IVD For *in Vitro* Diagnostic Use

AZF System Y-chromosome

Handbook

Real Time PCR test for detection of the microdeletions in AZF regions: AZFa (sY84, sY86), AZFb (sY127, sY134), AZFc (sY254, sY255) of the human Y chromosome

REF 01200-50

Σ **60**

NAME

AZF System Y-chromosome

INTRODUCTION

Y-chromosomal microdeletions are the second most frequent genetic cause of male infertility after the Klinefelter syndrome. In the last decade, many investigators have described the occurrence of microdeletions in infertile patients around the world and the molecular diagnosis of deletions has become an important test in the diagnostic workup of male infertility. Microdeletions occur in about one in 4000 men in the general population but its frequency is significantly increased among infertile men. Azoospermic men have a higher incidence of microdeletions than oligozoospermic men and consequently deletion frequency found in different laboratories may vary from 2 to 10% (or even higher, see Fig. below) reflecting the composition of the study population.

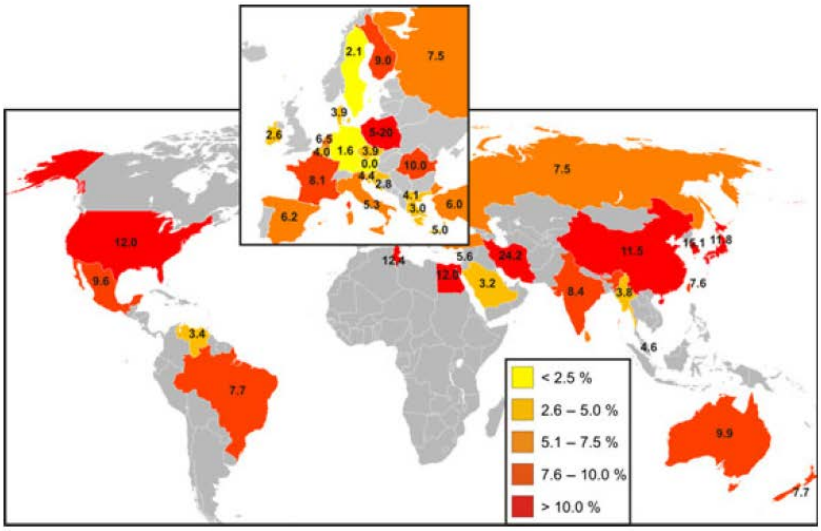
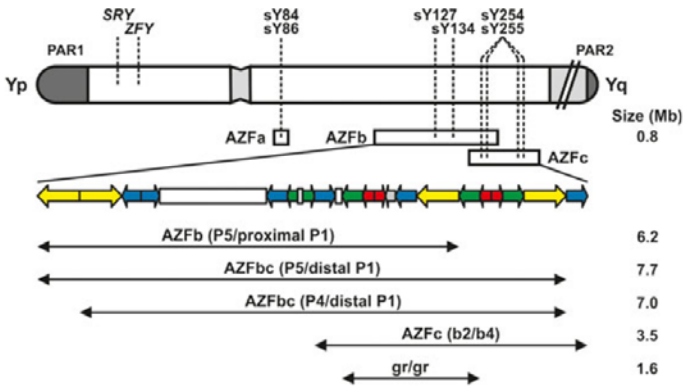


Fig. Worldwide frequencies of AZF deletions in infertile men (Simone et al, 2008)

The complete physical map and sequence of MSY (Male-Specific Region Of The Y Chromosome) has been available since 2003. According to the present knowledge, the following recurrent microdeletions of the Y chromosome are clinically relevant and are found in men with severe oligo- or azoospermia (see fig. below): AZFa, AZFb (P5/proximal P1), AZFbc (P5/distal P1 or P4/distal P1), AZFc (b2/b4). The most frequent deletion type is the AZFc region deletion (~80%) followed by AZFa (0.5–4%), AZFb (1–5%) and AZFbc (1–3%) deletion. Deletions which are detected as AZFabc are most likely related to abnormal karyotype such as 46,XX male or iso(Y).



INTENDED USE

Diagnosis of a microdeletion of the Y chromosome permits the cause of the patient's azoospermia/oligozoospermia to be established and to formulate a prognosis. The world literature, now based on several thousands of patients screened, indicates that, as a rule, clinically relevant deletions are found in patients with azoospermia or severe oligozoospermia with sperm concentrations <2 x 10^6/mL. 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, in the literature there are a number of examples of deletion carriers among non-idiopathic infertile men, for example, with a testicular tumor or after chemo-/radiotherapy, which would be considered to explain the spermatogenic failure. Therefore, the presence of any diagnosis accompanied by azoo- 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 varicocoelectomy because deletion carriers will most likely not benefit from the surgical procedure.

