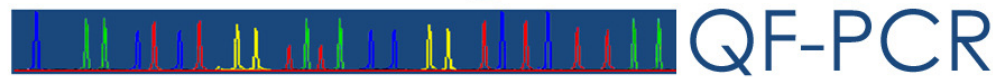# ANEUFAST™ QF-PCR

## 1.1 Aneufast™ Analysis Troubleshooting Guide

1.1.1 Maternal cell contamination of amniotic fluid samples

Prenatal samples may occasionally be contaminated by maternal cells. Heavily bloodstained amniotic fluid samples for instance are highly likely to be a mixture of fetal and maternal cells. In these cases it is possible that the presence of maternal DNA will hamper QF-PCR analysis. QF-PCR may, however, be attempted on this type of sample without any risk of misdiagnosis. If present, maternal cell contamination will result in characteristic STR profiles with extra allele peaks for all chromosome STRs that are not compatible with normal, trisomic, triploidy or mosaic cases (see further below).

The example below shows from top to bottom the detection of increasing levels of maternal cell contamination in four different amniotic fluids from normal XY male fetuses.
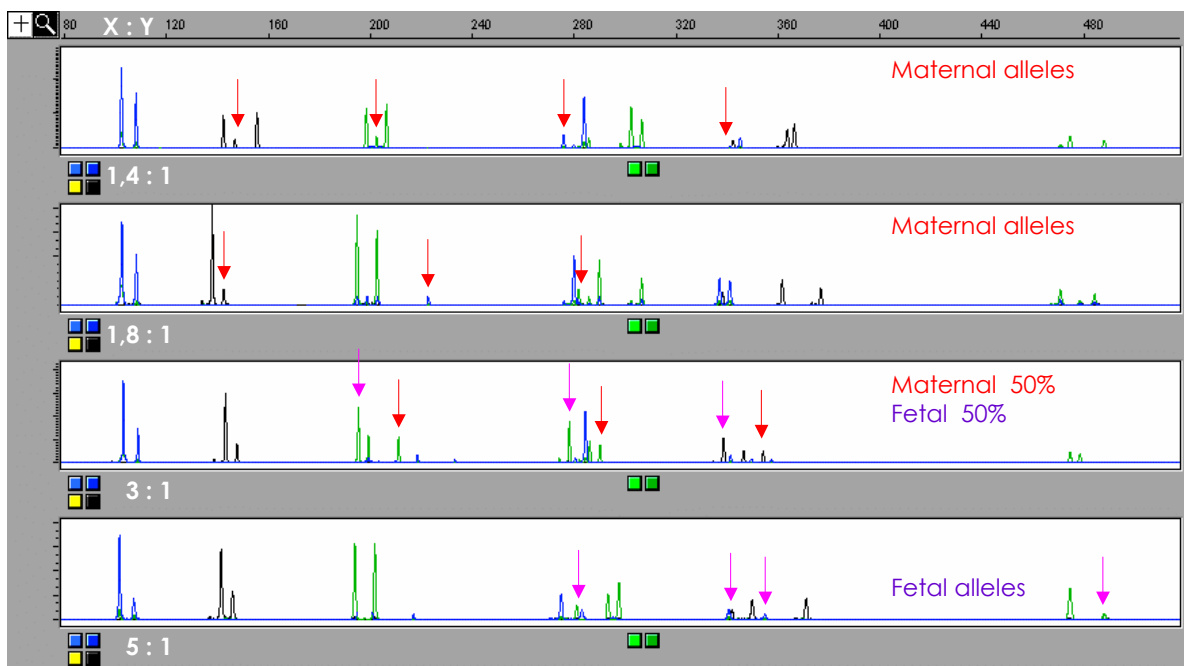
With male fetuses the fetal origin of the predominant cell line can be assessed by the ratio between the X- and Y- specific product of the AMXY; in DNA samples retrieved from female fetuses the proportion can be assessed comparing the STR profile with that obtained on maternal DNA. In the first lane, only a small amount of maternal contamination is detected, as a skewed AMXY ratio and some small extra STR alleles. In this case it is still possible to perform the fetal aneuploidy diagnosis as the STR ratios are not significantly altered by the presence of maternal DNA. On the other hand, the bottom sample shows the opposite situation with a predominant cell population of maternal origin, in which case appropriate fetal aneuploidy diagnosis is not possible.

