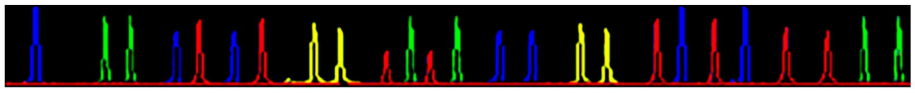
ANEUFAST™



QF-PCR

Multiplex QF-PCR Kit

For Rapid Diagnosis of Trisomy 21, 18, 13 and Sex Chromosomes Aneuploidies

User's Manual

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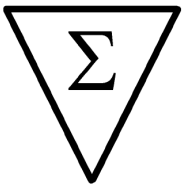

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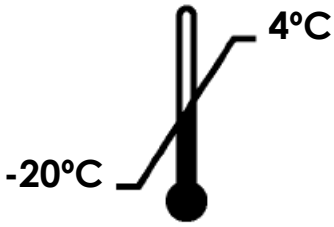


For professional *in vitro* diagnostic use only

Do not use if primary packaging is damaged

Keep Tubes Away from Direct Light

Read the User's Manual Carefully

REF mlg.anf.100

Product Overview

Purpose

The **Aneufast™** QF-PCR Kit contains six multiplex marker sets of short tandem repeats (STRs) that can be used for amplification of selected microsatellites and the Amelogenin-SRY.
This combination of markers allows the detection of aneuploidies involving chromosomes X, Y, 21, 18 and 13 with 100% sensitivity and specificity for non mosaic trisomies. **Aneufast™** is intended to be used to amplify DNA extracted from fresh prenatal samples such as Amniotic Fluids, chorionic villus samples (CVS) or fetal blood. It can also be used to analyse neonatal and adult blood or tissue samples.
Two multiplex QF-PCR sets (S1and S2) are used to perform initial Aneuploidy Diagnosis and the assays are designed to be analysed in a single electrophoresis; In addition, there are four chromosome- specific marker sets (M21, M13, M18 and MXY). They may be used as back-ups in case all the markers on S1 and S2 are non-informative (homozygous). However, they may also be applied individually for the diagnosis of trisomy 21, 13 , 18 and sex chromosome aneuploidies, respectively.

- ♦Markers included in **Aneufast™** have been extensively validated and applied on over 25.000 clinical specimens.
- ♦Additional data regarding the markers included in **Aneufast™** are retrievable in public databases accessible worldwide.

Five-Dye DNA Fragment Analysis

The **Aneufast™** QF-PCR Kit uses a five-dye fluorescent system for automated DNA fragment analysis. This allows multiplex amplification and electrophoresis of over fifteen loci simultaneously. The kit is intended for use on Applied Biosystems ABI PRISM® genetic analysis instrumentation. Fluorochromes include 6-FAM™, VIC™, NED™ and PET™ to be used in conjunction with GS 500 LIZ™ size standard (Applied Biosystems PN° 4322682)

Hot Start Taq Polymerase and optimised PCR buffer

In order to maximise specificity of Multiplex PCR, Hot Start Taq Polymerase is included in the optimised PCR reaction buffer. The enzyme is completely inactive at room temperature. This prevents mis-priming during PCR set up. Activation is obtained during the 15 min. at 95°C step before PCR cycling. This simplifies PCR set up and handling that can easily be done at room temperature.

Markers included in Aneufast™

Marker	Label	Het.	Chromosome Location	Known alleles in bp
AMXY	**6-Fam**	-	Xp22.1-22.31 - Yp11.2	X 104 Y 109
SRY	**6-Fam**	-	Yp11.2	Y 463
X22	**6-Fam**	0.91	Xq28 Yq (PAR2)	189-194-199-204-209-214-219-224-226-229-234-239-242-247-253
DXYS218	**PET**	0.65	Xp22.32 Yp11.3 (PAR1)	266-270-274-278-282-286-290-294
HPRT	**6-Fam**	0.75	Xq26.1	264-268-272-276-278-280-284-288-292-296-300-313
DXS6803	**VIC**	0.68	Xq12-Xq21.33	106-110-114-118-120-124-128
DXS6809	**VIC**	0.75	Xq	238-242-246-250-252-254-258-260-262-266-268-270-274
DXS8377	**NED**	0.85	Xq28	213-216-219-222-225-228-238-241-244-248-252
SBMA	**VIC**	0.75	Xq11.2-Xq12	166-169-172-175-178-181-184-187-190-193-196-199-202-205-208-211
D21S1414	**6-Fam**	0.85	21q21	328-330-334-338-342-346-350-352-354-356-358-360-362-443
D21S1411	**VIC**	0.93	21q22.3	246-262-266-274-278-282-286-290-294-298-302-306-316-319
D21S1446	**PET**	0.77	21q22.3-ter	200-204-208-212-214-218-220-224-228
D21S1437	**VIC**	0.78	21q21.1	120-124-128-132-136-140-144
D21S1008	**6-Fam**	0.70	21q22.1	196-200-204-208-212-216-220
D21S1412	**6-Fam**	0.73	21q22.2	384-388-392-396-400-406-410-414-418
D21S1435	**PET**	0.75	21q21	142-160-164-168-172-176-180-184-188
D18S391	**VIC**	0.75	18pter-18p11.22	144-148-152-156-160-164-168
D18S390	**VIC**	0.75	18q22.2	398-402-406-410-414-418-422-426-430
D18S535	**NED**	0.82	18q12.2	126-130-134-138-142-146-148-152-156
D18S386	**NED**	0.89	18q22.1	319-330-334-338-342-344-350-354-358-362-366-370-372-376-380-387
D18S858	**PET**	0.66	18q21.1	186-190-192-196-200-204
D18S499	**6-Fam**	0.72	18q21.32-q21.33	386-392-396-400-404-408
D18S1002	**6-Fam**	0.80	18q11.2	122-130-134-138-142
D13S631	**VIC**	0.78	13q31-32	192-196-200-204-208-212-215-218
D13S634	**VIC**	0.85	13q14.3	460-464-466-470-474-478-482-484-486-490-496-500
D13S258	**NED**	0.89	13q21	230-232-234-236-238-240-242-244-248-265-267-269-271-273-277-279-281
D13S305	**PET**	0.75	13q12.1-13q14.1	426-430-434-438-442-446-450-454-458
D13S628	**6-Fam**	0.70	13q31-q32	436-440-444-448-452-456-460-464
D13S742	**VIC**	0.75	13q12.12	254-258-262-266-268-270-274

The Heterozygosity reported in the Table refers to that in the Caucasian population. Allele sizes may vary up to 3 bp depending on the instrument and electrophoresis conditions employed. Sizes in this table have been obtained on ABI PRISM 3100-AVANT Genetic Analyser using the 36cm capillary array, POP4 polymer and GeneScan 36 POP4 default module.

About this User's Manual

This user's manual describes the following:

1- Materials and equipment required to use the Aneufast kit

2- How to use the kit to amplify DNA samples

3- How to perform automated detection

4- How to analyze results

Kit Storage

Fluorescent primers should be stored away from light.
The **Aneufast™** box is internally coated to increase light protection.

Aneufast™ is stable for up to one year if stored at -20°C.
PCR mixes can be stored as ready to use aliquots in PCR tubes at -20°C; this will keep freeze thaw cycles to a minimum reducing the risk of contamination and shortening QF-PCR set-up

1-Materials and equipment required to use the Aneufast™ kit

1.1 Laboratory Design

PCR amplification using fluorescently labelled primers is sensitive enough to amplify single target sequences. Thus particular care must be taken to avoid contamination. It is important to organise separate DNA extraction, PCR and analysis areas in the Lab. The main potential source of contamination is amplicons generated in previous runs. The PCR area should be dedicated to DNA extraction, Kit handling and PCR set up only.

PCR Set Up Area

IMPORTANT: The following items should never leave the PCR Setup Work Area:

♦Calculator
♦Gloves, disposable
♦Marker pen, permanent
♦Microcentrifuge
♦Microcentrifuge tubes, 1.5-mL, or 2.0-mL, or other appropriate clean tube
♦Microcentrifuge tube rack
♦Heated blocks or water baths
♦Pipette tips, sterile, disposable hydrophobic filter-plugged
♦Pipettes
♦Vortexer
♦ Thermalcycler

Work area for Amplified DNA

♦ABI Generic Analyser compatible with Five-Dyes Detection
♦ Heated block
♦ Sequencer disposables and consumables
♦Pipette tips, disposable hydrophobic
♦Pipettes
♦Vortexer

2- How to use Aneufast™ kit to amplify DNA samples by QF-PCR

2.1 DNA extraction

2.1.1 Background

QF-PCR is based on the assumption that within the early exponential phase of amplification, the amount of product is directly proportional to the amount of the target sequence present in the initial template. Crucial for the success of the assay is the amount of DNA used in relation to the number of amplification cycles.
Aneufast™ is optimised to work on low amounts of DNA such as small aliquots of freshly collected prenatal samples such as amniotic fluids, CVSs or fetal blood. However it can also be used to analyse DNA extracted from neonatal and adult blood or tissue samples, including buccal cells.
The suggested DNA extraction procedure allows similar DNA concentrations to be obtained on different samples, so that QF-PCR can be carried out in the same conditions.

2.1.2 Prenatal Samples

Fresh samples should be handled by trained staff and only small aliquots should be fractioned in Eppendorf tubes for DNA extraction and molecular diagnosis. The rapid test has been developed as a preliminary to conventional cytogenetic analysis. Therefore ideally the volume of amniotic fluid should not exceed 1.5 ml in order to avoid affecting cell culture. CVS samples must be prepared under inverted microscope by expert staff in order to carefully remove all maternal contaminating tissues and cells which could interfere with prenatal QF-PCR diagnosis. After centrifugation, all samples must be carefully inspected to exclude the possible presence of contaminating maternal blood cells. A full record of this must be kept until completion of study. For amniotic fluids, it is possible to analyse samples containing about 20% of visible blood in the cell pellets without noticing extra STR alleles in the QF-PCR profiles. Heavily bloodstained amniotic fluids should not be used for QF-PCR diagnosis, unless special precautions are undertaken to identify the source of the blood contamination, either maternal or fetal. It is possible to confirm or exclude the fetal origin of the predominant cell population if a maternal sample is also analysed and STR profiles compared.
For CVS, it is recommended to extract DNA from a small aliquot of cell suspension as prepared for cell culture or, in alternative, to analyse two small villi independently. This will reduce the risk of misinterpretation in cases of mosaicism.

