Multiplex QF-PCR Kit

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

User’s Manual
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 (S1 and 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), which 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 30,000 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 mispriming 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.
### Details of the 35 Markers included in the Aneufast™ QF-PCR Kit

<table>
<thead>
<tr>
<th>Marker</th>
<th>Label</th>
<th>Het.</th>
<th>Chromosome Location</th>
<th>Known alleles in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMXY</td>
<td>6-Fam</td>
<td>-</td>
<td>Xp22.1 Yp11.2</td>
<td>Chr.X 104 / Chr.Y 109</td>
</tr>
<tr>
<td>SRY</td>
<td>6-Fam</td>
<td>-</td>
<td>Yp11.2</td>
<td>Chr.Y 463</td>
</tr>
<tr>
<td>TAF9L</td>
<td>PET</td>
<td>-</td>
<td>Xq13 3p24</td>
<td>Chr.X 110 / Chr.3 107</td>
</tr>
<tr>
<td>X22</td>
<td>6-Fam</td>
<td>0.91</td>
<td>Xq28 Yq (PAR2)</td>
<td>189-194-199-204-209-214-219-224-226-229-234-239-242-247-253</td>
</tr>
<tr>
<td>DXYS267</td>
<td>PET</td>
<td>0.78</td>
<td>Xq21.31 Yp11.31</td>
<td>330-334-338-342-346-350-354</td>
</tr>
<tr>
<td>DXYS218</td>
<td>PET</td>
<td>0.65</td>
<td>Xp22.32 Yp11.3 (PAR1)</td>
<td>266-270-274-278-282-286-290-294</td>
</tr>
<tr>
<td>DXYS156</td>
<td>NED</td>
<td>0.68</td>
<td>Xq21.31 Yp11.31</td>
<td>134-139-144-149-154-159-164</td>
</tr>
<tr>
<td>HPRT</td>
<td>6-Fam</td>
<td>0.75</td>
<td>Xq26.1</td>
<td>264-268-272-276-278-280-284-288-292-296-300-313</td>
</tr>
<tr>
<td>DXS6803</td>
<td>VIC</td>
<td>0.68</td>
<td>Xq12-Xq21.33</td>
<td>106-110-114-118-120-124-128</td>
</tr>
<tr>
<td>DXS6809</td>
<td>VIC</td>
<td>0.75</td>
<td>Xq</td>
<td>238-242-246-250-252-254-258-260-262-266-268-270-274</td>
</tr>
<tr>
<td>DXS8377</td>
<td>NED</td>
<td>0.85</td>
<td>Xq28</td>
<td>213-216-219-222-225-228-238-241-244-248-252</td>
</tr>
<tr>
<td>SBMA</td>
<td>VIC</td>
<td>0.75</td>
<td>Xq11.2-Xq12</td>
<td>166-169-172-175-178-181-184-187-190-193-196-199-202-205-208-211</td>
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<tr>
<td>D21S1411</td>
<td>VIC</td>
<td>0.93</td>
<td>21q22.3</td>
<td>246-262-266-274-278-282-286-290-294-302-306-316-319</td>
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<tr>
<td>D21S1446</td>
<td>PET</td>
<td>0.77</td>
<td>21q22.3-ter</td>
<td>200-204-208-212-214-218-220-224-228</td>
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<tr>
<td>D21S1437</td>
<td>VIC</td>
<td>0.78</td>
<td>21q21.1</td>
<td>120-124-128-132-136-140-144</td>
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<tr>
<td>D21S1809</td>
<td>6-Fam</td>
<td>0.70</td>
<td>21q22.1</td>
<td>196-200-204-208-212-216-220</td>
</tr>
<tr>
<td>D21S1412</td>
<td>6-Fam</td>
<td>0.73</td>
<td>21q22.2</td>
<td>384-388-392-396-400-404-410-414-418</td>
</tr>
<tr>
<td>D21S1435</td>
<td>PET</td>
<td>0.75</td>
<td>21q21</td>
<td>142-160-164-168-172-176-180-184-188</td>
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<tr>
<td>D21S1442</td>
<td>6-Fam</td>
<td>0.76</td>
<td>21q11.11</td>
<td>136-144-148-152-156-160-166-170-174</td>
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<tr>
<td>D18S391</td>
<td>VIC</td>
<td>0.75</td>
<td>18p11.2</td>
<td>144-148-152-156-160-164-168</td>
</tr>
<tr>
<td>D18S390</td>
<td>VIC</td>
<td>0.75</td>
<td>18q22.2</td>
<td>398-402-406-410-414-418-422-426-430</td>
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<tr>
<td>D18S535</td>
<td>NED</td>
<td>0.82</td>
<td>18q12.2</td>
<td>126-130-134-138-142-146-148-152-156</td>
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<tr>
<td>D18S858</td>
<td>PET</td>
<td>0.66</td>
<td>18q21.1</td>
<td>186-190-192-196-200-204</td>
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<tr>
<td>D18S499</td>
<td>6-Fam</td>
<td>0.72</td>
<td>18q21.32</td>
<td>386-392-396-400-404-408</td>
</tr>
<tr>
<td>D18S1002</td>
<td>6-Fam</td>
<td>0.80</td>
<td>18q11.2</td>
<td>122-130-134-138-142</td>
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<tr>
<td>D18S976</td>
<td>NED</td>
<td>0.76</td>
<td>18p11.31</td>
<td>164-168-172-174-176-178-180-182-184</td>
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<tr>
<td>D13S631</td>
<td>VIC</td>
<td>0.78</td>
<td>13q31-32</td>
<td>192-196-200-204-208-212-215-218</td>
</tr>
<tr>
<td>D13S634</td>
<td>VIC</td>
<td>0.85</td>
<td>13q14.3</td>
<td>460-464-466-470-474-478-482-484-486-490-496-500</td>
</tr>
<tr>
<td>D13S305</td>
<td>PET</td>
<td>0.75</td>
<td>13q12.1-13q14.1</td>
<td>426-430-434-438-442-446-450-454-458</td>
</tr>
<tr>
<td>D13S628</td>
<td>6-Fam</td>
<td>0.70</td>
<td>13q31-q32</td>
<td>436-440-444-448-452-456-460-464</td>
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<td>D13S742</td>
<td>VIC</td>
<td>0.75</td>
<td>13q12.12</td>
<td>254-258-262-266-268-270-274</td>
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<tr>
<td>D13S797</td>
<td>NED</td>
<td>0.65</td>
<td>13q32-q33</td>
<td>417-430-434-438-442-446-450-454</td>
</tr>
</tbody>
</table>
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 the 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, 1.5mL, or 2.0mL, or other appropriate clean tubes
♦ Microcentrifuge tube rack
♦ Heated blocks or water baths
♦ Pipette tips, sterile, disposable hydrophobic filter-plugged
♦ Pipettes
♦ Vortexer
♦ Thermalcycler