1.1.2 Chromosome mosaicism

Although the **Aneufast™** QF-PCR Kit is not specifically designed to detect mosaicism, such conditions should be suspected in particular when unusual sex chromosome patterns are observed. **These patterns may be very difficult to interpret and substantive troubleshooting is recommended. Furthermore, in all suspected mosaic cases, the result should be confirmed by cytogenetic analysis, as the distribution of different cell lines in a sample can show great variability with different chromosome constitutions compatible with the same STR profiles.**

It is also worth emphasising that in mosaic cases, the comparison between the QF-PCR results on fresh uncultured samples and the percentages of different cell lines detected after culture by metaphase analysis and/or fluorescence in situ hybridisataion (FISH) on interphase cell nuclei, is of great diagnostic value. These results could help in determining more accurately the real chromosome constitution.

When CVS samples are analysed it is recommended to perform QF-PCR on two independent fronds. This will reduce the risk of misinterpretation in cases of confined placental mosaicism. CVS samples have the limitation of not always reflecting the fetal chromosome constitution and results have to be considered within the full clinical picture of each case.
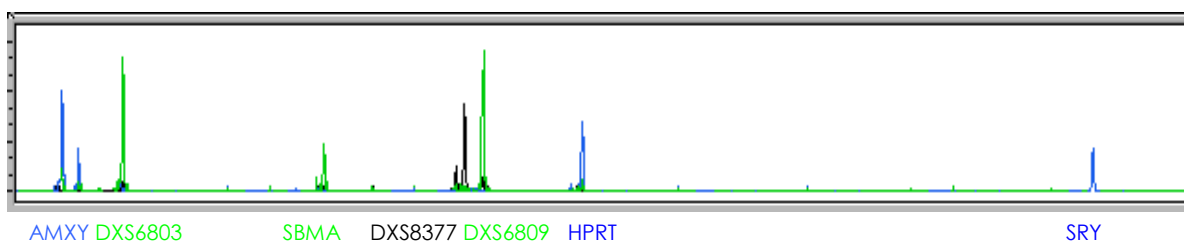
Example 1
Mosaicism X/XY
In these cases the normal XY cell line is detected by QF-PCR as Y-specific products for the AMXY and SRY; also all X-linked STRs will show single peaks. The pseudoautosomal markers X22 and DXYS128, when informative, will produce two unbalanced products.
The proportion between the two cell lines may be directly deduced by the ratio between the X- and Y- specific products of AMXY that should fall outside the limits of the normal ranges. The same result may also be observed as regards the pseudoautosomal markers X22 and XYS128. A

skewed X:Y ratio of 3:1 (or more), for instance, could be compatible with a X/XY mosaicism with the X monosomy present in ¾ of the cells (or more).

It is important to note that some X/XY mosaics may show STR patterns very similar to those in non-mosaic XXY cases. Thus, for example, an XXY case may look very similar to a mosaic X/XY, if the X and XY cell lines occur in equal proportions, and a 2:1 ratio of both AMYXY and the pseudoautosomal markers X22 and DXYS218. On the other hand, in cases of a non-mosaic XXY sex chromosome constitution the seven X-linked markers included in the **Aneufast™** MXY marker set should allow the detection of the two X chromosomes, when generating heterozygous patterns (ratios of 1:1).

AMXY DXS6803   SBMA   DXS8377 DXS6809 HPRT   SRY

Example: Electrophoretogram using the MXY back-up marker set detecting X/XY mosaicism. AMXY shows a skewed 3:1 ratio and the presence of a single X is reflected by the single product of the five X-linked markers DXS6803, SBMA, DXS8377, DXS6809 and HPRT.
The detection of Y- derived sequences by QF-PCR is highly sensitive; if present in very low proportion the XY cell line may not be detected after cell culture by cytogenetic analysis. Also, the proportion of the different cell lines may change during cell culture.

