Y (SRY) or amelogenin Y (AMELY). In the last years, a plethora of protocols have been developed that vary significantly in the use of

[Note: The remaining text contains detailed scientific content and data that are not fully transcribed here due to the nature of the document and the formatting requirements.]
selected primers, PCR chemistry and amplicon detection method. A compendium of established protocols is given in Table 1. Since the complete mapping of the male-specific region of the Y-chromosome (MSY) [14], many different STS-specific primers have been employed in the various protocols, enabling the detection of more detailed rearrangements in the palindromic/ampliconic regions of the MSY. However, for a relatively quick and versatile multiplex PCR-based YCMD detection method, it is important that the panel of STS primers is derived from regions of the MSY that are not polymorphic and that correlate well with their absence in men affected by oligo/azoospermia.

In this work we aimed to develop a multiplex PCR protocol which eliminates the need for downstream amplicon identification, such as agarose gel or capillary electrophoresis. On the basis of classical STS primers that cover 95% of all clinical relevant deletions reported in the literature as defined in the “EAA/EMQN guidelines for molecular diagnosis of Y-chromosomal microdeletions (2004)” [10], some of these primers were exchanged using sites in the genomic proximity. Empirically, primers and multiplex primer combinations were developed that result in a high separation of melting peaks during the melting curve program after PCR amplification, enabling in the presence of the new high-performance fluorescent dye EvaGreen™ the identification of all amplicons in a multiplex set only by means of their Tm values, hence making amplicon and deletion identification by amplicon size expendable.

Results

Validation of new AZF primer combinations

In an initial approach to develop a multiplex real-time PCR system for YCMD that utilizes only amplicon melting curve identification, we tested the classical Simoni et al. [10] multiplex system for its performance. We were not able to establish a functionate multiplex melting curve-based setup due to similarity of some amplicons in their melting temperature within a window of 0.5–1°C (i.e. ZFY, sy86, sy84 and sy255; see Materials & Methods), resulting in a convolution of the melting peaks that severely impeded the Tm identification of the amplicons. This made it necessary to evaluate other primer combinations, with the premise that substituting primers should be located in the genomic proximity of the established Simoni et al. [10] primers in regions that are not polymorphic and guarantee to identify over 95% of the most common AZF deletions. Using SybrGreen I chemistry, we tested over 30 different STS primers for the three different AZF regions in combinations, using the peak separability of the multiplex melting curve as the selection criterion. The classic ZFX/ZFY control gene, present in male and female DNA, had to be substituted by primers for AMELX/AMELY with 5°C less melting temperature. This made it more suitable within the framework of the remaining multiplex amplicons. The SRY control gene for male DNA of the Simoni et al. [10] setup could be included. The AZF-specific primers substituting the original ones were sy85/G34990 (instead of sy86/sy84) for the AZFa region and sY135 [instead of sY134] for the AZFb region. These primers were combined with sY254 (AZFc), sY255 (AZFc) and sY127 (AZFb) from Simoni et al. [10] to establish two different 4-plex setups as summarized in Table 2. High peak separability was achieved by selecting combinations in which the Tm values ranged from 77–87°C, with at least 2°C difference between each of the products. We had attempted to develop a 5-plex setup as in the original Simoni et al. [10] setup, but it was not possible to include

Table 1. Compilation of recent investigations employing single- and multiplex PCR for the detection of YCMD.