Quick DNA extraction from a small number of cells can be achieved by incubating cell pellets in the presence of a chelant reagent (Chelex 100). This can be purchased as ready to use InstaGene™ Matrix from BIO-RAD (cat. N°732-6030). This approach permits the addition of a Chelex volume appropriate to the number of cells. Thus a similar DNA concentration from different samples is obtained (Figure 1). Amniotic fluids at various gestational ages (e.g 14 and 20 weeks), CVSs or fetal and neonatal bloods can then be amplified under the same QF-PCR conditions. Furthermore, the whole procedure is performed in the same tube, thus greatly reducing the risk of mishandling, particularly when several samples are processed at the same time.

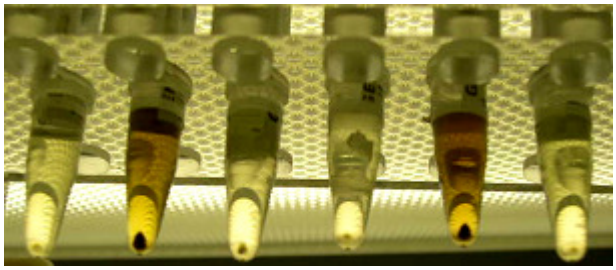


Figure1a: Cell pellets obtained by centrifugation of Amniotic Fluid Samples at different gestational ages (15-17 weeks). Note the heterogeneity in the amount of cells in the different pellets.

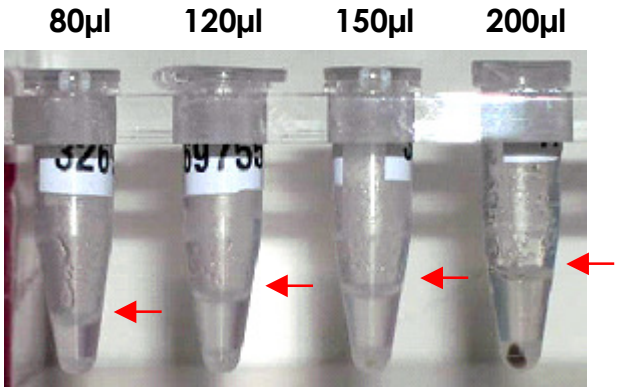


Figure1b: DNA at similar concentration can be obtained by adding different

volumes of Chelex depending on the amount of cells. The volume could vary between 50 µl (almost invisible pellets) to 350 µl (big AF pellets or 2-3mm CV fragment)

2.1.3 Neonatal and Adult Samples

Aneufast™ can be used to analyse samples collected from newborns and adults. In both cases not only blood but also for example buccal cells (either obtained by mouthwashes, mouthbrushes or using cotton swabs) are suitable for DNA extraction and QF-PCR amplification. For this purpose 0.5ml cell suspension should be used according to the protocol below.
If heparinised peripheral or fetal (cord) blood samples are withdrawn, 5µL aliquots should be used according to the protocol below.

2. 2 Protocol

This procedure is suitable for 0.2-1.5ml uncultured amniotic fluid, 100µl amniotic fluid cell culture, 5µL fetal or peripheral blood, 0.5 ml buccal cells or ≈ 0.2 mg of different fetal and adult tissues including Chorionic Villi.

- Keep Chelex resin in suspension on a magnetic stirrer.

1- Spin the sample in an Eppendorf tube for 5 minutes at 13,000 r.p.m.
2- Remove supernatant.

For Blood samples and heavily bloodstained amniotic fluids include red cell lysis and washing steps:

2.1- Add 1 ml H_2O to the cell pellet and vortex.
2.2- Incubate at room Temperature for 2 minutes.
2.3- Spin the sample in Eppendorf tube for 5 minutes at 13,000 r.p.m.
2.4- Remove supernatant; add 1ml H_2O and vortex.
2.5- Spin the sample in Eppendorf tube for 5 minutes at 13,000 r.p.m.
2.6- Repeat steps **2.4**& **2.5**.
2.7- Remove supernatant.

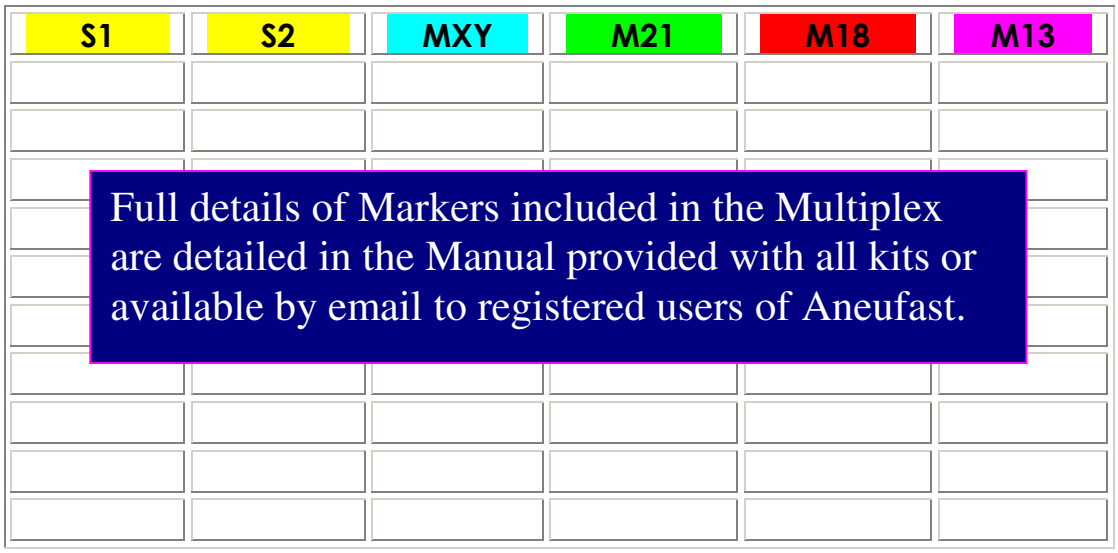
For clear Amniotic Fluids, CVSs, tissues and buccal cells proceed directly to the following steps:

3- Depending on the amount of cells add 50-350µl of Chelex to the pellet using a large bore tip.
4- Vortex 10 seconds.
5- Incubate for 8 minutes at 99°C or boiling water bath.
7- Vortex 10 seconds.
8- Spin in centrifuge 2 minutes at 13,000 r.p.m.
9- PCR ready single strand DNA is contained in the supernatant.

Carefully remove the supernatant for PCR without disturbing the resin pellet. Extracted DNA can be stored at 4°C for up to one week or until completion of the QF-PCR tests. Longer storage should be at -20°C.

2.3 Markers amplified with the Aneufast™ QF-PCR Kit

The markers included in each of the six sets are shown in the table below:



S1/ S2

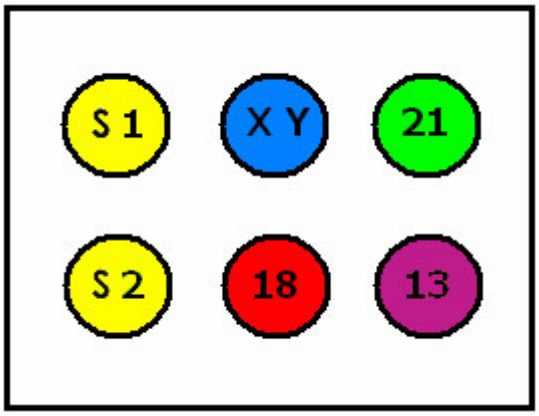
The two Multiplexes QF-PCR Sets S1 and S2 allow simultaneous analysis of four STRs on each of the autosomes 21, 18 and 13 in addition to sexing, using the two pseudoautosomal (X22 and DXYS218) STRs together with Amelogenin (AMXY) and SRY. These non -polymorphic sequences for sexing (AMXY and SRY) are independently amplified. Following collection of the products and simultaneous electrophoretic analysis, results from the S1 and S2 marker kits should be in agreement.

MXY, M21, M18 and **M13**

Chromosome- specific back-up marker sets are also available. MXY contains five STRs and two sexing markers on the sex chromosomes. M21, M13 and M18 contain five STRs on each of chromosomes 21 and 18, and four markers on chromosome 13. The back-up sets may be used either independently or in cases where all the S1 and S2 markers on any one of these chromosomes have been found to be uninformative (homozygous).
Extra markers not included in S1 and S2 are labelled*. Note that in each chromosome- specific set two markers amplified in S1 and S2 are repeated. This provides an opportunity to confirm sample identity. Any discrepant

results with respect to these markers, shared in common between the S1/ S2 and the chromosome-specific back-up marker sets, should be a matter of concern. The chromosome- specific back-up sets may also be used to confirm any abnormal results. The inclusion of more markers increases the accuracy of the diagnosis.

Aneufast™ QF-PCR Kit components



<u>S1/ S2 Sets:</u> Ready to use mixes for 100 reactions each

<u>XY, 21, 18, 13 Sets:</u> Ready to use mixes for 10 reactions each

2.4 PCR set up Protocol

Thaw vials and mix thoroughly by vortexing a few seconds.
Aliquot PCR Mix in each PCR tube in accordance with the table below:

Multiplex PCR Mix	10 µl
DNA	1-10ng
H_2O	up to 15 µl
Final PCR volume	**15 µl**

Aneufast™ mixes can be stored as ready to use 10 µl aliquots in PCR tubes DNA volume can vary between 1 and 5µl. If DNA is extracted following the suggested protocol, 4 µl should be used for PCR. H_2O must be added to the mix before aliquoting in accordance with the table below:

Multiplex PCR Mix	10 µl
H_2O	1 µl
Aliquot per tube	11 µl

<u>Warning</u>: In order to avoid possible contamination, Aneufast™ mixes must be aliquoted in the PCR Area with dedicated pipettes and filtered tips. One

drop of mineral oil on each PCR tube will also reduce the risk of contamination by amplicons generated in the previous PCR.