Patients with azoospermia who may be candidate for TESE/ICSI should be offered deletion screening because TESE should not be recommended in cases of complete deletion of the AZFa region. Micro-TESE in azoospermic carriers of deletions of the AZFb or AZFbc regions with proximal breakpoint in the P4 palindrome may be eventually attempted. However, the patient should be fully informed about the very low/virtually zero chance to retrieve spermatozoa. A standard biopsy (without microsurgical equipment) should never be attempted in these cases. Therefore, the diagnosis of a deletion has prognostic value and can influence therapeutic options.

Genetic counselling is mandatory to provide information about the risk of conceiving a son with impaired spermatogenesis. In case of partial AZFa or AZFb and AZFc deletion, the counselling (with AZF testing) is relevant also for other male members of the family as transmission of these type of deletions has been reported in the literature.

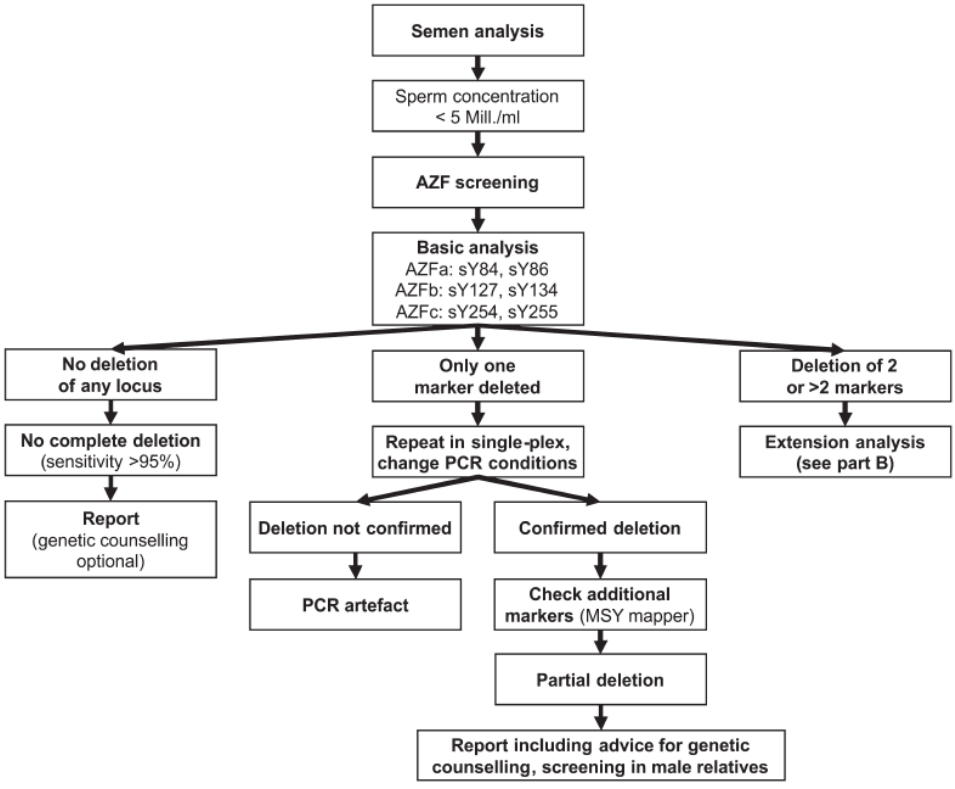


Fig. Flow chart with indications of AZF screening (American Society of Andrology and European Academy of Andrology guidelines, Andrology, 2014, 2, 5–19)

PRINCIPLE OF ASSAY

AZF System Y-chromosome is a qualitative tests that allow the detection by Real Time PCR based on the amplification of the genome specific region using specific primers. In Real Time PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the reaction allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run.