| Reference       | Primers          | Multiplex | Detection               | Real-time Dye |
|-----------------|------------------|-----------|------------------------|---------------|
| UMENO et al. [24] | classical        | 6-plex    | Hitachi Microchip       |               |
| AKINN-SEIFER et al. [41] | classical+kit   | 2 × 6-plex | Agarose gel electrophoresis (?) |               |
| ZHANG et al. [22] | selected         | 6-plex    | Agarose/Capillary electrophoresis | SybrGreen I/FAM |
| LIN et al. [42]  | partial AZFc     | 6-plex    | Agarose gel electrophoresis |               |
| BOR et al. [43]  | classical        | 5 × 5-plex | Agarose gel electrophoresis |               |
| THORNHILL et al. [44] | classical     | 2 × 4-plex | Agarose gel electrophoresis |               |
| BOR et al. [25]  | classical        | 5 × 5-plex | Capillary electrophoresis | FAM/HEX       |
| MARTINEZ et al. [45] | classical | 3 × 3-plex | Agarose gel electrophoresis |               |
| KLEIMAN et al. [46] | selected for transcripts | 4-3, 1-plex | Agarose gel electrophoresis |               |
| CHARPENEL et al. [26] | classical | 4 × 3-plex, 1 × 4-plex | Agarose gel electrophoresis |               |
| KENT-FIRST et al. [47] | classical+AZFd | 9 × 4-8-plex | Agarose gel electrophoresis |               |
| HENEGARIU et al. [48] | classical+selected | 7-5, 7-5, 5-plex | Agarose gel electrophoresis |               |
| MITRA et al. [49] | classical+selected | 2 × 4-plex | Agarose gel electrophoresis |               |
| PLASESKI et al. [21] | selected | 11-plex | Capillary electrophoresis | FAM/HEX       |
| FODOR et al. [27] | selected         | 5-plex    | Capillary electrophoresis |               |
| LIN et al. [42]  | classical+selected | 6-plex    | Agarose gel electrophoresis |               |
| HUCKLENBROICH et al. [50] | selected | 3-2-plex | Agarose gel electrophoresis |               |
| JABASINI et al. [51] | selected for haplotyping | 7-5-plex | Hitachi Microchip |               |
| BUCH et al. [23]  | selected         | 16 × 1-plex | Melting curve | SybrGreen I |
| Promega Corp., WI, USA YCMD Detection System 2.0 (2009) | proprietary | 6-5, 4-plex | Agarose gel electrophoresis |               |

Primer noted are either those of Simoni et al. [10] (“classical”) or specifically designed for the region to be amplified (“selected”). If quantitative real-time PCR were conducted, dye chemistry is noted under ‘Real-Time Dye’. All protocols but one (Buch et al. [23]) base their analysis on the electrophoretic separation of amplicons.

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Table 2. Primers used for the two different multiplex setups.

| Setup 1 | Primers | Concentration [nM] | Origin | Amplicon Tm [°C] | Amplicon Size [bp] | Location [bp] |
|---------|---------|--------------------|--------|-----------------|-------------------|---------------|
| SRY (Control) | GAATATCCCGCTCCTCCGGA/GCTGGTGCTCCTCCTTGAG | 125 | Simoni et al. [10] | 86.50–86.68 | 470 | 2655107–2655576 |
| y254 (AZFc, DAZ-1) | GGGGTATCAGAGCTTAAACTGG-GAGCTG | 50 | | 81.51–81.83 | 380 | 25316193–25316572; 25372576–25372955; 26952264–26952643; 26986963–26987342 |
| y133 (AZFb) | ATTCCTCTTCCTCCTCAGT-TTTGTGTTGTTCTCAAAAGGGA | 500 | NEW | 83.86–84.11 | 177 | 23499309–23499485 |
| y85 (AZFa) | GCTATTTCTCTGCTATTGCATT-TTGCAATTGCTAT-TGAAGT | 375 | NEW | 76.61–76.81 | 80 | 14638102–14638181 |

**Setup 2**

| AMELxy (Control) | ATCAGAGCTTTAACGTTGG-AAGCTG/CCCTGGGCTCCTG- TAAAGAATAGTG | 250 | NEW | 79.79–80.02 | 106 (X); 112 (Y) | 11314994–11315099 (X); 6737888–6737999 (Y) |
| y255 (AZFb, DAZ-1) | GTTACGAGTGCGTGTTATG/CTGCTGATTGCCAGCAC | 125 | Simoni et al. [10] | 84.35–84.52 | 124 | 25314817–25314940; 25374208–25374331; 26950888–26951011; 26988595–26988718 |
| y127 (AZFb) | GGCTCACAAACGAAAAGAAAGTTGCAATTAAGGGGA | 300 | Simoni et al. [10] | 82.21–82.28 | 274 | 22570417–22570690 |
| G34990 (AZFa) | CATTCCGTTTATCAGCCAG/CAGTGACTCAGGGTATTAAGT | 750 | NEW | 76.41–76.56 | 83 | 15032211–15032293 |
| Alternatively: AMELxy (Control) | ATCAGAGCTTTAACGTTGG-AAGCTG/CCCTGGGCTCCTG- TAAAGAATAGTG | 500 | NEW | 79.33–79.56 | 105 | 6737888–6737992 |

**Table 2. Primers used for the two different multiplex setups.**

Given are their sequences, the final concentration in the PCR reaction, the origin (either Simoni et al. [10] or newly designed), the amplicon melting temperatures (given as regions), amplicon size and the Y-chromosomal location based on Human Genome GRCh37/hg19 assembly (Feb. 2009).