Work area for Amplified DNA

♦ ABI Genetic Analyser compatible with Five-Dyes Detection
♦ Heated block
♦ Sequencer disposables and consumables
♦ Pipette tips, disposable hydrophobic
♦ Pipettes
♦ Vortexer
2- How to use the 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. It is recommended that any DNA extraction procedure is extensively evaluated before being applied in diagnostic procedures. In optimal amplification conditions and using standard electrophoretic parameters (refer to the Applied Biosystems Genetic Analyser user’s manual), acceptable and reproducible results are obtained at input DNA amounts of 1 to 10 ng. 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, as an 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. Nº 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.

![Figure 1a](image1a.png)

Figure 1a: 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.

![Figure 1b](image1b.png)

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

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

2.1- Add 1 mL H₂O 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 H₂O 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:

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>MXY</th>
<th>M21</th>
<th>M18</th>
<th>M13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMXY</td>
<td>SRY</td>
<td>SRY</td>
<td>D21S1411</td>
<td>D18S386</td>
<td>D13S631</td>
<td></td>
</tr>
<tr>
<td>DXYS267</td>
<td>X22</td>
<td>AMXY</td>
<td>D21S1435</td>
<td>D18S391</td>
<td>D13S634</td>
<td></td>
</tr>
<tr>
<td>D21S1414</td>
<td>DXYS218</td>
<td>HPRT</td>
<td>D21S1437*</td>
<td>D18S858*</td>
<td>D13S742*</td>
<td></td>
</tr>
<tr>
<td>D21S1446</td>
<td>HPRT</td>
<td>TAF9L*</td>
<td>D21S1412*</td>
<td>D18S499*</td>
<td>D13S628*</td>
<td></td>
</tr>
<tr>
<td>D21S1442</td>
<td>D21S1411</td>
<td>DXYS156*</td>
<td>D21S1809*</td>
<td>D18S1002*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S535</td>
<td>D21S1435</td>
<td>SBMA*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S391</td>
<td>D13S634</td>
<td>DXS6803*</td>
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<td></td>
</tr>
<tr>
<td>D18S976</td>
<td>D13S258</td>
<td>DXS6809*</td>
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<td></td>
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<td>D13S797</td>
<td>D18S386</td>
<td>DXS8377*</td>
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<td>D13S631</td>
<td>D18S390</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13S305</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**S1/ S2**