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, as deletions involve well-defined regions including many STS loci. Therefore, **Sacace™ AZF System Y-chromosome** kit was developed to detect two STS loci in each AZF region and choice of STS primers was based on the experience of many laboratories, the results of external quality control and the guidelines of American and European Andrology societies. These primers include:

For AZFa: sY84, sY86

For AZFb: sY127, sY134

For AZFc: sY254, sY255

The **Sacace™ AZF System Y-chromosome** kit contains also a control primer pair that amplifies a unique region in both male and female DNA (ZFX/ZFY). These control primer pairs are internal controls for the multiplex amplification reactions and test the integrity of the genomic DNA sample. Finally, **Sacace™ AZF System Y-chromosome** kit also includes a primer pair that amplifies a region of the SRY gene, acting as control amplification for the testis-determining factor on the short arm of the Y chromosome and for the presence of Y-specific sequences when the ZFY gene is absent (e.g. in XX males).

The use of this primer set will enable the detection of almost all clinically relevant deletions and of over 95% of the deletions reported in the literature in the three AZF regions and is sufficient for routine diagnostics.

MATERIALS PROVIDED

- **PCR-mix-A ,** 215 µL, (mix of ZFX/Y, sY86(AZFa), sY127(AZFb), sY254(AZFc) primers);
- **PCR-mix-B,** 215 µL, (mix of SRY, sY84(AZFa), sY134(AZFb), sY255(AZFc) primers);
- **Taq polymerase,** 40 µL;
- **Diluent**, 2 x 2 mL;
- **Positive Control C+**, 150 µL

Contains reagents for 60 tests.

STORAGE INSTRUCTIONS

AZF System Y-chromosome kit must be stored at -20°C. The kits can be shipped at 2-8°C and stored as indicated immediately on receipt.

STABILITY

AZF System Y-chromosome kit is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation

- DNA extraction kit
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification

- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Freezer, refrigerator
- Tube racks

WARNINGS AND PRECAUTIONS

IVD For *in Vitro* Diagnostic Use Only

***In Vitro* Diagnostic Medical Device**

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities’ regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

AZF System Y-chromosome Kit can analyze genomic DNA extracted from:

- *whole blood* collected in EDTA tubes;

Specimens can be stored at +2-8°C for no longer than 24 hours, or freeze at -20°C to -80°C.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial genomic DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.
Sacace Biotechnologies recommends to use the following kit:

⇒**Genomic column DNA Express** – spin column extraction kit (Sacace, REF K-1-1/E)
⇒**SaMag Blood DNA extraction kit** (Sacace, REF SM001);
⇒**QIAamp DNA Blood mini kit** (Qiagen, REF 51104);

Please carry out DNA extraction according to the manufacturer's instruction.

PROTOCOL

AZF System Y-chromosome kit do not include reagents required for sample preparation and DNA extraction. Blood samples and biological materials must be processed by using the recommended kits or those with similar performances of quality and quantity of extracted DNA. Use of blood samples collected in tubes containing heparin is not recommended.
The analysis of the genomic DNA specimens using **AZF System Y-chromosome** kits includes the following stages:

1. Preparing the Real Time PCR;
2. Real Time PCR analysis;
3. Data analysis with the software of Real Time PCR instrument;
4. Results analysis and conclusions.

EXPERIMENTAL PROTOCOL

The total reaction volume is **35 µl**, the volume of DNA sample is **7 µl**.

All obtained DNA samples should be examined in two tubes – one with **PCR-mix-A.** and the other one with **PCR-mix-B.**

1. Prepare required quantity of PCR tubes according to the number of samples plus 2 controls for each mix.
2. Prepare for each PCR-mix one new tube and add for each sample
   - **24,5 µl * N+2** of **Diluent**,
   - **3,5 µl * N+2** of **PCR-mix-A (or B)**
   - **0,3 µl * N+2** of **Taq Polymerase.**

Do not store the prepared reaction mixture.

PCR run should include amplification reactions for four control points: negative control and positive control for two reaction mixtures (**PCR-mix-A** and **PCR-mix-B**).

3. Transfer **28 µl** of the prepared mixture to each sample tube.
4. Add **7 µl** of **DNA** obtained from clinical samples or control using tips with aerosol barrier.
5. Carry out the control amplification reactions:

NCA -Add **7 µl** of **Diluent** to the tube labeled NCA (Negative Control of Amplification).

C+ -Add **7 µl** of **Positive Control** to the tube labeled C+ (Positive Control of Amplification).

6. Insert the tubes in the Real-time PCR instrument. Perform the amplification reaction immediately after DNA samples and controls are added to the reaction mixture.