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one more amplicon delivering an additional melting peak without decreasing peak separability.

A detailed genomic location map of the new primers is given in Figure 1, with the primers employed in our two multiplex setups in red and substituted primers from Simoni et al. [10] in black. The numbers in brackets define their genomic locations based on the GRCh37/hg19 assembly (Feb. 2009).

Increasing melting curve resolution by EvaGreen™

In principle, the two multiplex setups could be established using SybrGreen I chemistry. However, in a further effort to increase melting peak resolution, we tested two other DNA-binding dyes in respect to signal strength and peak separability. Syto-13 had been extremely superior in performance of EvaGreen™, giving very high fluorescence signals during the amplification phase (data not shown), and strong signals with prominent melting peaks during the melting program, as shown with the AMELxy setup compared to the other two dyes (Figure 2). Syto-13 displayed a very low overall signal range, SybrGreen I performed better, with all (four) peaks present, but EvaGreen™ exhibited a signal range almost an order of magnitude higher. Therefore, we selected EvaGreen™ as the appropriate DNA-binding dye for YCMD detection by real-time multiplex PCR.

Performance of the two different 4-plex setups to detect YCMD

We tested each of the two different multiplex setups on isolated genomic DNA from a fertile man (positive control: PC), and men bearing either an AZFa, AZFb+c or AZFc deletion. A non-template control (water) served for contamination testing. The results for the SRY and AMELxy multiplex setups are given in Figures 3 and 4, respectively. Each of the corresponding melting curves (black lines) exported from the PCR system was imported into the ‘qPCR’ package. The function meltcurve was invoked which conducts an automatic first derivative melting curve transformation (red lines) and peak identification, including calculation of the respective Tm values. For each of the two setups, all investigated AZF deletions could be clearly identified by the absence of the corresponding peaks, as noted by asterisks in the single melting graphs. The positive control gave four discriminable peaks in all two setups, and the control amplicons (SRY: 86.74–87.08°C; AMELxy: 79.88–79.97°C; see Table 2) were present as sharp peaks in all positive controls or AZF melting curves. We verified the amplicons that pertain to the individual melting peaks by subsequent microcapillary electrophoresis (Bioanalyzer) and could identify each of the amplicons by the corresponding present or absent bands, depending on type of AZF deletion. All Tm values (given as ranges) and amplicon sizes are summarized in Table 2.

Robustness and sensitivity analysis of the two multiplex setups

To test the developed YCMD multiplex system for robustness in respect to peak strength and separability, the two setups were tested on four different genomic DNAs from men without AZF deletions. Irrespective of genomic origin, the melting peaks from all four samples could be clearly distinguished (Figure S1A). There were small individual differences in peak height/area, but in all of the samples, individual peaks were well separated. In addition, microcapillary electrophoresis displayed a homogeneous pattern of the corresponding amplicons (Figure S1B). The sensitivity of the system was evaluated by using genomic DNA from a positive control and conducting real-time PCR with subsequent melting curve analysis on increasing two-fold dilutions (Figure S1C).
individual melting peaks could be accurately identified up to an initial DNA concentration of 0.35 ng/µl after 40 cycles of amplification.

A further establishment of robustness was conducted by using 18 new samples (11 controls, 6 AZFc and 1 AZFbc) for multiplex setups performed by a different female co-worker (Figure S3, S4, S5). With the exception of one reaction (Setup 3, Con_03), in all investigated setups (Setup 1, Setup 2 and Setup 2 with alternative AMELy primer, denoted as Setup 3) four clearly separable peaks were visible for the control reactions. In agreement with the corresponding deleted regions, AZFc and AZFbc samples exhibited three or two peaks.

