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Devyner®Prenatal QF-PCR test kit

For Research Use Only

Protocol Guide

1. Introduction to Devyner QF-PCR

Intended Use

The Devyner QF-PCR kit is an in vitro diagnostic product for determination of aneuploidies in chromosomes 13, 18, 21, X and Y.

Included in the Kit

The Devyner QF-PCR kit contains ready-to-use reagents for PCR amplification of genetic markers.

Test Procedure

DNA extraction: The Devyner QF-PCR kit has been validated using Devyner DNA extraction, AmpliSens® RIBO-prep and QIAamp DNA Blood Mini Kits.
Amplification: The Devyner QF-PCR kit has been validated using Veriti™ 96-Well Thermal Cycler.
Detection: Applied Biosystems Genetic Analyzers (ABI 310, 3100, 3130, 3500, 3730) that support detection of Any5Dye.

Principle of the Procedure

Quantitative fluorescent PCR (QF-PCR) analysis includes amplification, detection and analysis of short tandem repeat (STR) markers and non-polymorphic markers. Fluorescently labelled primers are used for amplification of chromosome specific markers and thus the copy number of each marker is indicative of the copy number of the chromosome.

The resulting PCR products are separated and analyzed using an automated genetic analyzer. The relative amount of each allele is quantified by calculating the ratio of the peak heights or peak areas. A normal diploid sample has the contribution of two of each of the somatic chromosomes. Two alleles of a chromosome specific STR marker are detected as two peaks in a 1:1 ratio when the marker is heterozygous and as one peak when the marker is homozygous. The detection of an additional allele as three peaks in a 1:1:1 ratio or as two peaks in a 2:1/1:2 ratio indicates the presence of an additional STR sequence possibly corresponding to an additional chromosome, as in the case of trisomy.

2. Warnings and Precautions

A.Devyner QF-PCR has been validated using a total PCR reaction volume of 25 µL. changing the reaction volume will compromise the kit performance.
B.Avoid microbial contamination of reagents when removing aliquots from reagent vials. The use of sterile disposable aerosol barrier pipette tips is recommended.
C.Do not pool reagents from different lots or from different vials of the same lot.
D.Do not use a kit after its expiry date.
E.Do not use opened or damaged kit reagent vials.

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F.Work flow in the laboratory should proceed in a unidirectional manner, beginning in the reagent preparation area and moving to the DNA extraction area and then to the amplification area and finally to the detection area. Pre-amplification activities should begin with reagent preparation and proceed to DNA extraction. Reagent preparation activities and DNA extraction activities should be performed in separate areas. Supplies and equipment should be dedicated to each activity and not used for other activities or moved between areas. Gloves should be worn in each area and should be changed before leaving that area. Equipment and supplies used for reagent preparation should not be used for DNA extraction activities or for pipetting or processing amplified DNA or other sources of target DNA. Amplification and detection supplies and equipment should remain in the amplification and detection area at all times.

G.Handling of kit components and samples, their use, storage and disposal should be in accordance with the procedures defined by national biohazard safety guidelines or regulations.

H.Wear powder free disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and kit reagents.

3. Required Material

3.1 Included in the Kits

Configurations

The Devyner QF-PCR test kits contain reagents for analysis of 25, 50 or 100 samples.

3.2 Required but not provided

Reagent Preparation

· Consumables for the Thermal Cycler
· Micropipette/dispenser with aerosol barrier tips or displacement tips
· Disposable protective gloves (powder free)

DNA Extraction

· Reagents and equipment according to manufacturer instructions for use
· Micropipette/multipipette with aerosol barrier tips

Amplification

· Thermal Cycler: Applied Biosystems® Veriti® 96-Well Thermal Cycler. For use of alternative thermal cyclers the following ramping rates must be applied: heating 0,8 ˚C/s, cooling 1,6 ˚C/s
· Micropipette/dispenser with aerosol barrier tips or displacement tips

Detection

· Applied Biosystems Genetic Analyzer (ABI 310, 3100, 3130, 3500, 3730)
· Performance optimized polymers: POP-4™ or POP-7™
· Hi-Di™ Formamide, Genetic Analysis Grade
· 1x Genetic Analyzer Buffer
· Micropipette/multipipette/dispenser with aerosol barrier tips or displacement tips

Size Standard

Devney®Keysar GK-500 Size Standard

3.3 Dye Set Calibration

ABI 3100, 3130, 3730:

Use Dye Set. G5.