The two Multiplexes QF-PCR Sets S1 and S2 allow simultaneous analysis of five STRs on each of the autosomes 21, 18 and 13 in addition to three pseudoautosomal (DXYS267, X22 and DXYS218) and one X-linked STRs. Two non-polymorphic sequences, Amelogenin (AMXY) and SRY, are independently amplified for sexing. 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 six STRs and two sexing markers on the sex chromosomes. In addition, the paralogous sequence TAF9L on chromosome 3 and X allow accurate assessment of chromosome X dosage in all cases independently from frequency calculation (see further below). 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.

**Aneufast™ QF-PCR Kit components**

**2.4 PCR set up Protocol**

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

<table>
<thead>
<tr>
<th>Multiplex PCR Mix</th>
<th>10 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1-10ng</td>
</tr>
<tr>
<td>H₂O</td>
<td>up to 15 µL</td>
</tr>
<tr>
<td>Final PCR volume</td>
<td>15 µL</td>
</tr>
</tbody>
</table>

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. H₂O must be added to the mix before aliquoting in accordance with the table below:

<table>
<thead>
<tr>
<th>Multiplex PCR Mix</th>
<th>10 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1 µL</td>
</tr>
<tr>
<td>Aliquot per tube</td>
<td>11 µL</td>
</tr>
</tbody>
</table>

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Warning: 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

Warning: 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.

Hot Start Taq Polymerase

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 95ºC.

1- Program the Thermalcycler according to the following parameters:

<table>
<thead>
<tr>
<th>Taq Activation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>25-28 Cycles</td>
<td>Hold</td>
<td>Hold</td>
<td>Hold</td>
<td>Hold</td>
</tr>
<tr>
<td>95ºC 15 min.</td>
<td>95ºC 40 sec.</td>
<td>59ºC 1 min. 30 sec.</td>
<td>72ºC 40 sec.</td>
<td>60ºC 30 min.</td>
<td>4-20ºC</td>
</tr>
</tbody>
</table>

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 4ºC.

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 of 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 4ºC.

2- Use 40 µL of this mix to inject

   1.5 µL of each Aneufast™ S1 and S2 product 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 set.

4- Denature the sample tubes/plate with Formamide and Size Standard for 2 minutes at 95ºC.

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 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 for your genetic analyser should be used with Aneufast™. The QF-PCR kit is compatible with GeneScan® Analysis version 3.1 or higher, Genotyper® and all versions of GeneMapper®, Genotyper® macros or GeneMapper® settings for automated allele call and genotyping are also available. 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. These types of problems are illustrated and discussed in detail 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; and 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.

<table>
<thead>
<tr>
<th>Dye/Sample Peak</th>
<th>Sample File Name</th>
<th>Marker</th>
<th>Allele</th>
<th>Size</th>
<th>Height</th>
<th>Area</th>
<th>Data Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>G,22</td>
<td>_002.fsa</td>
<td>0135634</td>
<td>460</td>
<td>450.10</td>
<td>174</td>
<td>1426</td>
<td>5819</td>
</tr>
<tr>
<td>G,23</td>
<td>_002.fsa</td>
<td>0135634</td>
<td>475</td>
<td>475.34</td>
<td>186</td>
<td>1460</td>
<td>5893</td>
</tr>
</tbody>
</table>

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.

Samples with a normal copy number for a given chromosome will show heterozygous or homozygous patterns for all 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 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.