Mosaicism X/XX
A monosomy X cell line is usually suspected together with an XX cell line, when the X-linked HPRT and the pseudoautosomal markers X22 and DXYS218 show unbalanced fluorescent products, with ratios outside the normal or trisomic range. If the monosomy X cell line is present in less than about 20% of the cell population, it may remain undetectable.
In cases where both cell lines are present in the same proportion (50%), all markers will produce trisomic diallelic patterns (ratio 2:1) similar to those produced by the XXX sex chromosome constitution. On the other hand, the detection of trisomy X by QF-PCR is 100% reliable by the occurrence of a trisomic triallelic pattern for at least one of the STR markers.

In a case where all informative markers on the sex chromosomes included in the **Aneufast™** MXY marker set give trisomic diallelic results, the possibility of a mosaic X/XX (50%) cannot be excluded.

1.1.3 Mosaicism for Autosomal Trisomies

Autosomal disomy/trisomy mosaicism should be suspected in cases where all informative STRs on the same chromosome produce either two unbalanced peaks with ratios outside normal and trisomic values, or a normal heterozygous pattern with an extra minor peak.

Example 2
Mosaic trisomy 21
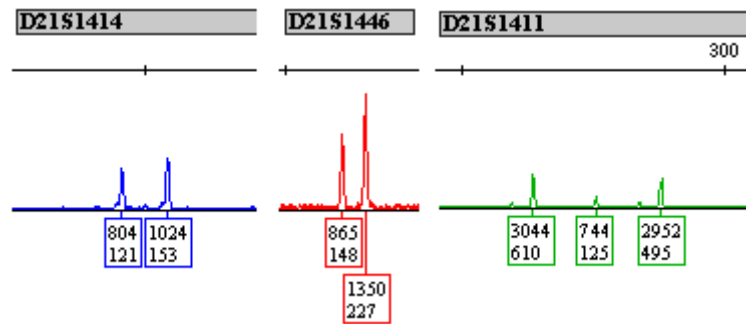
Unbalanced alleles are seen for both D21S1414 and D21S1446. As regards D21S1446 the ratio (1:1,6) falls outside the normal or trisomic range. Note also the higher height of the longer allele of both markers, which is unusual, as preferential amplification normally takes place for the shorter marker. The presence of an extra chromosome in a low proportion of cells is indicated by the occurrence of an extra allele detected with D21S1411. The sensitivity in detecting the trisomic cell line is of course higher if the extra chromosome produces an allele of different length

1.1.4 PCR artefacts during STR amplification

Example 1
Stutter Bands
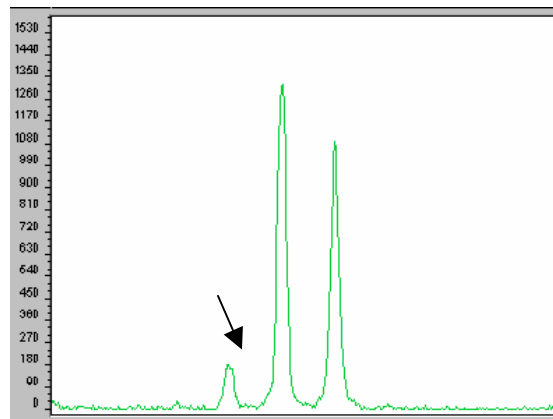
Taq polymerase slippage during PCR amplification of repeated sequences can produce extra products that are exactly one repeat smaller than the STR allele; these are called stutter bands. The proportion of stutter bands is characteristic for each STR marker and usually does not exceed 15% of the area of the corresponding allele (see figure). This artefact is lower (and almost undetectable) for penta and tetranucleotides, but increases in tri- and dinucleotide repeats.

Aneufast™ does not include dinucleotide repeats, as these sequences produce high numbers of stutter bands that hamper accurate allelic quantification and in some cases may not be easily distinguished from true alleles.