4. Storage and Handling Requirements

A.

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Store all components below -18 °C.

B.

The separated reaction mixes may be stored at +2 to +8 °C for at least 7 days and at below -18 °C for at least 90 days. Avoid repeated freeze-thawing.

C.

Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

D.

Do not mix reagents from different kit lot numbers.

5. Sample Requirements

Clinical Samples

The Devyner QF-PCR kit is for use with human genomic DNA extracted from whole blood, amniotic fluid and chorionic villus biopsy samples.

Procedure and Storage

According to manufacturer's instructions for use.

Controls

It is recommended that suitable controls such as normal DNA and negative control (no DNA) are included in each run.

6. Instructions for Use

6.1 Workflow Devyner QF-PCR

The separated reaction mix should be prepared before preparing the samples, if the complete process is performed in one day. Only if the samples are prepared the day before amplification or earlier, the opposite order is advisable.

Devyner QF-PCR has been validated using a total PCR reaction volume of 25 µL. Changing the reaction volume will compromise the kit performance.

Ensure that the Mix is completely thawed before use.

1. Vortex the tubes and centrifuge each tube briefly to collect the content.
2. Add 20 µL to separate PCR reaction tubes.
3. Cap the reaction tubes and centrifuge briefly to collect the contents.
4. Continue to step 6.2

The separated reactions is stable at +2-8 °C for at least 7 days and at below -18 °C for at least 90 days. Avoid repeated freeze-thawing.

6.2 Sample Preparation and PCR Amplification

DNA Extraction

According to manufacturer's instructions for use.

It is recommended that alternative DNA extraction methods and sample materials are thoroughly evaluated with the Devyner QF-PCR kit prior to the results being used for diagnostic use. For recommended PCR conditions and analysis settings (see below), results are consistently obtained at DNA concentrations between 30 and 50 ng/PCR reaction.

Addition of Sample

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Samples and controls should be added in a dedicated area separated from reagent preparation, amplification and detection areas.

1. Add 2 µL of clinical sample (30 - 50 ng genomic DNA/µL sample) to each PCR reaction tube containing separated reaction mix (from step 6.1)
2. Cap the tubes and centrifuge briefly to collect the content.

Amplification

Turn on the Thermal Cycler at least 30 minutes prior to amplification.

For use of alternative thermal cyclers the following ramping rates must be applied: heating 0,8 ˚C/s, cooling 1,6 ˚C/s.

Amplification Area:

Program the Thermal Cycler for amplification according to the following thermal profile (consult the User´s Manual for additional information on programming and operation of the thermal cycler):

95 °C 15 min
94 °C 30 sec; 58 °C 1 min 30 sec; 72 °C 1 min 30 sec for 45 cycles
72 °C 30 min
4 °C FOREVER

PCR Protocol:			
Steps:		**Tem.:°C**	**Time**
Initial denaturing		**95°C**	**15 min**
Denaturing	**45 cycle**	**94°C**	**30 sec**
Annealing		**58°C**	**90 sec**
Extension		**72°C**	**90 sec**
Final Extension		**72°C**	**30 min**
Hold		**4°C**	∞

1. Set reaction volume to 25 µL.
2. Start the amplification (duration approximately 3.5hrs).
3. Following amplification, remove the tubes containing completed PCR amplification reaction from the thermal cycler and place into a suitable holder. Centrifuge briefly to collect the content. Remove the caps carefully to avoid aerosol contamination. Do not bring amplified material into the pre-amplification areas. Amplified material should be restricted to amplification and detection areas.

6.3 Detection

Sample Preparation

Refer to the respective ABI Genetic Analyzers User Manual for instructions on maintenance and handling. Prior to running the Devyner QF-PCR kit, the instrument must be spectrally calibrated to support detection of the dye set DEV-5.