2.4.1 Performing PCR

<u>Warning</u>: According to good laboratory practice internal quality control samples of known genotype should be processed in each assay to assess the effectiveness of the procedure

<u>Hot Start Taq Polymerase</u>

In order to increase the PCR specificity, Hot Start Taq Polymerase is included in the reaction buffer. The enzyme is totally inactive at room temperature. This allows easy set up of PCR reaction without ice. Activation is achieved with 15 min. hold at 95°C.

1- Program the Thermalcycler according to the following parameters:

Taq Activation	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**Storage**
Hold	**25-28 Cycles**			**Hold**	**Hold**
95°C 15 min.	95°C 40 sec.	60°C 1 min. 30 sec.	72°C 40 sec.	60°C 30 min.	4-20°C ∞

2- Place tubes in Thermalcycler and close the lid.
3- Start the PCR.

Using the suggested DNA extraction procedure and volume, efficient amplification is carried out for 28 repeating cycles. For different DNA extraction and amounts, the optimal PCR cycle number should be worked out in order to keep amplification within its exponential phase.

4- PCR products are stable at room temperature overnight, longer storage before electrophoresis should be at 4°C.

Warning: After PCR is complete, tubes should never be opened in the PCR set up area. This is essential in order to avoid contamination at any future PCR amplification.
Particular care should be taken in disposing amplified products according to good laboratory practice and local legislation.

3- How to perform automated electrophoresis and detection

Aneufast™ is designed to be used in conjunction with Applied Biosystems Genetic Analysers supporting Five-Dye Data Collection.

3.1 Software requirements for Five-Dye Data Collection

ABI Collection™
Make sure your Applied Biosystems Data Collection™ Software supports Five-Dye data for DNA fragment analysis applications. Refer to the Genetic Analyser User's Manual.
Additionally, a matrix file or spectral calibration should be generated using the 6-FAM™, VIC™, NED™, PET™ and LIZ™ matrix standards (DS-33) according to the Genetic Analyser instructions.

3.2 Running Samples

Warning: Amplified products should be handled in the analysis area with dedicated pipettes and tips to avoid contamination in successive PCR amplifications.

3.2.1 Preparing samples for Electrophoresis

GeneScan™-500 LIZ™ Size Standard (ABI P/N 4322682) should be used with **Aneufast™**.

1- In a 1.5ml tube, prepare the necessary amount of size standard for all the samples to be analysed by combining:
- 40µl Hi-Di™ Formamide (ABI P/N 4311320)
- 0.3 µl GeneScan™-500 LIZ™
This mix can be prepared in excess and kept stored at 4°C.
2- Use 40 µl of this mix to inject
1,5 µl of each **Aneufast™** S1 and S2 products collected in the same tube.
3- Use 20 µl of this mix to inject
1,5 µl of each **Aneufast™** Chromosome M21, M18, M13, MXY back-up marker sets.
4- Denature the sample tubes/plate with Formamide and Size Standard for 2 minutes at 95°C.
5- Load samples on the Genetic Analyser according to the User's Manual.

3.2.2 Capillary Electrophoresis

The Aneufast™ QF-PCR Kit generates amplicons between 105 and 490 bp, which are efficiently separated by electrophoresis through 36cm capillaries, using standard microsatellite modules.
Refer to the ABI PRISM™ Genetic Analyzer and Data Collection Software User's Manual for detailed information on polymer, software and set up for Five Dye microsatellite analysis on your instrument.
Aneufast Run Modules for compatible versions of Data Collection Software can be downloaded from www.aneufast.com or www.qf-pcr.com

1- Create a Five-Dye sample sheet using the Data Collection Software.
2- Select the appropriate run module.
3- Start the Run.
Note: Injection time and/or voltage can be adjusted to the amount of PCR product. Increasing/decreasing of the injection time/voltage will allow more or less products to run through the capillary. Amplified products can be reinjected and re-analysed several times.

4- How to analyse results

4.1 Analysis Software

Applied Biosystems fragment analysis software suitable to your genetic analyser should be used with **Aneufast™**. The QF-PCR kit is compatible with GeneScan™ Analysis version 3.1 or higher and all versions of GeneMapper™. GeneMapper™ panels, bins and analysis method for automated peak labelling are available to download on our web site www.aneufast.com.
Refer to the ABI PRISM GeneScan® Analysis Software or GeneMapper user's manual for detailed information on importing collection data, setting up analysis parameters and analysing results.

4.2 Analysis of QF-PCR products

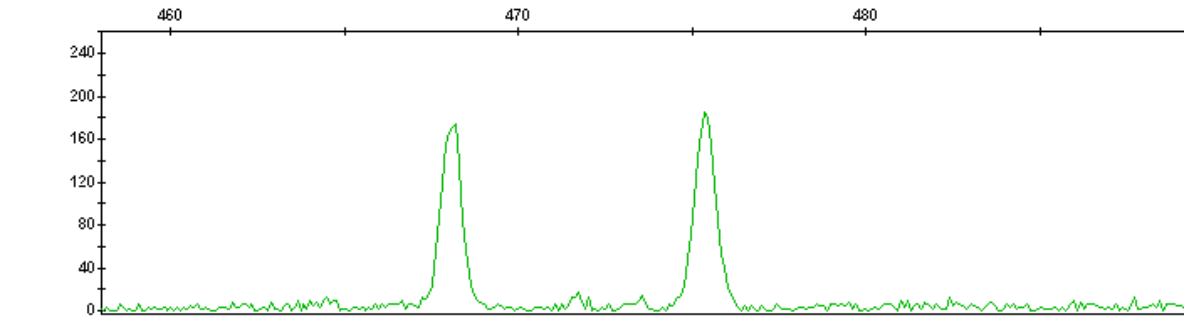
4.2.1 Overview

In the great majority of cases the analysis of **Aneufast™** QF-PCR products is straightforward providing rapid and unequivocal results after the S1 and S2 analysis. However, sometimes results may be puzzling. This could be due to the underlying biology (such as mosaicism with different chromosome constitution in different cell lines), or amniotic fluid samples contaminated by maternal blood; this type of problems are illustrated and discussed in details in the troubleshooting section on www.aneufast.com.

Each marker is identified by the size and colour of the corresponding amplicons. Allele size range is shown in the Overview; markers with alleles of similar size are labelled with different fluorochromes. FAM, VIC, NED and PET dyes are used to label primers; these fluorochromes are detectable respectively as Blue, Green, Yellow-Black and Red on the electrophoretograms. LIZ dye (Orange) is only used for the Size Standard, which undergoes electrophoresis together with the QF-PCR products.
Once the **Aneufast™** panel and bin set have been downloaded (or generated), GeneMapper software can be used for automated identification and analysis of the PCR product; Refer to the GeneMapper User's Manual for detailed information on how to perform automated analysis.

4.2.2 The principle of QF-PCR

QF-PCR amplification of STR markers generates a fluorescent product that is directly proportional to the amount of target sequence present in the initial template.
The amount of fluorescent PCR product is a numerical value corresponding to the area of the peaks in an electrophoretogram. The peak height is also a measure of fluorescent activity. Thus it is directly proportional to the amount of fluorescent products. The results window of ABI analysis software shows electrophoresis results (electrophoretograms) and generates tables, showing all relevant information.
The figure below highlights the most important data to be taken into account in analysing Aneufast™ products.

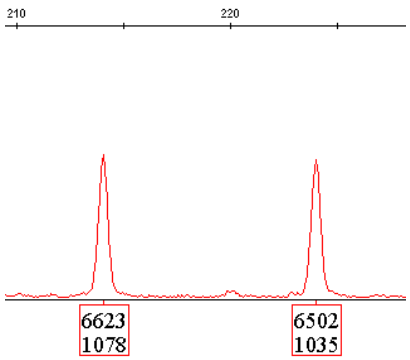


	Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
	G,22	_D02.fsa	D13S634	468	468.18	174	1426	5819
	G,23	_D02.fsa	D13S634	475	475.34	186	1460	5893

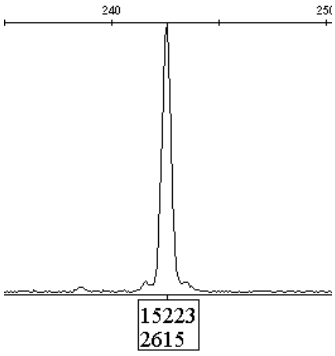
Size is the length of the amplicons in bp. Area and Height are absolute values, measuring fluorescent activity and therefore the amount of the PCR product.

4.2.3 Detection of Normal Disomy

In normal individuals heterozygous for the STRs, the same amount of fluorescence is generated for both alleles. Therefore, the ratio between the area (and height) of the fluorescent peaks is 1:1 (see figure).
In homozygous individuals STR alleles have the same repeat number and size, therefore quantification is not possible and the marker is uninformative.



Normal Heterozygous
1:1



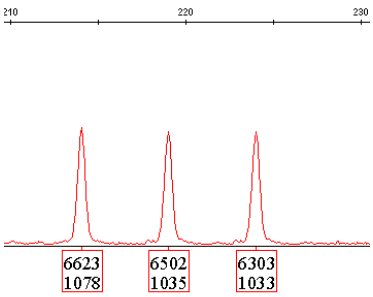
Homozygous
Uninformative

Allele plots generated by GeneMapper 3.7.
Peaks are labeled with Area (top) and Height (Bottom).