Amplification

Create a temperature profile on your instrument as follows:

	Plate- or modular type Instruments[1]			**Rotor-type Instruments[2]**		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**94**	1 min 30 s	1	**95**	1 min 30 s	1
2	**94**	15 s	5	**95**	15 s	5
	64	40 s		**60**	40 s	
	72	40 s		**72**	40 s	
3	**94**	15 s	40	**95**	15 s	40
	64	40 s *fluorescent signal detection**		**60**	40 s *fluorescent signal detection**	
	72	40 s		**72**	40 s	

[1] For example, SaCycler-96™ (Sacace); CFX-96 / iQ5™ (BioRad); Mx3005P™ (Agilent); ABI® 7500 Real Time PCR (Applied); LightCycler® 96 (Roche).*

[2] For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)

Fluorescence is detected in FAM/Green, JOE/Yellow/HEX, Rox/Orange and Cy5/Red fluorescence channels.

DATA ANALYSIS

The fluorescent signal intensity is detected in 4 channels as shown in the table below:

PCR-mix-A		PCR-mix-B	
Locus	Channel	Locus	Channel
ZFY/X	Joe/Hex/Yellow	*SRY*	Joe/Hex/Yellow
sY86 (AZF_A)	Fam/Green	sY84 (AZF_A)	Fam/Green
sY127 (AZF_B)	Rox/Orange	sY134 (AZF_B)	Rox/Orange
sY254 (AZF_C)	Cy5/Red	sY255 (AZF_C)	Cy5/Red

INSTRUMENT SETTINGS

SaCycler-96 SETTINGS

Set the threshold manually as in the following table:

Channel	Threshold
FAM	100
HEX	20
ROX	20
Cy5	20

CFX-96 / iQ5 SETTINGS

Set the threshold manually as in the following table:

Channel	Threshold
FAM	100
HEX	50
ROX	50
Cy5	50

RotorGeneQ SETTINGS

Set as in the following table:

Channel	Threshold	More Settings/ Outlier Removal	Dynamic tube/ Slope Correct
FAM/Green	0,025	10%	ON
JOE/Yellow	0,02	10%	ON
ROX/Orange	0,025	10%	ON
Cy5/Red	0,025	20%	ON

For other plate-type instruments:

Set the threshold manually at a level 10-20% of the fluorescence of the positive control in the last amplification cycle.

RESULTS INTERPRETATION

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates).

The result of amplification is considered **positive** if the fluorescence curve is characteristic of real-time PCR (S-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the below table.

The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

Channel	Mix-A	Mix-B	Interpretation
JOE/HEX/ Yellow	**+ (Ct<28)**	**+ (Ct<28)**	Valid result. Amplification of Internal control. Correct DNA extraction and PCR protocol.
	-	**-**	Result is not valid. PCR analysis should be repeated starting from DNA extraction stage
FAM/ Green	**+ (Ct<28)**	**+ (Ct<28)**	Presence of region AZFa
	-	**-**	Deletion in region AZFa
ROX/ Orange	**+ (Ct<28)**	**+ (Ct<28)**	Presence of region AZFb
	-	**-**	Deletion in region AZFb
Cy5/ Red	**+ (Ct<28)**	**+ (Ct<28)**	Presence of region AZFc
	-	**-**	Deletion in region AZFc

Sample is considered positive (+) for AZF region if the detected Ct value is less than 28, otherwise it is considered negative (-) for AZF indicating presence of deletion in region AZF.

The result is **invalid** if *Ct* value is not determined (absent) in the channel JOE/HEX/Yellow. In such cases, PCR analysis should be repeated starting from DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.

A positive signal only on the JOE/Yellow channel with the Mix-A means that the presence of woman DNA in the sample.

For FAM, ROX, Cy5 channels in case there is discordance between Mix-A and Mix-B results (Mix-A positive and Mix-B negative or vice versa) the result is doubtful and amplification experiment should be repeated.

Results are accepted as significant only if both positive and negative controls of amplification passed correctly (see below the table for controls).

Control	Stage for control	*Ct* value in the channel for fluorophore			
		FAM	JOE	ROX	Cy5
NCA	PCR	NEG	NEG	NEG	NEG
C+	PCR	POS (<23)	POS (<23)	POS (<23)	POS (<23)

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KEY TO SYMBOLS USED

REF	List Number		Caution!
IVD	For *In Vitro* Diagnostic Use		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**IC**	Internal Control
LOT	Lot Number		

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* iQ5™ is a registered trademark of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
*ABI® is a registered trademark of Applied Biosystems
* LightCycler® 96 is trademark of Roche

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