Sample Preparation for Capillary Electrophoresis

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1. Prepare a loading cocktail by combining and mixing 2 µL of the size standard (e.g. 500 SIZER ORANGE) with 100 µL Hi-Di™ Formamide (sufficient mix for 6 wells/tubes).
2. Vortex for 15 seconds.
3. Dispense 15 µL of the loading cocktail into the required number of wells of a microwell plate or into individual tubes (ABI310) to be placed on the Genetic Analyzer.
4. Add 1,5 µL of the sample PCR product to the corresponding well/tube containing loading cock-tail.
5. Seal the plate/tubes.

Instrument Preparation

Create a sample sheet using the data collection software with the following settings:
Sample ID
Dye Set: Any5Dye

- Recommended run Module: See below for different polymers and instruments

Run Modules

The amount of PCR product injected into the capillaries can be adjusted by increasing/decreasing the injection time and/or injection voltage.

ABI 310

Run Parameters	POP-4
Capillary length	47 cm
Run temperature	60 ˚C
Injection voltage	15 kV
Injection time	5 - 15 s
Run voltage	15 kV
Run time	40 min

ABI 3100/3130

Run Parameters	POP-4/POP-7
Capillary length	36 cm
Run temperature	60 ˚C
Injection voltage	1,5 kV
Injection time	20s
Run voltage	15 kV
Run time	1500 s

ABI 3500

Run Parameters	POP-7
Capillary length	50 cm
Run temperature	60 ˚C
Injection voltage	1,6 kV
Injection time	15 s
Run voltage	19,5 kV
Run time	1500 s

7. Results and Analysis

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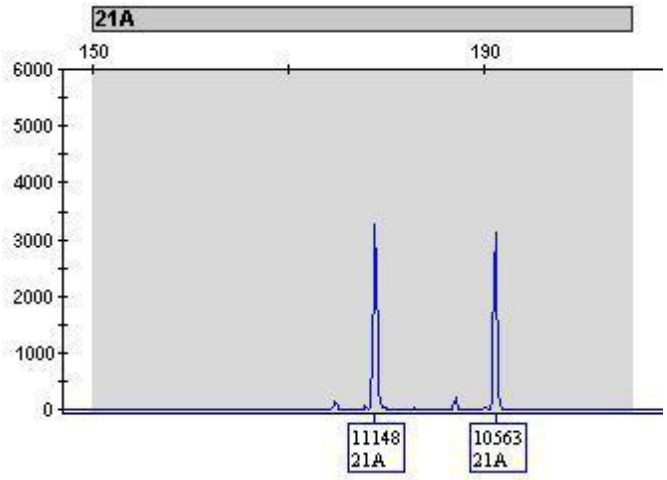
Best practice guidelines for diagnosis of aneuploidy by QF-PCR have been established by the Clinical Molecular Genetics Society (UK) and Association for Clinical Cytogenetics (UK) and are available at: http://www.cmgs.org.uk

Principles of QF-PCR

Chromosome specific, repeated DNA sequences known as small tandem repeats (STRs) are amplified by PCR. By using fluorescently labelled primers visualization and quantification of the fluorescently labelled PCR products may be performed. Quantification can be achieved by calculating the ratio of the specific peak areas of the respective STR using an automated DNA sequencer. STRs vary in size between subjects, depending on the number tetra repeats present on each allele. DNA amplified from normal subjects who are heterozygous (have alleles of different lengths) for a specific STR marker is expected to show two peaks of different length with the same peak areas (Figure 7.1). STR markers that are heterozygous are considered to be informative.

Figure 7.1. Heterozygous marker (area ratio 1:1)



DNA amplified from subjects who are trisomic will exhibit either three peaks with similar area (Figure 7.2), or only two peaks, one of them with twice as large area as the other (Figure 7.3).