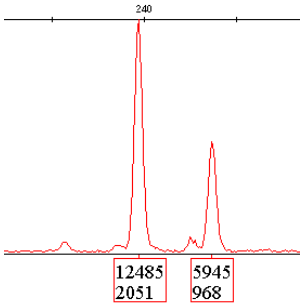
Samples with a normal copy number for a given chromosome will show heterozygous or homozygous patterns for all the STRs used. Assessment of normal copy number should be based on at least two informative markers on each chromosome

4.2.4 Detection of Trisomy 21, 18, 13 and Triploidy

In a trisomic sample, the three copies of a chromosome can be detected with the corresponding chromosome- specific STRs as three peaks having the same fluorescent intensity and a ratio between the areas of 1:1:1 (Trisomic Triallelic).
If two chromosomes have the same repeat number, quantitative PCR will produce two unbalanced fluorescent peaks with an area ratio of 2:1 (Trisomic Diallelic). Triploid samples will produce trisomic diallelic and triallelic patterns for informative STRs on all chromosomes.



Trisomic Triallelic
1:1:1



Trisomic Diallelic
2:1

Trisomic Samples will produce trisomic Triallelic and Diallelic or homozygous patterns for all markers on the same chromosome. The diagnosis of Trisomy is acceptable if at least two markers on the same chromosome have trisomic patterns being the others homozygous.

Due to the occasional preferential amplification of the smaller allele, the ratios between fluorescent peaks may vary within limits shown in the table below.

STR Peak Ratio	Interpretation
0.8 - 1.4 : 1	Normal
≤ 0.6 – ≥1.8 : 1	Trisomy
1.6 : 1 for alleles differing ≥ 20b.p.	Normal

Ratio Ranges within STR alleles.

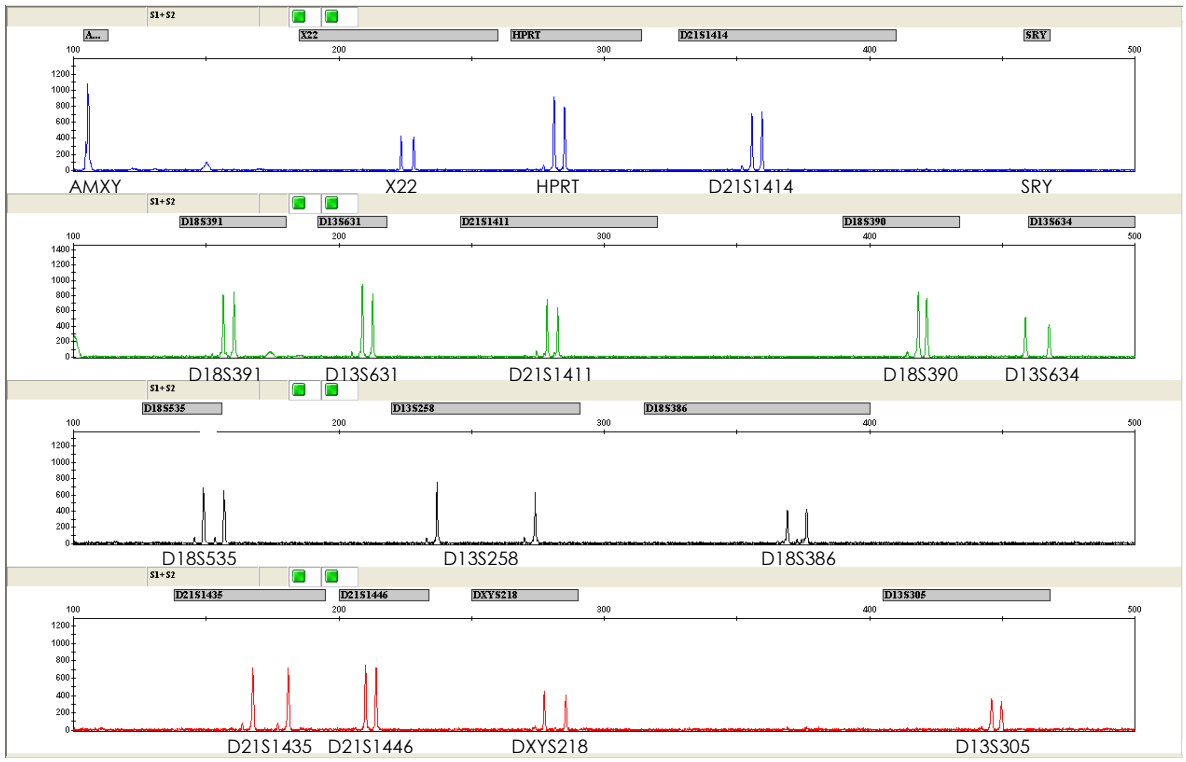
Ratios are calculated by dividing the area of the smaller by the area of the longer allele. Occasionally, STR alleles differing by more than 20 bp in length may generate ratios outside the normal values. This is due to preferential amplification of the smaller PCR product. If at least two more informative STRs are available in the same PCR within the normal range, this result can be considered a PCR artefact. If all other markers on the same chromosome are homozygous uninformative, the **Aneufast™** chromosome-specific marker set M21, M13 or M18 should be used to add more markers and confirm the result.

4.3 Analysis Examples

4.3.1 Detection of normal chromosome complement

Example 1

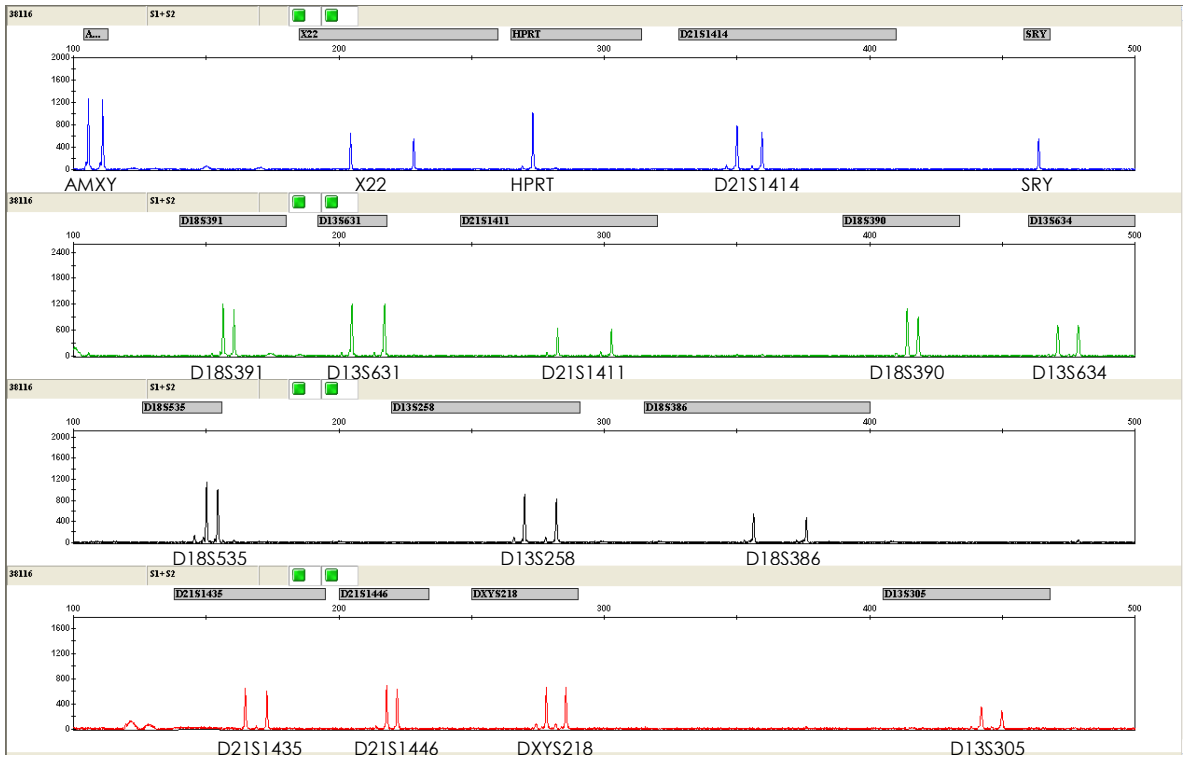
GeneMapper 3.7 electrophoretogram showing **Aneufast™** S1 and S2 detecting a normal XX female sex chromosome constitution.



Only the X- specific product of the AMXY is present and SRY is not amplified. Both pseudoautosomal markers (X22 and DXYS218) and the X-linked HPRT are normal heterozygous, reflecting a normal XX sex chromosome complement. Four markers on chromosome 21 (D21S1414, D21S1411, D21S1446, D21S1435), 18 (D18S535, D18S391, D18S386, D18S390) and 13 (D13S631, D13S634, D13S258, D13S305) are normal heterozygous, confirming the presence of normal chromosome copy number for these autosomes.

Example 2

Electrophoretogram showing **Aneufast™** S1 and S2 detecting a normal XY male sex chromosome constitution.



Both the X- and Y- specific products of the AMXY are present with a normal ratio of 1:1. The XY male sex chromosome constitution is confirmed by the occurrence of the SRY product. In this example, the presence of two sex chromosomes is also further confirmed by the normal heterozygous pattern of both pseudoautosomal markers X22 and DXYS218. The four markers on chromosome 21 (D21S1414, D21S1411, D21S1435 and D21S1446) are normal heterozygous with a ratio of 1:1between the two fluorescent peaks, and the same patterns are seen for D18S391, D18S390, D18S535 and D18S386 on chromosome 18. The markers on chromosome 13 (D13S631, D13S634, D13S258 and D13S305) are also normal heterozygous.