Test of AMELxy control primers and portability of the multiplex setup to a 96-well block based system

In our developed multiplex system, the ZFX/ZFY control primers which are male/female specific and test for contamination with female genomic DNA in the Simoni et al. [10] setup are substituted by the AMELX/AMELY primer pair in multiplex Setup 2 sharing the same features. However, in contrast to the ZFX/ZFY primers which produce a 495 bp amplicon from the X- and Y-chromosomal regions, AMELX/AMELY amplicons can be distinguished by capillary electrophoretic sizing by means of their 6 bp size difference depending on X- or Y-chromosomal origin (see double band at 106/112 bp in Figure 4). As an alternative, multiplex Setup 2 containing an AMELY-specific primer (105 bp amplicon) can be used if testing for female contamination is considered superfluous (Table 2; see Discussion). We tested the gender specificity of both primer pairs on female genomic DNA from blood and verified the AMELxy primer to amplify the female AMELX region, while the AMELY primer did not (Figure S2A).

As the multiplex system was established on a glass capillary-based real-time PCR system (LightcyclerTM), it was important to prove general applicability by validating the performance on a 96-well block based PCR system (Stratagene MX3000). The reaction volume was extended from 20 µl to 25 µl while keeping all primer concentrations fixed at those in the initial setup (Table 2). For each

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**Figure 1. Y-chromosomal locations of the primers used for the two multiplex setups.** Schematic representation of the Y-chromosome similar to Simoni et al. [10] showing the positions of the multiplex primers for the detection of AZFa, AZFb and AZFc deletions. The models from Vogt et al. [3] and Repping et al. [52] are shown on the left and right, respectively. The locations of the STS primers used for the two multiplex real-time setups in this work are given in red together with their genomic positions based on the GRCh37/hg19 assembly (Feb. 2009).

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of the two multiplex setups, we amplified genomic DNA from three individuals without AZF deletions, from men with AZFa/AZFb/AZFc deletions and a non-template control (water). Similar to our results obtained with the Lightcycler system, each of the three control samples (Figure S2B+C, upper rows) exhibited 4 clearly identifiable and separable melting peaks. In the AZFa, AZFb+c and AZFc deleted samples (Figure S2B+C, middle rows), the corresponding peaks are missing and the non-template control samples are all negative (Figure S2B+C, lower rows).

Discussion

The establishment of YCMDs as a major cause of impaired spermatogenesis, triggered by the discovery of the DAZ gene [17] and the description of three discrete AZF loci [3], has led to increasing interest in the genomic analysis of male infertility. With the popularization of the PCR (polymerase chain reaction) methodology, rapid testing for the presence or absence of YCMD by amplification of the respective AZF loci is achievable in matter of a few hours. Although YCMD were initially screened by singleplex PCRs on sequence-tagged sites of the AZF regions [18,19,20], several multiplex PCR systems for YCMD detection have recently been developed. The simultaneous amplification of more than one AZF locus in the presence of control genes confers robustness to the interpretation of the results by ensuring a successful and sensitive amplification reaction. In light of possible false negative results when the PCR reaction fails, this avoids misinterpretations in respect to the patient’s fertility status.

In addition to the plethora of existing multiplex protocols that are based on PCR amplification and subsequent analysis of the amplicons by agarose gel/capillary electrophoresis, real-time PCR approaches exist which monitor the multiplex amplification. We have compiled existing multiplex systems for the identification of YCMD in Table 1. Using real-time PCR offers the possibility of detecting initial cross-contamination, avoiding prolonged amplification in the saturation phase of the PCR, identifying low quality DNA (by high crossing points) and quantifying ratios of AZF genes or their copy numbers [21,22]. In all established protocols, post-PCR amplicon separation by either agarose gel or capillary electrophoresis is employed to identify the presence or absence of an AZF deletion (see Table 1, ‘Detection’). We are only aware of one work in which the YCMD identification is based solely on singleplex amplicon detection by melting curve analysis [23] (see Table 1). This is an interesting approach, as the presence/absence of YCMD can be checked by the absence/presence of the corresponding amplicons, and the validity of the amplicons is quickly verified by the typical melting point (Tm) of the PCR product. The major advantage, however, is that any following identification of the amplicons by electrophoretic size separation is made expendable, hence saving considerable time and investment.

Since a multiplex setup has several advantages over singleplex methods, such as reducing the number of reactions/input DNA and inclusion of an internal control gene for the monitoring of successful amplification, we aimed to develop a real-time based multiplex approach based only on melting curve amplicon identification. Furthermore, the method should be robust, sensitive, portable between different PCR hardwares (with little optimization needed) and give rise to automatic amplicon detection.