Figure 7.2. Trisomic marker (area ratio 1:1:1)

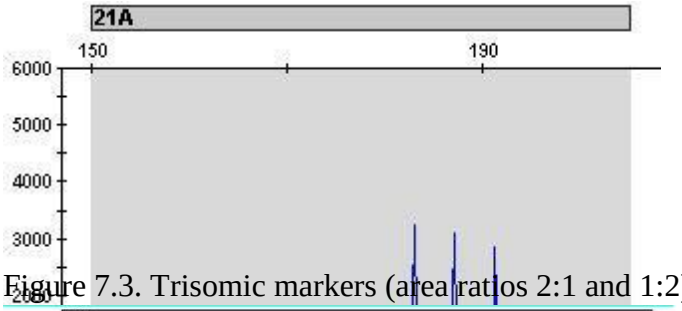
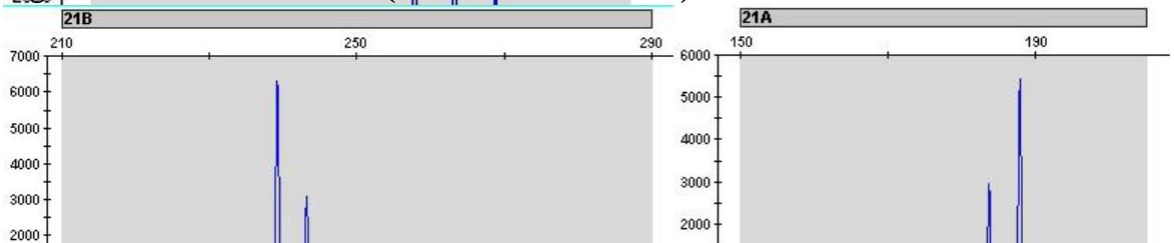


Figure 7.3. Trisomic markers (area ratios 2:1 and 1:2)



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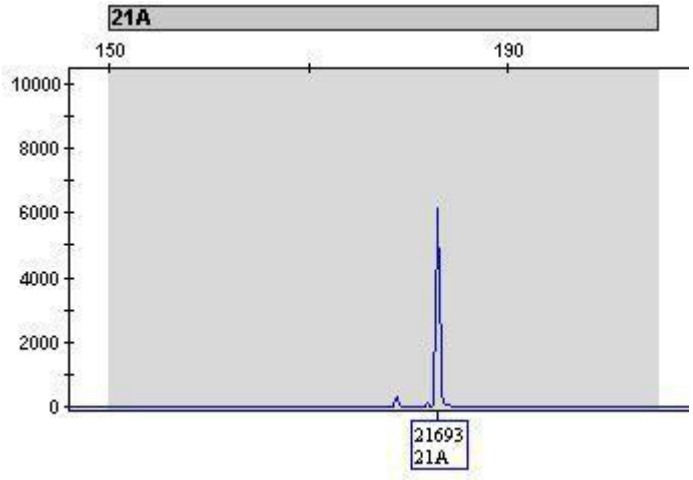
The presence of only one peak in a specific marker indicates homozygosity (have alleles of same length) and is considered to be uninformative. Subjects who are homozygous or monosomic for a specific STR marker will display only one peak (Figure 7.4).

Figure 7.4. Homozygous/monosomic marker



X chromosome counting markers

The T1 and T3 markers are non-polymorphic X chromosome counting markers that may be used to determine the number of X chromosomes when monosomy X is suspected. The X chromosome counting markers define sequences present on the X chromosome and an autosomal chromosome that are amplified using identical primers. The amplified marker fragments are separated according to length and the X chromosomal copy number is determined by fragment area ratio calculation.

In a normal female an X chromosome counting marker area ratio of 1:1 is expected (Figure 7.5). In normal males and females with monosomy X a 2:1 ratio is expected (Figure 7.6).

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Figure 7.5: Normal female T1 and T3 markers (area ratio 1:1).