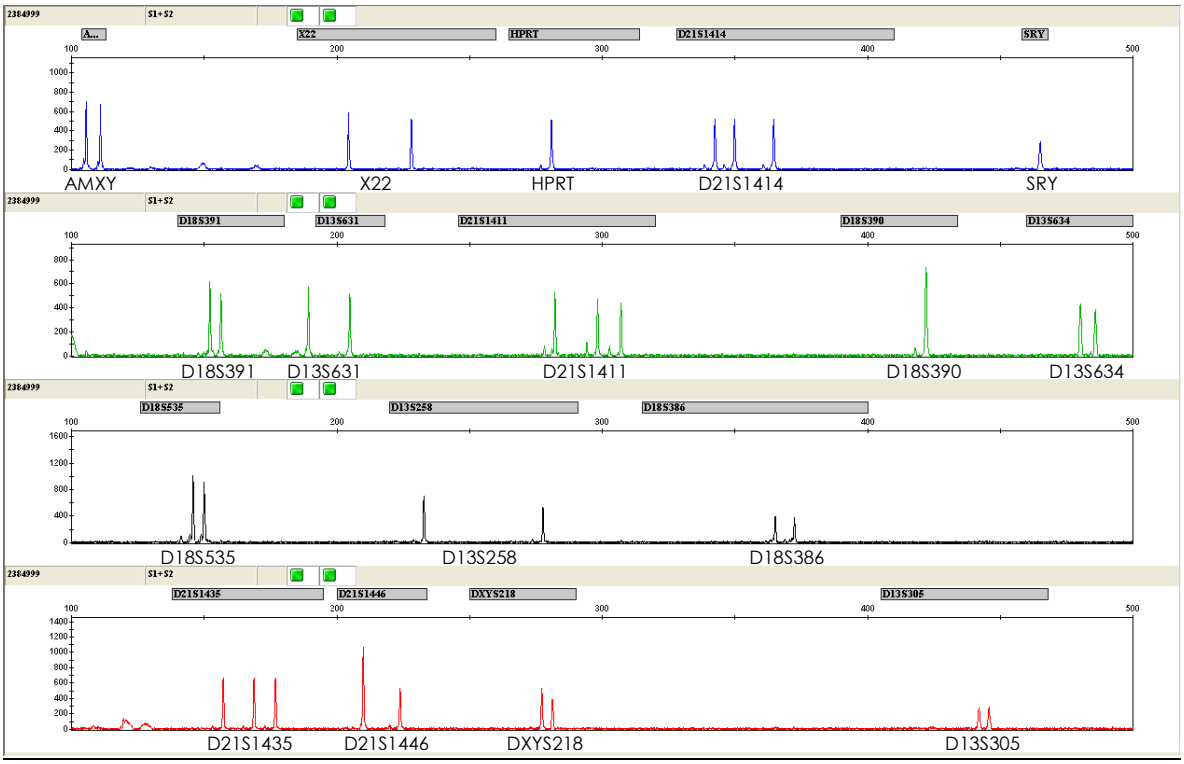
Important Note:

Diagnosis of normal samples is acceptable if at least two markers on each chromosome have clear heterozygous patterns within the normal range. In cases where only one marker is uninformative with an apparent normal result, extra STRs should be added by using the corresponding back-up chromosome- specific **Aneufast™** marker set. The inclusion of at least six markers on one chromosome should provide results for almost all cases. After adding these extra markers, rare samples heterozygous for only one sequence may be reported as normal.

4.3.2 Detection of Autosomal Trisomies and Triploidy

Aneufast™ can identify not only Trisomy 21, Trisomy 18 and Trisomy 13 but also Triploidy (69,XXX or 69,XXY)

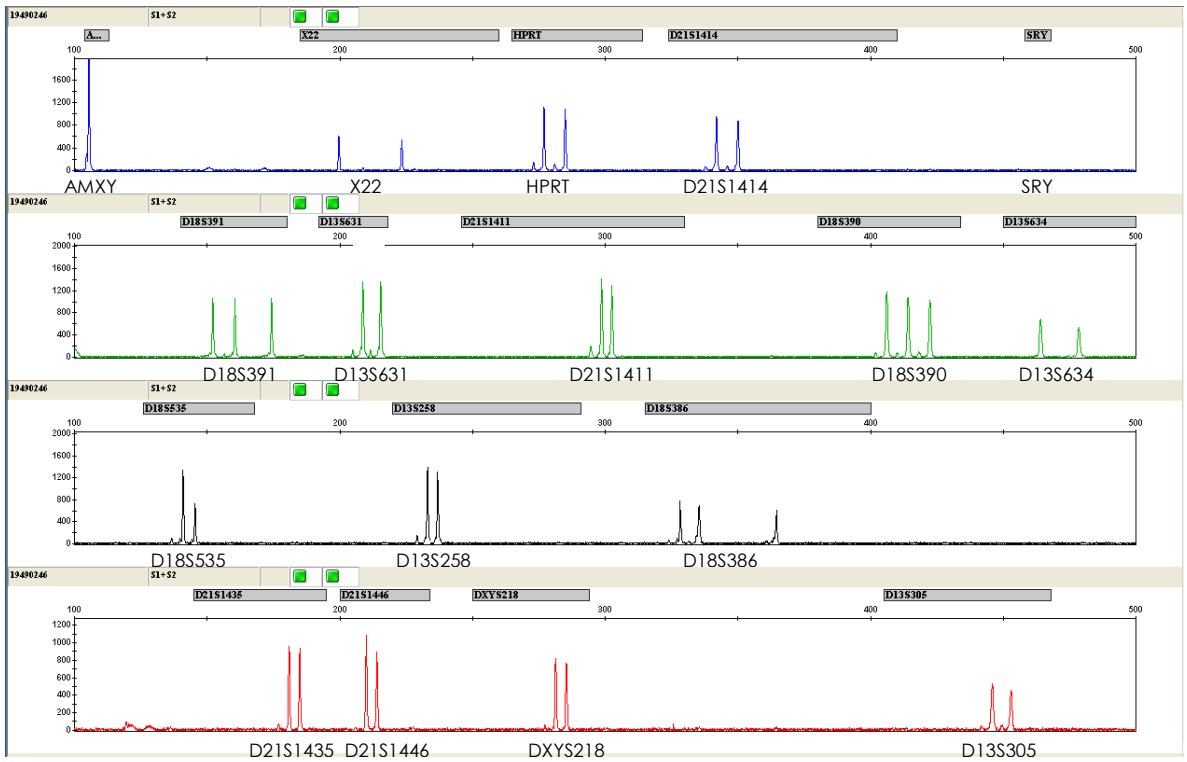
Example 3
Detection of Trisomy 21



Three markers on chromosome 21 show trisomic triallelic patterns (D21S1414, D21S1411, D21S1435), D21S1446 is trisomic diallelic. All four STRs on chromosome 13 and three markers on chromosome 18 are informative for the normal disomic chromosome complement. Both the X- and Y- specific products of the AMXY are present with a normal ratio of 1:1 together with SRY- specific PCR product. The XY male chromosome constitution is also confirmed by the normal heterozygous pattern of both pseudoautosomal markers (X22 and DXYS218) and the single product for the X- linked HPRT.

Example 4

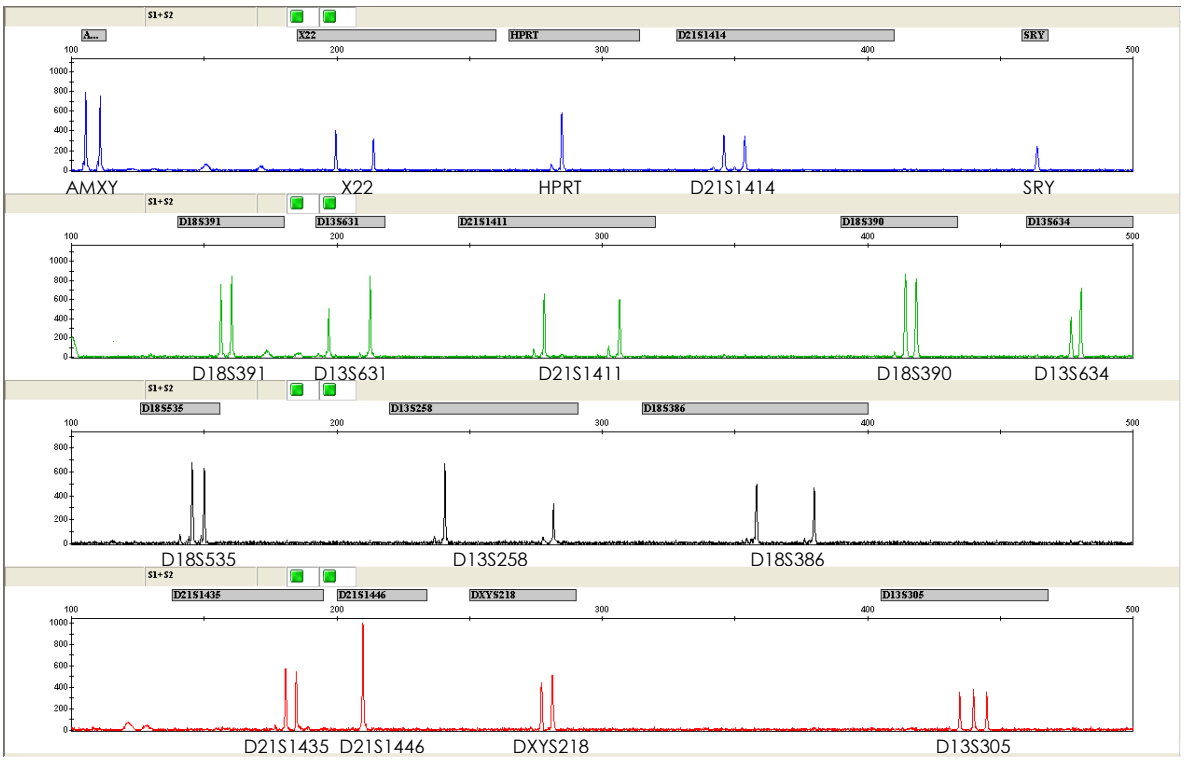
Detection of Trisomy 18



In this example, Trisomy 18 is identified as a trisomic triallelic pattern for D18S391, D18S390 and D18S386; the other marker on this chromosome is trisomic diallelic (ratio of 2:1 for D18S535). All markers on chromosomes 21 and 13 are heterozygous disomic normal. The female XX sex chromosome constitution is determined by the occurrence of the X- specific product of AMXY only, in absence of the SRY product and also by the heterozygous pattern of the X -linked HPRT as well as the two pseudoautosomal markers (X22 and DXYS128).