Similar to other investigations [23–26], and because there are no specific rules governing the STS marker selection, we initially chose to select the classical multiplex primer setup from Simoni...
et al. [10], covering at least 95% of all AZF loci that have been reported. As is widely established, it was possible to conduct a multiplex PCR with this setup and identify the amplicons by electrophoretic separation. However, due to the high similarity in the Tm values of ZFY, sY86, sY84, and sY255 (85.2°C, 86.1°C, 86.0°C, 84.7°C, respectively), an identification only by analysis of the melting peaks was not possible due to peak convolution. The substitution of the ZFX/ZFY control gene with the corresponding AMELX/AMELY control gene as in [27], sY86/sY84 by sY85/G34990 (AZFa), and sY134 by sY133 (AZFb), provided the basis for two different 4-plex setups in which the amplicon peaks displayed 2°C separability (see Table 2). The genomic proximity of the substituting primers for the AZFa and AZFb region ensures a similar outcome to the Simoni et al. [10] setup, as they are located within the same breakpoints [28–30]. However, it was not possible to include two control primer pairs in each multiplex reaction, such as ZFX/ZFY and SRY in Simoni et al. [10], because peak separability could not be maintained in the case of a 5-plex setup. We therefore split the multiplexes into two different 4-plex setups with SRY (male specific) and AMELX (male/female-specific), using one control in each multiplex. With these two setups, the following scenarios can be identified: (i) Contamination with female DNA would give an increase in the 106 bp X-chromosomal derived amplicon. This is not clearly distinguishable, but the same accounts for the original Simoni et al. [10] setup. (ii) Accidentally using female DNA would give a negative SRY setup, and a positive AMELx setup without AZF-specific signals. In this unlikely case, absence of male genomic DNA could be verified further by absence of the AMELx amplicon, which can be used as an alternative in Setup 2 (Table 2). (iii) In the rare case of an XX-male, the SRY control from Setup 1 should be positive, although recent results show that XX-males might even be negative for this gene [31]. (iv) In case of an AMELy deletion, encountered infrequently in the Asian population [32], the AMELx amplicon would serve as an internal control for a successful amplification.

The decision to exchange the commonly used SybrGreen I dye by the relatively new EvaGreen® dye was based on the known poor performance of SybrGreen I in melting curve analysis [33,34] and our own observation that EvaGreen® delivered much higher fluorescence values during amplification and strong peaks during melting curve analysis. We then put considerable effort in titrating the primers with the goal of obtaining fairly equal amounts of the individual amplicons [35], an essential prerequisite for robust peak identification.

With this developed multiplex system, we were able to accurately identify the missing amplicons as a consequence of
YCMD deletions in genomic DNA from men with AZFa, AZFb+c and AZFc deletions. The identification of the YCMD was robust, sensitive and independent of PCR hardware. For the safe-minded, and as shown by the microelectrophoretic separations of the multiplex PCRs in this work, it is possible for an initial validation (i.e. when setting up this system in a laboratory) to correlate and validate the melting peaks from the PCR machine with the electrophoretic sizing of the amplicons. But we want to emphasize that this is only necessary once, as the Tm values alone suffice. Furthermore, by using the ‘qpcR’ package for the open-source statistical environment R, we show in this work the potential for automatic peak identification of the multiplex melting curves, including Tm calculation.

In recent years, other genetic alterations have been identified which are involved in male infertility, such as Y-chromosomal haplogroups that are associated with reduced fertility [36], partial deletions in the AZFc region [37] or point-mutations in AZF located genes [38]. One drawback of our multiplex system, which is restricted by the number of amplicons whose melting peaks can be clearly distinguished, is that it can only identify larger deletions, as in the well established Simoni et al. [10] protocol.

We believe that our method is a great improvement to existing multiplex PCR methods for the detection of YCMD, since it is the first real-time based multiplex approach in this field, with all advantages of the latter such as amplification monitoring, an internal control in each reaction, decreased PCR setup number and most importantly, the elimination of any downstream amplicon analysis such as gel/capillary electrophoresis. A potential automation of Tm and hence YCMD identification, as exemplified in this work, may increase the benefit of this system even further.