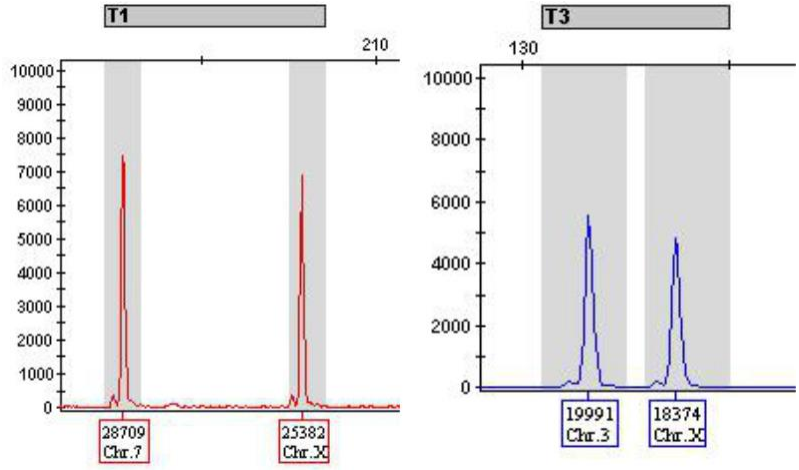
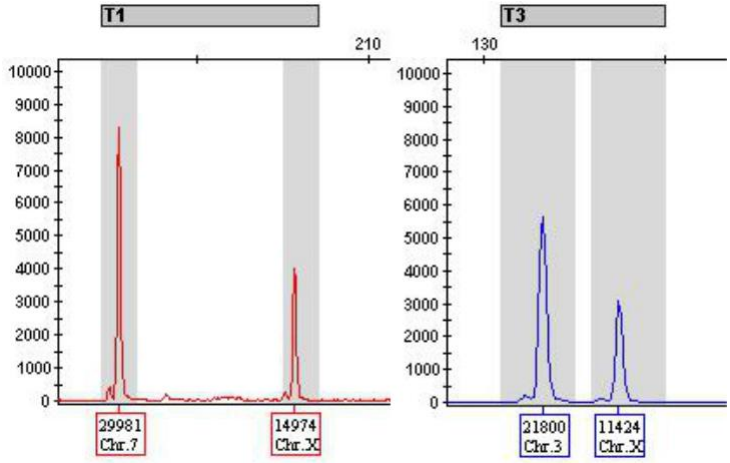


Figure 7.6: Normal male T1 and T3 markers (area ratio 2:1).



Non-Polymorphic Markers

The AMELXY and SRY markers amplify non-polymorphic sequences on the X (AMELX) and Y (AMELY and SRY) chromosomes and can be used to determine the presence or absence of a Y chromosome. AMELXY may be used to assess the relative number of X to Y chromosomes.

Pseudoautosomal XY Markers

The XY2 and XY3 markers are polymorphic STR markers present on both the X and Y chromosomes.

The ZFYX marker is a non-polymorphic (non-STR) marker present on both the X and Y chromosomes.

These markers may be used to assess the total number of sex chromosomes when informative. It is not possible to determine which allele represents the X or Y chromosome.

Marker Overview Devyner QF-PCR

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ID	Location[1]	Marker	Marker size range (bp)[2]	Dye Colour
13A	13q12.12	D13S742	222 - 334	Green
13B	13q21.32-q21.33	D13S634	365 - 435	Blue
13C	13q31.1	D13S628	420 - 475	Yellow
13D	13q13.3	D13S305	435 - 505	Green
13K	13q21.1	D13S1492	100 - 175	Red
18B	18q12.3	D18S978	195 - 230	Yellow
18C	18q12.3	D18S535	300 - 350	Blue
18D	18q22.1	D18S386	338 - 430	Green
18J	18p11.31	D18S976	440 - 495	Red
18M	18p11.32	GATA178F11	350 - 410	Yellow
21A	21q21.3	D21S1435	150 - 208	Blue
21B	21q21.1	D21S11	215 - 290	Blue
21C	21q22.3	D21S1411	245 - 345	Yellow
21D	21q22.13	D21S1444	440 - 495	Blue
21H	21q21.3	D21S1442	362 - 420	Red
21I	21q21.1	D21S1437	105 - 152	Yellow
X1	Xq26.2	DXS1187	120 - 170	Green
X3	Xq26.2-q26.3	XHPRT	265 - 308	Red
X9	Xq27.1-q27.2	DXS2390	312 - 357	Red
SRY	Yp11.31	SRY	236	Yellow
XY2	Xq21.31, Yp11.31	DXYS267	175 - 217	Green
XY3	Xp22.33, Yp11.32	DXYS218	215 - 260	Red
AMELXY	Xp22.2 ,Yp11.2	AMELX, AMELY	X=104,Y=110	Blue
ZFYX	Yp11.31, Xp22.11	ZFY, ZFX	157 - 166	Yellow
T1	7q34, Xq13	-	7=181,X=201	Red
T3	3p24.2, Xq21.1	-	3=133,X=137	Blue

1. UCSC
2. Based on observed and calculated marker sizes using ABI3130, POP-7 polymer and 500 SIZER ORANGE. Please note that marker size ranges may vary depending on the instrument, polymer type and size marker used during electrophoresis.