Example 5

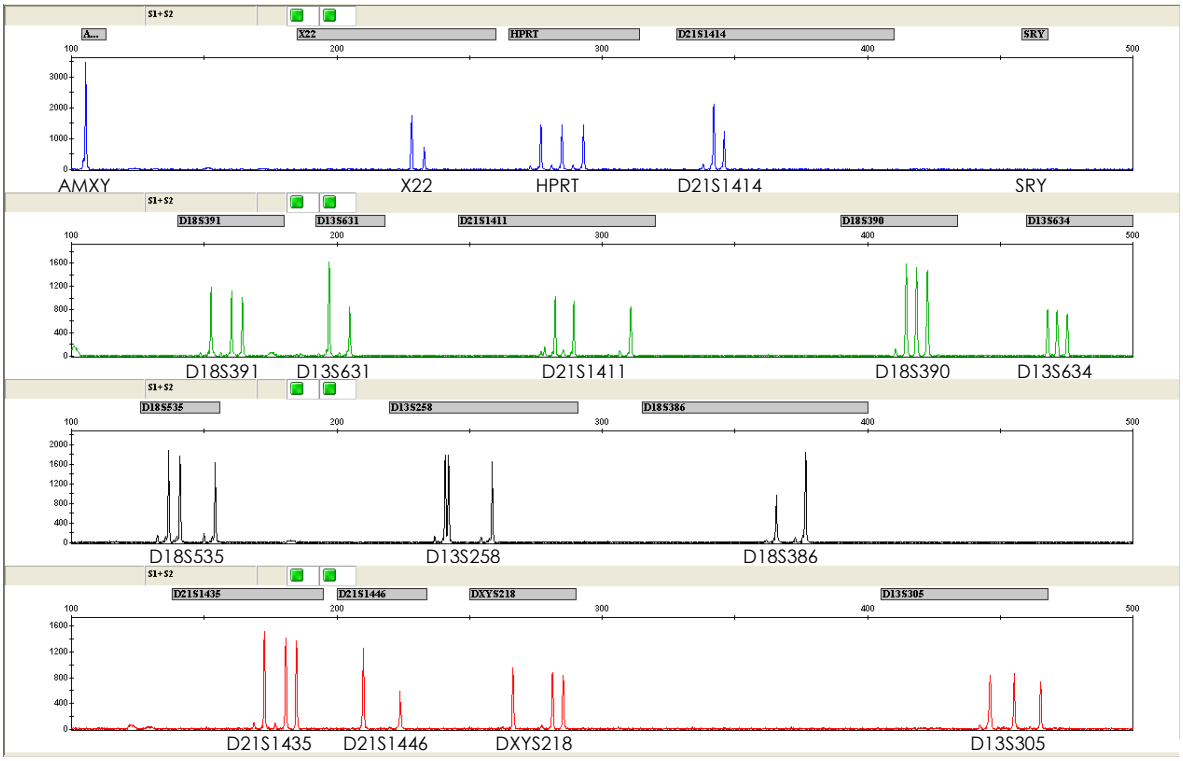
Detection of Trisomy 13



In this example Trisomy 13 is detected due to the trisomic diallelic pattern for D13S631, D13S634 (ratio 1:2) and D13S258 (ratio 2:1), while D13S305 is trisomic triallelic. Three out of four markers on chromosome 21 and all markers on chromosome 18 are informative, showing a normal chromosome copy number (ratios 1:1). The XY male sex chromosome constitution is identified by the occurrence of both the X- and Y- specific products of AMXY (with a normal ratio of 1:1) in addition to the SRY product. The normal male sex chromosome constitution is confirmed by the heterozygous pattern of both the two pseudoautosomal markers X22 and DXYS128 and the single X-linked HPRT allele.

Example 6

Detection of Triploidy



Electrophoretogram showing **Aneufast™** S1/S2 detecting the 69, XXX chromosome constitution. There is only a single X- specific product of AMXY with the absence of Y products. Three X chromosomes are detected as trisomic diallelic pattern for the pseudoautosomal marker X22 and the trisomic diallelic profile for the X-linked HPRT and DXYS218. All four markers on chromosomes 21, 18 and 13 are also indicative of trisomy for these chromosomes.

Important Note:

Aneufast™ S1/ S2 include four STRs on each autosome. Diagnosis of Trisomy 21, 13 or 18 should be based on at least two informative markers with clear trisomic patterns on the respective chromosome. In cases where only one marker shows a trisomic pattern (the remaining three being homozygous) **Aneufast™** chromosome- specific back-up marker sets M21, M13 or M18 should be used to add more STRs. Suspected trisomies indicated by a single marker should not be reported. In the unlikely event of the back-up marker set also being uninformative, alternative methods such as cytogenetic analysis should be used to confirm the suspected abnormal result.

Following initial aneuploidy detection with the **Aneufast™**S1/S2 kit, sample identity should always be confirmed by retesting the sample. In these cases

the use of the chromosome- specific extra marker sets M21, M13 and M18 will also allow more STRs to be assessed.

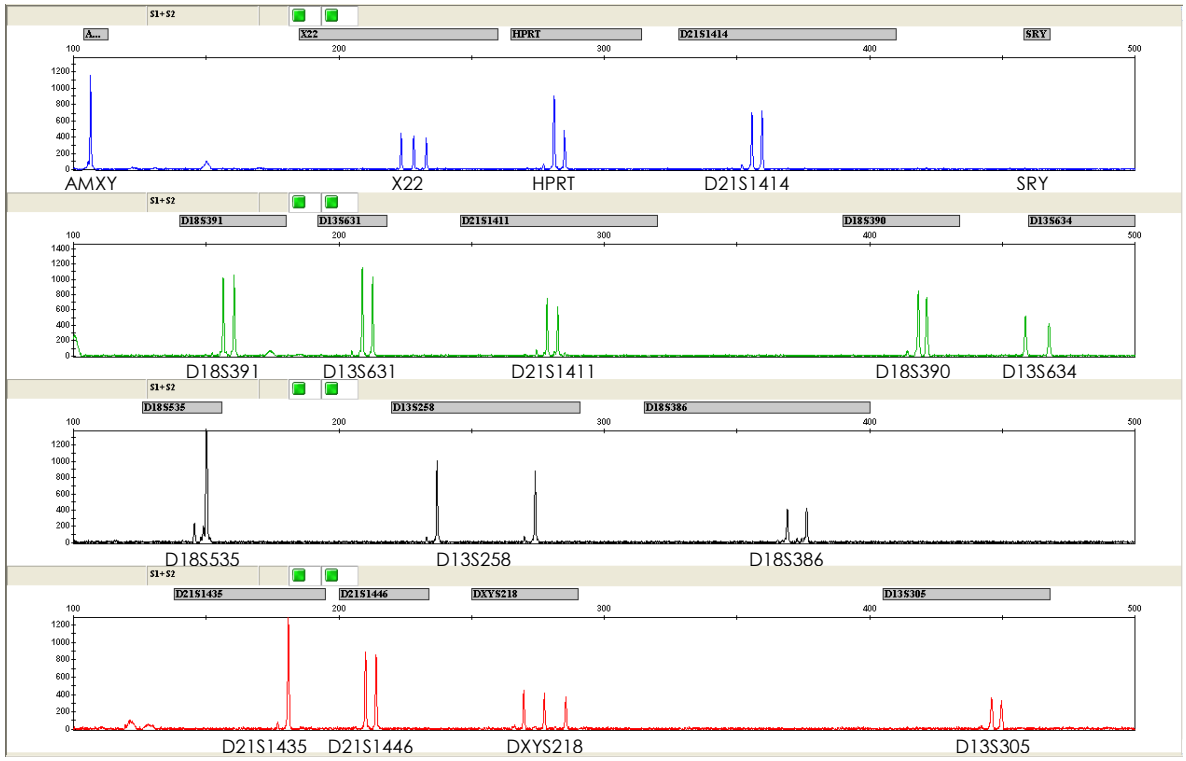
4.3.3 Aneufast™ Chromosome- Specific back-up marker sets

Extra markers not included in S1/S2 are highlighted. Note that in each of the multiplex marker set M21, M13 and M18, two STRs amplified in S1/ S2 are also included. This allows confirmation of the identity of the sample.
From top to bottom:
Results of the chromosome 21- Specific back- up marker set **M21** used for detecting Trisomy 21. Note the triallelic results for three of the markers and the trisomic diallelic (1:2 and 2:1) result for the other two markers.
Results of the chromosome 18- Specific back- up marker set **M18** used for detecting Trisomy 18. Note the triallelic result for one STR (with reduced height of the longer allele) and the 1:2 or 2:1 trisomic diallelic result for the other four markers.
Results of the chromosome 13- Specific back- up marker set **M13** used for detecting Trisomy 13. Note the triallelic trisomic result for one STR and the trisomic diallelic (1:2 and 2:1) result for the other three markers.