**Materials and Methods**

**Isolation of genomic DNA**

Genomic DNA from control (n = 5), AZFa (n = 1), AZFbc (n = 4) and AZFc (n = 7) samples was isolated from testicular tissue obtained from a therapeutic testicular sperm extraction procedure (TESE), after direct surgical immersion of a rice grain sized biopsy sample in 2 ml RNeater® (Ambion, Austin, TX), using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). A second set of control (n = 11), AZFc (n = 6) and AZFbc (n = 1) samples was additionally investigated by a different female scientist to test against individual bias. Written informed consent and approval was obtained from the Ethical Commission of the Medical Board Hamburg (Approval OB/X/2000), and the studies conducted in accordance with the guidelines of the Helsinki Declaration regarding...
the use of human tissues. For more details on this procedure, see [39]. DNA purity (ratio 260 nm/280 nm) was assessed on an Ultraspec 3000 spectrophotometer (GE Healthcare, Freiburg Germany). Genomic DNA integrity was checked by 1% agarose/TAE gel electrophoresis. Female DNA from blood was obtained with the DNaseasy Tissue Kit (Qiagen) according to the manufacturer’s protocol. Genomic DNA from a male with an AZFa deletion was isolated from blood with the FlexiGene DNA Kit (Qiagen).

Selection of suitable multiplex primers for the detection of YCMD

From an empirical (by quantitative PCR) validation of over 30 primer pairs deposited in the NCBI UniSTS database (http://www.ncbi.nlm.nih.gov/unists/), we selected primer combinations which resemble a similar setup as in the widely established Simoni et al. protocol [10]. However, the classical setup is only applicable when subsequent amplicon size analysis by gel electrophoresis is conducted. The amplicons can not be discriminated by melting curve analysis alone as their $T_m$ values differ only in a region of 0.5–1°C. This was especially the case for primers specific to ZFY (control gene, $T_m = 85.2\degree C$), sY84 (AZFa, $T_m = 86.1\degree C$), sY84 (AZFb, $T_m = 86.0\degree C$) and sY255 (AZFc, $T_m = 84.7\degree C$) whose combined amplicons lacked separability in melting curve analysis. Using the NCBI UniSTS database, alternative primers were selected for genomic sites in near proximity to the original primers. These were validated through quantitative PCR by combining them with any of the remaining primers to establish maximal separability of the melting curve peaks ($T_m$ values). Primer sequences were rechecked for sequence specificity by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) and UCSC Genome cPCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). Validated substitutes for the primers above which displayed high separability in their $T_m$ values were AMELxy (control), sY85 (AZFa), G34990 (AZFa), and sY133 (AZFb). These primers were combined with the remaining primers sY254 (AZFc), sY255 (AZFc) and sY127 (AZFb) from Simoni et al. [10] to establish two different 4-plex setups as summarized in Table 2.

Quantitative real-time PCR (qPCR) setup and parameters

The two different multiplex setups were tested with three different DNA-binding dyes (SybrGreen I, Invitrogen, Darmstadt, Germany; Syto-13, Invitrogen, Darmstadt, Germany; EvaGreen™, Biotium, Hayward, USA) in order to investigate dye-dependent peak resolution during melting curve analysis. Quantitative real-time PCR was conducted in a Lightcycler™ 1.0 instrument (Roche, Basel, Switzerland) in glass capillaries at 20 μl volume containing 1× Premix Ex TaqTM (Takara Bio Europe, France) with either 0.5× SybrGreen I (from 10000× stock), 2.5 μM Syto-13 (from 5 mM stock) or 0.2× EvaGreen (from 20× stock) and optimally titrated (with respect to equal melting peak area) final primer concentrations in the PCR reactions as indicated in Table 2. PCR cycling was conducted with an initial denaturation at 95°C for 1 min, 35 cycles with 95°C 10 s/60°C 30 s/72°C 30 s, and final cooling to 40°C for 1 min. A melting curve was run after cycling by an initial ramping from 40°C to 70°C at 2°C/s and a subsequent higher resolution melting program from 70°C to 95°C at 0.1°C/s. The raw fluorescence melting curve data (temperature versus fluorescence) was exported from the Lightcycler v3.5 software. To extend applicability of our method from a capillary-based real-time PCR system to a block-based (96-well) system, PCR reactions with setups similar to above but in 25 μl volume were conducted on a Stratagene Mx3000P PCR instrument (Agilent Technologies, Palo Alto, CA, USA) and the melting data exported by the accompanying software.