Marker peaks with sizes outside given ranges may be detected and should not be analyzed in the context of Devyner QF-PCR. We recommend the use of Devyner QF-PCR Resolution for the resolution of specific chromosomes.

Performing Analysis

When performing manual analysis the marker peaks in an electrophoretogram should be identified according to the specific marker size ranges presented in the marker overview. Please note that marker size ranges may vary depending on the instrument, polymer type and size marker used during electrophoresis. The analysis of heterozygous markers dis-playing two allele peaks is performed by calculation of peak area ratios (peak1/peak2). Peak1 is the peak area of the shorter

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length fragment and peak2 is the peak area of the longer length fragment. See ratio criteria below for interpretation.
For markers displaying three allele peaks the ratio is always calculated starting with the area of the shortest length fragment (peak1), i.e. peak1/peak2 and peak1/peak3, respectively.
Homozygous markers are considered uninformative.

Ratio Criteria (RC)

RC 1 is used when peak distance is <24bp
RC 2 is used when peak distance is ≥24bp

Markers displaying two (2) allele peaks

Ratio	1:2	Inconclusive	1:1	Inconclusive	2:1
RC 1	<0,65	0,65-0,74	0,75-1,44	1,45-1,75	>1,75
RC 2	<0,65	0,65-0,74	0,75-1,54	1,55-1,75	>1,75

Markers displaying three (3) allele peaks

Ratio	Inconclusive	1:1:1	Inconclusive
RC 1	<0,74	0,75-1,44	>1,45
RC 2	<0,74	0,75-1,54	>1,55

Height Ratio

Height ratio may be calculated when an area ratio is classified as inconclusive. The same RC is applied as for area ratio calculation

Results Interpretation

To interpret a result as normal for a particular chromosome, at least two informative markers consistent with a normal genotype are required with all other markers being uninformative. To interpret a result as abnormal for a particular chromosome, at least two informative markers consistent with an abnormal genotype are required with all other markers being unin-formative.
Normal allelic pattern is determined by:
Marker showing two peaks of similar height/area and the peak ratio is classified as 1:1.
Abnormal allelic pattern is determined by:
a) Marker showing two peaks of differing height/area and the peak ratio is classified as 2:1 or 1:2.
b) Marker showing three peaks of similar height/area and the peak ratio is classified as 1:1:1
Monosomy X pattern is determined by:
a) All X and XY markers showing homozygous allelic pattern.
b) The AMELY and SRY peaks are not detected.
c) Marker T1 showing two peaks of differing height/area and the peak ratio is classified as 2:1.
d) Marker T3 showing two peaks of differing height/area and the peak ratio is classified as 2:1.

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If peak ratios are classified as inconclusive, results may be resolved by using the Devyner QF-PCR Resolution kits.
Marker peaks with sizes outside given marker size ranges may appear and should not be analyzed in the context of Devyner QF-PCR. We recommend the use of Devyner QF-PCR Resolution for the resolution of specific chromosomes.

Troubleshooting

If a marker displays inconclusive results or is not detected a number of reasons are possible:
» Mosaicism
» Copy number variation (CNV)
» Microvariants due to specific repeat mutations
» Partial chromosome trisomy
» Stutter peak overlapping specific allele peaks
» Crosstalk between dye-channels
» Electrophoretic spike
» Preferential amplification causing skewing
» Contaminating DNA: second genotype, PCR amplicons
» Primer site polymorphism/alteration
» Somatic microsatellite mutation
» DNA concentration is too high or too low
» DNA is degraded or of poor quality
» Submicroscopic duplication/deletion of individual markers
In rare cases, amplification failure due to mutation of the primer site has been reported for the AMELY sequence.
In rare cases, ratio skewing due to mutation of the primer site has been reported for the T1 marker sequences.
If both normal and abnormal allelic patterns are obtained for a particular chromosome, it is recommended that follow-up studies are performed to identify the reason.
Copy number variants (CNVs) have been reported for markers 13A, 13B, 13C, 13D, 18B, 18C, 18D, 21B, 21C, 21I, X3, XY3, AMELXY, ZFYX and T1.
If electrophoretograms are of poor quality the data should not be interpreted. The PCR product may be re-injected and re-analyzed.