Example 2
Preferential Amplification
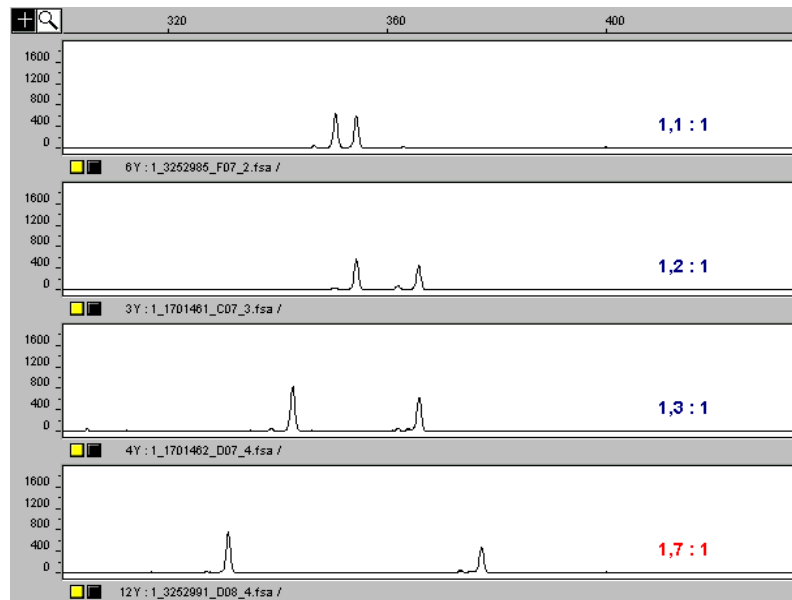
STR markers included in **Aneufast™** are highly polymorphic, implying a high number of informative alleles and a wide size range of the corresponding PCR products. In cases where two alleles fall 20 or more bp apart, it is possible that the shorter fragment is favoured during PCR amplification. This phenomenon may occur especially for X22 and D18S386 markers. However the optimised **Aneufast™** buffer together with a low number of PCR cycles should limit this happening to within the accepted ranges for normal and trisomic diallelic samples. High preferential amplification may result from the addition of too much DNA to the PCR reaction. This could be counterbalanced by reducing the number of PCR cycles.

The example illustrates the increased amplification of the shorter allele in relation the longer in three different cases with ratios 1,1:1; 1,2:1, 1,3:1 and 1,7:1.

Example 3
<u>Incomplete Final Extension</u>
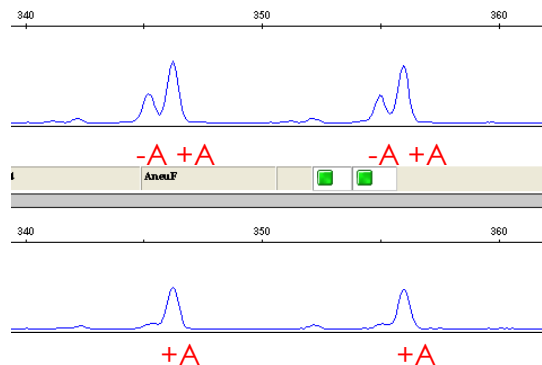
A well known phenomenon during PCR amplification is the Taq addition of an extra A nucleotide at the end of the PCR products. It is impossible to avoid the Taq adding this extra base. The ideal, therefore, is to facilitate its complete addition at the end of all PCR products; this is achieved by the final incubation at 60ºC at the end of **Aneufast™** thermal cycling. Occasionally incomplete A addition may occur in cases where too much DNA is used for PCR amplification. This will result in allele peaks split in two, with the smaller product differing exactly one bp from the main allele. Even in the presence of incomplete A addition, accurate quantification can still be achieved by considering the sum of both peaks as the STR allele area. In the right conditions, however, this artefact should not occur with **Aneufast™**. Its presence could reflect too many PCR cycles for the amount of DNA used.

1.1.5 Rare Polymorphisms and Mutations of Aneufast™ Markers

<u>Amelogenin</u> (AMXY)

The Y- specific product of the AMXY can occasionally be absent in normal males. This polymorphism is extremely rare in Caucasian males (0,04%) but has shown to be present in up to 2% of normal males of Malay and Indian ethnicity. Sex determination of a DNA sample using **Aneufast™** includes a fragment amplified on the SRY gene. The male sex chromosome constitution should always show both AMY and SRY products; however due to this possible polymorphism with absence of the Y-specific AMXY product, occasionally only the SRY sequence is

present. In these rare cases the normal male sex chromosome constitution can only be assessed if both pseudoautosomal STRs (X22 and DXYS218) are informative and normal heterozygous.