<table>
<thead>
<tr>
<th>STR Peak Ratio</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 - 1.4 : 1</td>
<td>Normal</td>
</tr>
<tr>
<td>≤ 0.6 – ≥1.8 : 1</td>
<td>Trisomy</td>
</tr>
<tr>
<td>1.6 : 1 for alleles differing ≥ 20b.p.</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Ratio Ranges within STR alleles.

Ratios are calculated by dividing the area of the smaller allele 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 4.0 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. All pseudoautosomal markers (X22, DXYS267 and DXYS218) and the X-linked HPRT are normal heterozygous, reflecting a normal XX sex chromosome complement. Five markers on each chromosome 21, 18 and 13 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 3 pseudoautosomal markers X22, DXYS267 and DXYS218. Four markers on chromosome 21 (D21S1414, D21S1411, D21S1442 and D21S1446) are normal heterozygous with a ratio of 1:1 between the two fluorescent peaks, and the same patterns are seen for D18S391, D18S976, D18S535 and D18S386 on chromosome 18. All markers on chromosome 13 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 seven 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**

Four markers on chromosome 21 show trisomic diallelic patterns (D21S1414, D21S1411, D21S1435 and D21S1446). D21S1442 is trisomic triallelic. All five STRs on chromosome 18 and three markers on chromosome 13 are informative for the normal disomic chromosome complement. Only the X-specific product of the AMXY is present and SRY is not amplified (XX female sex chromosome constitution). Three pseudoautosomal markers and the X-linked HPRT are normal heterozygous.
**Example 4**  
Detection of Trisomy 18

In this example, Trisomy 18 is identified as a trisomic diallelic pattern for D18S391, D18S390, D18S535 and D18S976 (ratio of 2:1); the other marker on this chromosome is trisomic triallelic (D18S386). Four markers on chromosomes 21 and 13 are heterozygous normal (ratio 1:1). The presence of both the X and Y specific products of AMXY, together with the SRY product, determine the male XY sex chromosome constitution. Normal sex chromosome complement is confirmed by the heterozygous pattern of two pseudoautosomal markers (X22 and DXYS267).
Example 5
Detection of Trisomy 13

In this example Trisomy 13 is determined by the trisomic diallelic pattern for D13S631, D13S258, D13S797 and D13S305, while D13S634 is trisomic triallelic. All five markers on chromosome 21 and three 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 all three pseudoautosomal markers (X22, DXYS128 and DXYS267) 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 SRY product. Three X chromosomes are detected as trisomic diallelic pattern for DXYS218 and DXYS267 and the trisomic triallelic profile for the X22 and the X-linked HPRT. Five markers on chromosomes 21 and 13 are also indicative of trisomy for these chromosomes as well as four markers on chromosome 18.

**Important Note:**

**Aneufast™ S1/S2** include five 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 analysed.

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 sets 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 four markers and the trisomic diallelic (2:1) result for the remaining one.

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 X monosomy

As shown in this example, when tested with Anufast™ S1/S2, X chromosome monosomy is indicated by the single fluorescent products for the three pseudoautosomal markers (X22, DXYS267 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 four STRs (thus indistinguishable from an X chromosome monosomy) is about 0.5 %. Following the addition of all the extra markers included in the MXY back-up marker set, the likelihood for a normal female to be found homozygous for all the STRs, is reduced to about 0.5 per 100,000. In these cases accurate X chromosome dosage can be further assessed by the TAF9L marker included in the Anufast™ MXY assay (see further below), which allows assessment of X monosomy independently from any likelihood calculation.
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 the pseudoautosomal markers X22 and DXYS218 while DXYS267 is trisomic diallelic. The presence of two X chromosomes is further confirmed by the heterozygous pattern of the X-linked HPRT marker.

Four out of five markers on chromosomes 21 and 18 as well as all markers on chromosomes 13 are informative, indicating the normal disomic copy number. Four more X-linked and one pseudoautosomal markers are available in the Aneufast™ MXY assay together with the paralogous sequence TAF9L to confirm the initial XXY result.
Example 9
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 diallelic patterns of the two pseudoautosomal STRs X22 and DXYS218. The X-linked HPRT and DXYS267 are trisomic triallelic. The normal chromosome 21, 18 and 13 copy numbers is detected with five, four and three markers respectively. Using the Aneufast™ MXY chromosome-specific marker set, four more X-linked and one pseudoautosomal markers are available together with the paralogous sequence TAF9L to confirm the initial Trisomy X result.
Example 10
Detection of the XYY sex chromosome constitution