4.3.4 Detection of Sex Chromosome Aneuploidies

Example 7

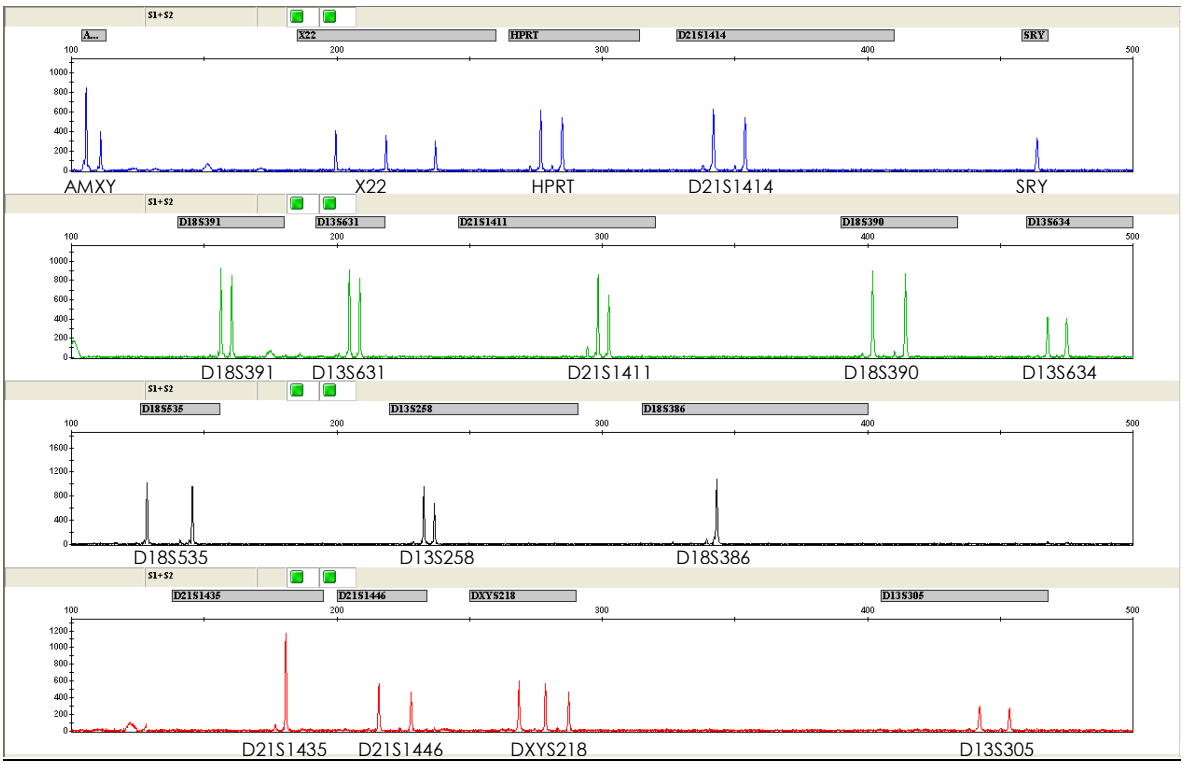
Detection of Trisomy X



A female sex chromosome constitution is detected as a single X chromosome- specific peak of the AMXY in absence of the SRY product. In this example, the three doses of X chromosome are detected as trisomic patterns of the two pseudoautosomal STRs X22 and DXYS218. The X-linked HPRT is trisomic diallelic. The normal chromosome 21 and 18 copy number is detected with three out of four markers in a ratio of 1:1. Four markers on chromosome 13 are also informative, indicating a normal disomic chromosome constitution. Using the **Aneufast™** MXY chromosome- specific marker set, four more X-linked markers are available to confirm the initial Trisomy X result.

Example 8

Detection of the XXY sex chromosome constitution

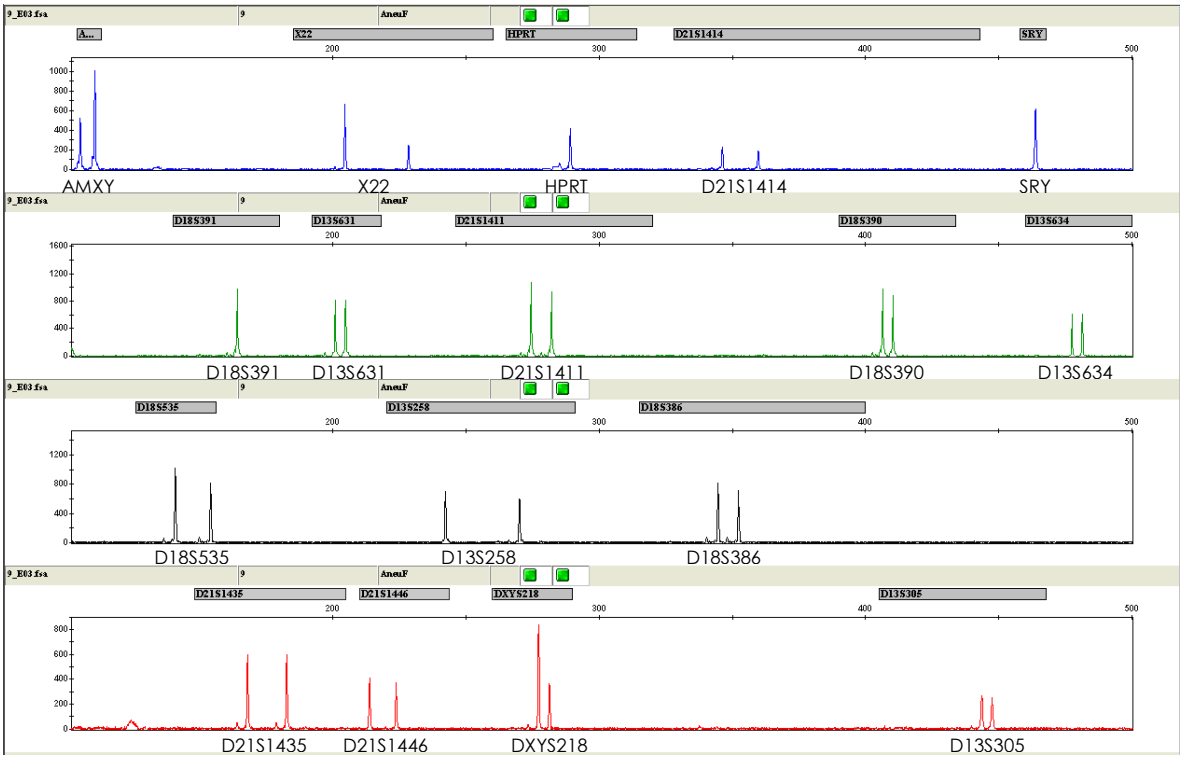


The X- specific product of AMXY is in double dose, compared to the Y (ratio of 2:1). Three sex chromosomes are detected as trisomic triallelic patterns of both the pseudoautosomal markers X22 and DXYS218. The presence of two X chromosomes is further confirmed by the heterozygous pattern of the X-linked HPRT marker.

Three out of four markers on chromosomes 21 and 18 as well as all markers on chromosomes 13 are informative, indicating the normal disomic copy number. Four more markers on the X are available in the Aneufast™ MXY chromosome- specific marker set to confirm the initial XXY result.

Example 9

Detection of the XYY sex chromosome constitution



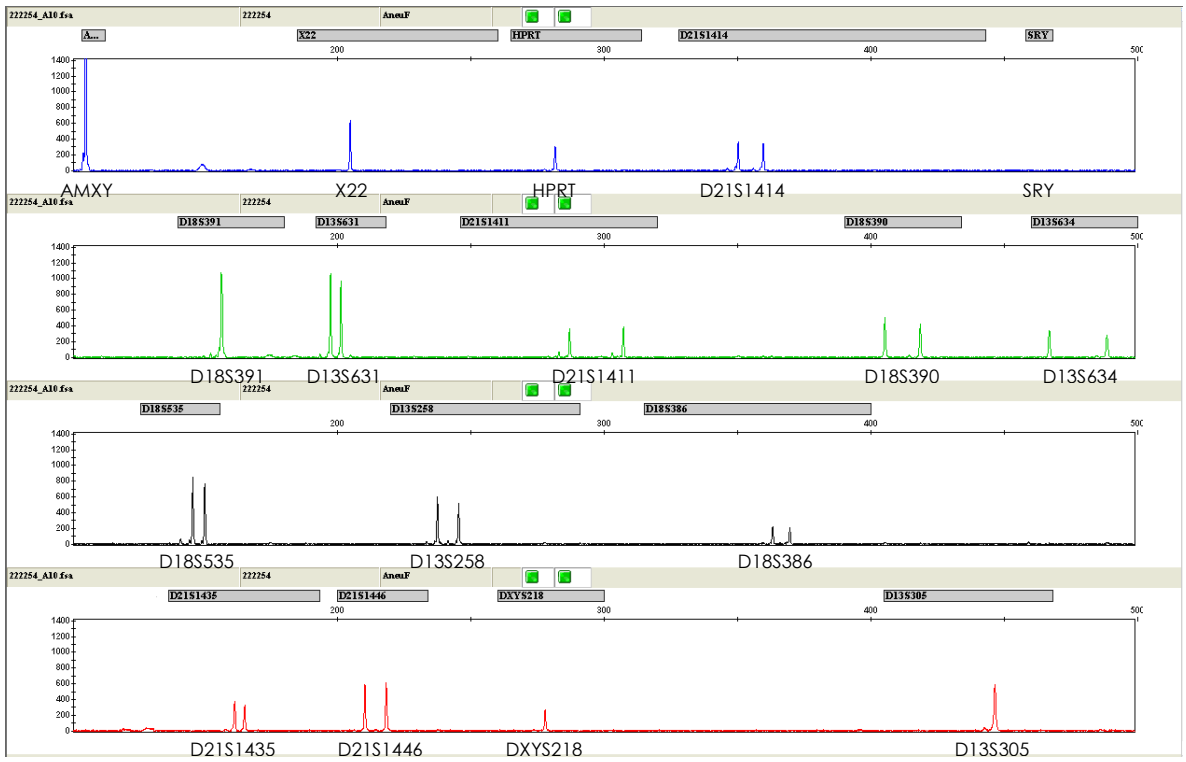
The Y- specific product of the AMXY occurs in double dose, compared to the X-specific (ratio of 1:2). The presence of three sex chromosomes is confirmed by the trisomic diallelic pattern for the two pseudoautosomal X22 and DXYS218 markers (ratio 2:1). The SRY product is not quantifiable and only confirms the presence of chromosome Y. In this example, four markers on chromosomes 21 and 13 as well as three on chromosome 18 are informative indicating a normal disomic constitution for these chromosomes.

Important Note:

Polymorphic duplications and deletions of the Y-specific product of the Amelogenin have been described. Thus, all XYY results should be confirmed by the two pseudoautosomal STRs X22 and DXYS218; these are the only suitable markers. If informative, they should produce trisomic diallelic patterns.