The X- and Y- specific products of the Amelogenin have also been found duplicated in a low proportion of normal males. These, extremely rare, AMXY patterns are identical to those generated from XXY and XYY males. Note, however, that all normal XY males with Amelogenin polymorphic duplications should readily be distinguished from XXY and XYY males by evaluation of the seven X-linked and the two pseudoautosomal markers X22 and DXYS218 included in the **Aneufast™** S1, S2 and MXY marker sets.

Duplications of STR markers

Occasionally, STRs may show trisomic Triallelic or Diallelic patterns in normal individuals. This rare phenomenon (observed in about 0.1% of prenatal samples) may be due to polymorphic duplications of the microsatellite sequence. These patterns are usually detected as Trisomy for one marker with normal results for all other informative STRs on the same chromosome.

In this situation, the **Aneufast™** chromosome- specific M21, M13 or M18 marker sets should always be used to include all available markers. Because of the presence of these polymorphisms, it is unacceptable to issue abnormal results with a single informative marker.

It is also advisable to test DNA from a blood or buccal sample from both parents to confirm the inheritance of the polymorphism. In "de novo" cases, the possibility of a partial chromosome imbalance involving the STR sequence must be excluded by cytogenetic analysis. In these cases it is important to distinguish between polymorphism of no clinical significance and duplications that may be associated with learning and/or physical disability.
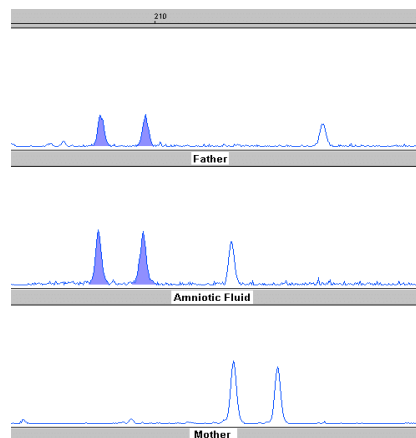
Example: The trisomic triallelic pattern observed from the amniotic fluid DNA sample is due to the inheritance of the same two STR alleles on one paternal chromosome (highlighted).

Mutations in STR primer binding sites

For primer sets included in **Aneufast™**, mutations in STR primer binding sites are extremely rare (1/15,000) ; and this complication is only included here for completeness. Such mutations may affect correct annealing of the primer to its complementary sequence; if the mismatch position corresponds to the 3' end of either forward or reverse primer, it can cause complete failure of amplification of one allele. These mutations in STR markers are generally undetectable, as they are indistinguishable from homozygosity. In trisomic cases, however, null alleles could generate a normal heterozygous pattern for an STR. For this reason alone it is advisable not to issue a normal result based on a single informative marker. After adding all chromosome- specific STRs included in **Aneufast™** M21, M13, M18 and MXY marker sets, the likelihood for a normal or trisomic sample to be found homozygous for all sequences (thus undetectable) is almost zero.

If the mutation falls in the STR primer binding site, depending on its position, it could affect the annealing (and extension) efficiency. This could result in STR profiles generally incompatible with normal (1:1 ratio) or trisomic (2:1 ratio) patterns.
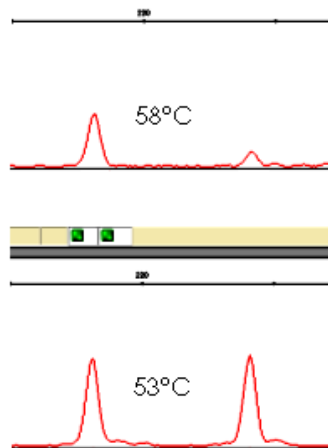
Example: Reduced efficiency of amplification of the longer allele. Repeating PCR amplification with an annealing temperature 5ºC lower may help to confirm that the unusual result is due to a mutation in the STR primer binding site.