<table>
<thead>
<tr>
<th>AMXY</th>
<th>D21S1442</th>
<th>X22</th>
<th>HPRT</th>
<th>D21S1414</th>
<th>SRY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D21S1411</th>
<th>D18S391</th>
<th>D13S631</th>
<th>D18S535</th>
<th>D13S634</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D18S390</th>
<th>D13S797</th>
<th>D18S976</th>
<th>D13S305</th>
<th>D13S634</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Y-specific product of the AMXY is 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 three pseudoautosomal X22, DXYS218 and DXYS267 markers. The SRY product is not quantifiable and only confirms the presence of chromosome Y. In this example, four markers on chromosomes 21 and 18 as well as all five STRs on chromosome 13 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 pseudoautosomal STRs X22, DXYS218 and DXYS267; these are the only suitable markers together with the DXYS156 included in the MXY assay to confirm this chromosome constitution. If informative, they should produce trisomic diallelic patterns.
4.3.5 Aneufast™ MXY Chromosome-Specific back-up marker set

The Aneufast™ MXY specific back-up marker set should be used to confirm any initial S1/S2 results indicative of sex-chromosome aneuploidy, as well as when homozygosity of sex chromosome markers included in the initial S1/S2 marker sets precludes appropriate diagnosis.

The Aneufast™ MXY includes primers to amplify chromosome-specific sequences of the paralogous gene TAF9L. This gene has a high degree of sequence identity between Chromosome 3 and Chromosome X. However, nucleotide changes occur within each locus and can be used to generate chromosome-specific PCR products. The primers included in the MXY assay exploit a 3bp deletion to generate one Chromosome 3 specific product that is 3 bp shorter than the corresponding product on Chromosome X. Relative quantification between Chromosome 3 and Chromosome X allows diagnosis of X monosomy to be performed in all cases independently from frequency calculation.
Detection of X chromosome aneuploidies by QF-PCR analysis of TAF9L

From top to bottom:

- **X monosomy** (Turner Syndrome) is determined by the double dose of Chromosome 3-specific product compared to the X. In this case, single alleles should be detected for all STR markers on the sex chromosomes in absence of Y-specific sequences of the AMXY and SRY.
- Two peaks of equal fluorescent intensity (ratio 1:1) indicate the presence of two X chromosomes (normal female or Klinefelter Syndrome)
- Skewed ratio (0.7:1) in favour of the X specific product indicates the presence of three X chromosomes. In this case, STR markers on the sex chromosomes should result in trisomic patterns.
Example 11
Detection of the 45,X sex chromosome constitution using the MXY Assay

The detection of single fluorescent products for all extra MXY STR 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 nine highly polymorphic markers. The presence of a single X chromosome in these cases is further assessed by the 2:1 ratio observed for the TAF9L marker, which allow diagnosis to be performed independently from likelihood calculations.
Example 12
Detection of the XXY sex chromosome constitution using the MXY Assay

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. The presence of three sex chromosomes is further confirmed by the trisomic triallelic pattern of the pseudoautosomal DXYS156. TAF9L also indicates the presence of two X chromosomes.
Example 13
Detection of the XXX sex chromosome constitution using the MXY Assay

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 all four extra markers. Note that HPRT and SBMA are trisomic triallelic, thus excluding eventual mosaicism (see next section). TAF9L marker shows a ratio of 0.7:1 confirming the extra dose of chromosome X
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, STR polymorphism or mutations. Detailed examples of interpretations in such cases can be found on www.aneufast.com in the analysis troubleshooting section.

5 Aneufast™ performance evaluation

A total of 1132 selected prenatal samples were tested using the Aneufast™ kit without previous knowledge of their Karyotypes. Sample handling and results analysis were carried out as shown in this manual. Normal females were detected in 482 cases and normal males in 536. Eight samples revealed clear evidence of maternal cell contamination and no results could be obtained other than fetal sex. Trisomy 21 was detected in 39 cases, 19 samples showed Trisomy 18 and 7 cases showed Trisomy 13; triploidy was detected in 5 cases. All samples with sex chromosome aneuploidies were also identified and these included 21 cases of X monosomy, 5 trisomy X, 5 47,XXY and 5 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 other than the amplified sequence. 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.
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Suggested Reading