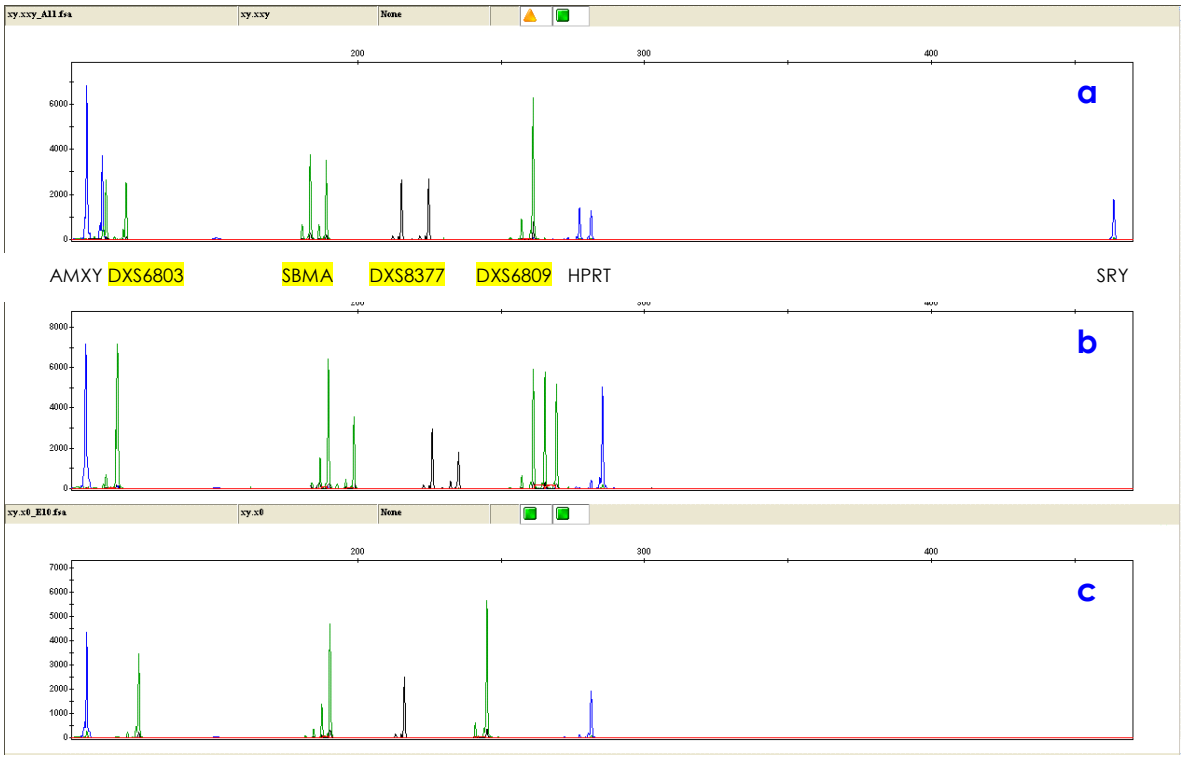
Example 10

Detection of X monosomy



As shown in this example, when tested with **Aneufast™** S1/S2, X chromosome monosomy is indicated by the single fluorescent products for the two pseudoautosomal markers (X22 and DXYS218) and the X-linked HPRT, in the absence of Y-specific products of AMXY and SRY. The likelihood for a normal female to be found homozygous for three STRs (thus indistinguishable from a monosomy X chromosome constitution) is about 1.5 %. On the other hand, the likelihood for a normal female to be homozygous for all the STRs included in the **Aneufast™** MXY back-up marker set, is reduced to about 1 per 20.000.

4.3.5 Aneufast™ MXY Chromosome-Specific back-up marker set



Electrophoretograms showing the detection of sex chromosome aneuploidies using the **Aneufast™** MXY specific back-up set. Extra X-linked markers are highlighted. This multiplex marker set should be used to confirm any initial S1/S2 results indicative of sex- chromosome aneuploidy as well as when homozygosity of all sex chromosome markers included in the initial S1/S2 marker sets, precludes appropriate diagnosis.

From top to bottom:

a) The detection of the XXY sex chromosome constitution: The X- specific product of AMXY is in double dose, compared to the Y (ratio 2:1) and the SRY product confirms the presence of the Y chromosome. In this example, three out of four extra X-linked markers as well as the HPRT are heterozygous (ratio 1:1) confirming the presence of two X chromosomes.
b) The detection of the Trisomy X sex chromosome constitution: The female sex chromosome constitution is detected as a single X chromosome-specific peak of the AMXY in absence of the SRY product. In this example, the three doses of the X chromosome are detected as trisomic patterns for three out of four extra markers. Note that DXS6809 is trisomic triallelic, thus excluding eventual mosaicism (see next section).
c) The detection of the X monosomy sex chromosome constitution: The detection of single fluorescent products for all four extra MXY markers in

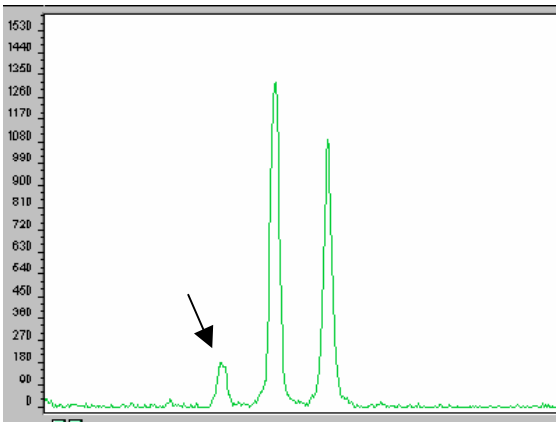
absence of Y- derived sequences (AMXY and SRY) reflects the presence of a single X chromosome. This result complements the S1/S2 with a total of seven highly polymorphic STRs analysed. It is extremely unlikely for a normal XX female to be homozygous for all seven sequences.

In the great majority of cases Aneufast™ QF-PCR Kit results are straightforward. Occasionally unusual patterns may be observed. These are quite often typical of different conditions such as PCR artefacts, maternal cell contamination, chromosome mosaicism, STRs polymorphism or mutations. Detailed examples of interpretations in such cases can be found on www.aneufast.com in the analysis troubleshooting section.

4.4 PCR artefacts during STR amplification

Example 1

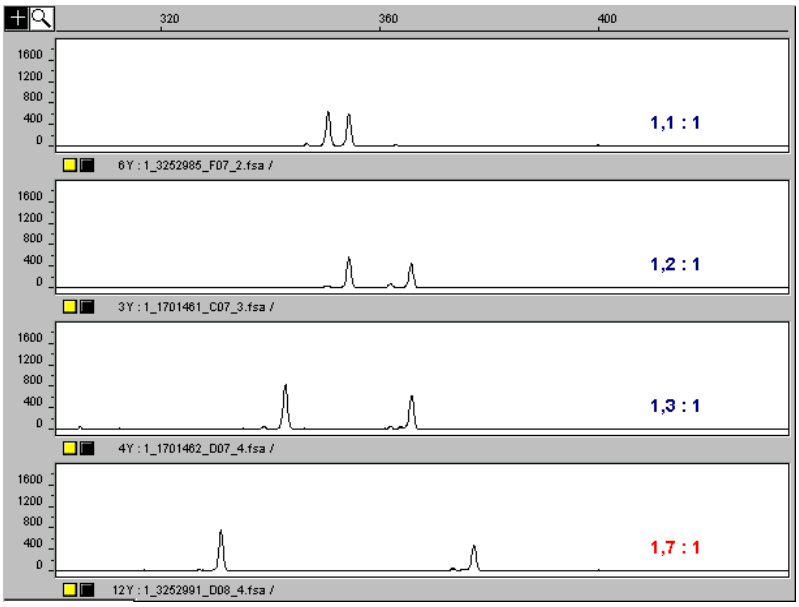
Stutter Bands



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

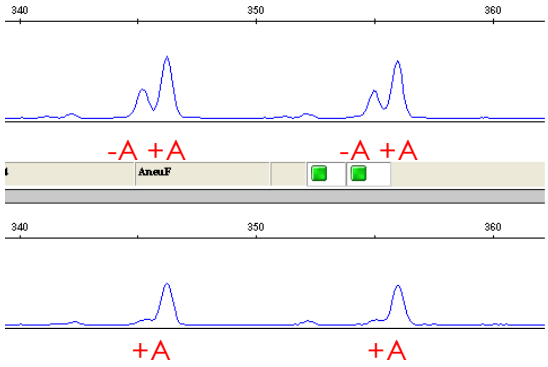
Preferential Amplification



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

Incomplete Final Extension



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 60°C 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 shorter product differing 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.

5 Aneufast™ performance and evaluation

A total of 439 prenatal samples were tested using the **Aneufast™** kit without previous knowledge of their Karyotypes. Samples handling and results analysis were carried out as shown in this manual. Normal females were detected in 190 cases and normal males in 195. Three samples revealed clear evidence of maternal cell contamination and no results could be obtained other than fetal sex. Trisomy 21 was detected in 16 cases, 10 samples showed Trisomy 18 and 5 cases showed Trisomy 13; triploidy was detected in 3 cases. All samples with sex chromosome aneuploidies were also identified and these included 6 cases of X monosomy, 5 trisomy X, 3 47,XXY and 3 47,XYY. All results obtained were found in agreement with cytogenetic analysis so that **Aneufast™** showed overall 100% sensitivity and specificity.

6 QF-PCR limitations

The Quantitative Fluorescent PCR assay cannot detect variation in sequences others than the amplified. It will not detect any abnormality in any other chromosome. It may not detect rearrangements and mosaicism involving the tested chromosomes.
The result only refers to the analysed sample; it may not reflect the fetal chromosome constitution in case of confined placental mosaicism or in samples contaminated with maternal cells.

Disclaimer

Results obtained with any IVD Kit should only be employed and interpreted within the whole clinical picture. Molgentix S.L. cannot be considered responsible for any clinical decisions taken.

This product does not provide a licence to perform PCR under any patent that may be owned by any third party including Hoffman-La Roche (F. Hoffman-La Roche Ltd, Diagnostic, CH-4070 Basel, Switzerland) and Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501).

Suggested Readings

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