PCR Artefacts

Stutter peaks (Figure 7.7) are detected as extra peaks that are one repeat or a multiple of repeats smaller than the actual STR allele. Stutter peaks may be included in the ration calculation. The stutter peak area is typically less than 15% of the corresponding STR peak area.

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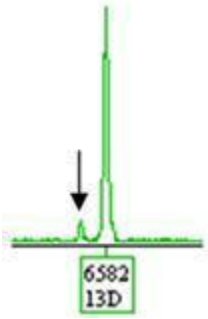
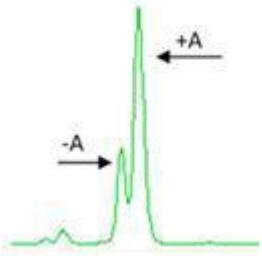
Figure 7.7. Stutter peak as indicated by the arrow.

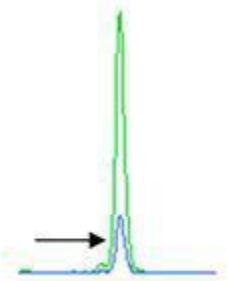


-A peaks (Figure 7.8) are detected as extra peaks that is one base pair shorter than the full length (+A peak) PCR product. -A peaks may be included in the ratio calculation.
Figure 7.8. -A and +A peaks as indicated by the arrows.



Electrophoretic Artefacts

Crosstalk/bleed through between dye channels may occur during detection (Figure 7.9). Cross-talk appears as equally sized peaks in neighbouring dye channels and should be excluded from the analysis.
Figure 7.9. Crosstalk peak (from green to blue channel) as indicated by the arrow.



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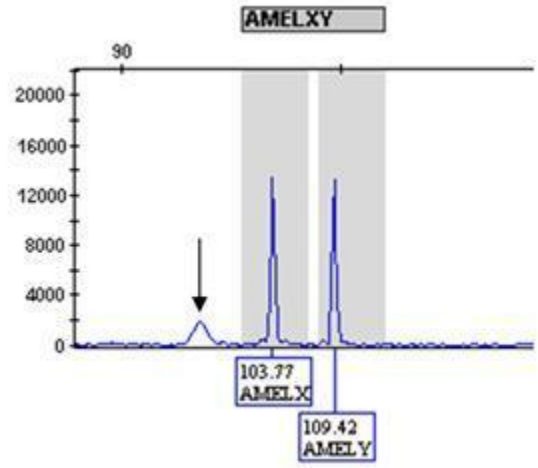
Dye blobs may appear in the sample analysis range (Figure 7.10). In general, dye blobs appear as broad, undefined peaks of a single colour and tend to occur relatively early in the data.
Figure 7.10.Dye blob as indicated by the arrow.



8. Procedural Limitations

A.
Use of this product should be limited to personnel trained in the techniques of PCR and capillary electrophoresis.

B.
The Devyner QF-PCR kit has been validated using the Applied Biosystems® Veriti® 96-Well Thermal Cycler. It is recommended that alternative thermocycler instruments are thoroughly evaluated with the Devyner QF-PCR kit prior to the results being used for diagnostic use.

C.
The Devyner QF-PCR kit has been validated using QIAamp DNA Blood Mini Kit for extraction of DNA from human whole blood and amniotic fluid; QIAamp DNA Mini Kit for extraction of DNA from chorionic villus biopsies. Performance with other matrices and DNA extraction kits has not been validated and may result in false negative or false positive results.

D.
The Devyner QF-PCR kit should be used only for the detection of specific chromosomal aneuploidies according to the instructions for use. The assay has not been validated for detection of structural rearrangements, mosaicisms or abnormalities in any other chromosome. Results obtained with Devyner QF-PCR can only be directly applied to the tissue or specific sample material tested. Contamination by maternal cells or placental mosaicism may result in discrepancies between results obtained with Devyner QF-PCR and other techniques.