Melting curve analysis, automatic peak detection and $T_m$ identification

Exported raw fluorescence melting curve data was imported into the ‘qpcR’ package [40] for the open-source statistical environment R (www.r-project.org). The function meltrange, which provides automatic raw fluorescence data smoothing, first derivative calculation and automatic peak detection with $T_m$ identification, was used to analyze the raw melt data and identify present or absent amplicons in control and AZF samples. All melting curves in this work were made by the corresponding box parameter of this function.

Validation of amplicons by microcapillary electrophoresis

To verify the validity of the multiplex amplicons, PCR reactions were size-separated after cycling on an Agilent Bioanalyzer (Model 2100; Agilent Technologies, Palo Alto, CA, USA) using the DNA 1000 capillary chip and checked for their absence in AZF deleted samples.

Supporting Information

Figure S1 Robustness and sensitivity analysis of the two multiplex setups. (A) The two different multiplex setups were tested on four different genomic DNAs from patients without AZF deletions. Independent of DNA origin, the melting curves display a highly similar pattern. (B) Microelectrophoretic (Bioanalyzer) separation of the amplicons obtained in (A). (C) Testing of the two multiplex setups on a positive control (no AZF deletion) on six 2-fold dilutions. The DNA concentrations are given in the legend on the right side. Robust melting patterns were obtained with DNA concentrations higher than 0.35 ng/μl.

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Figure S2 Test of new AMELxy/AMELy control primers for gender specificity and evaluation of the two multiplex setups on a block-based (96-well) quantitative PCR system (Stratagene 3000). (A) Female genomic DNA extracted from blood was subjected to quantitative PCR using the AMELxy and AMELy (alternative) primer pairs included in the multiplex setups. As expected, AMELxy gave a positive signal due to homology with the AMELx gene, while AMELy was negative. (B) and (C) Evaluation of the SRY and AMELxy multiplex setup on a 96-well quantitative PCR system (Stratagene 3000), respectively. Each of the setups was tested on three positive controls (genomic DNA from men without YCMD, upper row), samples from men with AZFa, AZFb and AZFc-deletions (middle row) and negative control (lower row).

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Figure S3 Test for robustness of Setup 1 by using a second set of control (n = 11), AZFc (n = 6) and AZFbc (n = 1) samples (Lightcycler 1.0). Setup 1 was tested on an independent set of control (Con_01–Con_11), AZFc (AZFc_01–AZFc_06) and AZFbc (AZFbc_01) samples by a different female scientist. Melting curves of control samples displayed clear visibility of four peaks. Correspondingly, AZFc and AZFbc samples exhibited three or two peaks, respectively. Bottom right: Boxplot from the melting points ($T_m$ values) of all amplicons.

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Figure S4 Test for robustness of Setup 2 by using a second set of control (n = 11), AZFc (n = 6) and AZFbc (n = 1) samples (Lightcycler 1.0). Setup 2 was tested on an
independent set of control (Con_01–Con_11), AZFc (AZFc_01–AZFc_06) and AZFbc (AZFbc_01) samples by a different female scientist. Melting curves of control samples displayed clear visibility of four peaks. Correspondingly, AZFc and AZFbc samples exhibited three or two peaks, respectively. Bottom right: Boxplot from the melting points ($T_m$ values) of all amplicons. (TIF)

**Figure S5** Test for robustness of Setup 3 by using a second set of control (n = 11), AZFc (n = 6) and AZFbc (n = 1) samples (Lightcycler 1.0). Setup 3 (Setup 2 with alternative AMELY primer) was tested on an independent set of control (Con_01–Con_11), AZFc (AZFc_01–AZFc_06) and AZFbc (AZFbc_01) samples by a different female scientist. Melting curves of control samples displayed clear visibility of four peaks. Correspondingly, AZFc and AZFbc samples exhibited three or two peaks, respectively. With the exception of one run (Con_03), all melting peaks could be easily distinguished. Bottom right: Boxplot from the melting points ($T_m$ values) of all amplicons. (TIF)

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**Author Contributions**
Conceived and designed the experiments: VK. ANS. Performed the experiments: VK HCO JG. Analyzed the data: VK. ANS. Contributed reagents/materials/analysis tools: HCO JG. Wrote the paper: ANS.

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