



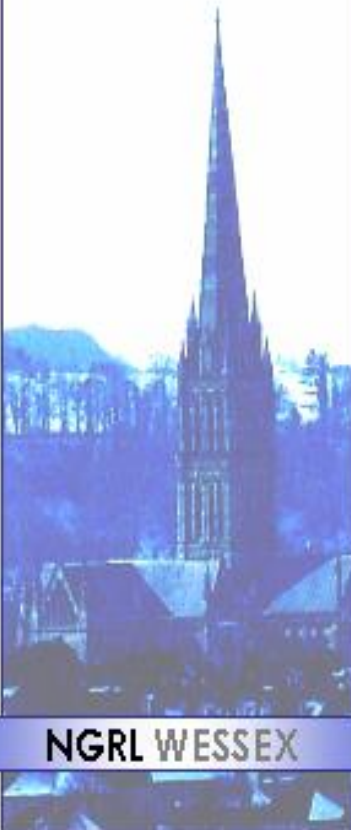
National Genetics Reference Laboratory  
(Wessex)

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**NHS**

**Technology  
Assessment**


**ChromoQuant™ (version 1)**  
*in vitro* diagnostic test kit for  
analysis of common  
chromosomal disorders



**NGRL WESSEX**

**September 2005**



<b>Title</b>	ChromoQuant™ (version 1) <i>in vitro</i> diagnostic test kit for analysis of common chromosomal disorders
<b>NGRL Ref</b>	NGRLW_CQv1_1.0
<b>Publication Date</b>	September 2005
<b>Document Purpose</b>	Dissemination of information about first CE marked kit for detection of aneuploidy by QF PCR
<b>Target Audience</b>	Laboratories performing or setting up QF PCR testing for detection of aneuploidy in prenatal samples
<b>NGRL Funded by</b>	

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### Peer Review and Approval

This document has been subject to peer review and Cybergene<sup>AB</sup> have been given the opportunity to comment on the content of the report.

### Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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## ABSTRACT

At the time of writing (September 2005) ChromoQuant™ is the first and only prenatal diagnostic test kit available worldwide which is CE marked and therefore compliant with the *In Vitro* Medical Devices Directive (98/79/EC). This QF PCR based kit is produced and marketed by Cybergene<sup>AB</sup>. The kit is CE marked for the prenatal diagnosis of trisomy 13, 18, 21 and sex chromosome aneuploidy. NGRL (Wessex) has performed a technology assessment of ChromoQuant version 1 by analysing retrospectively collected DNA samples (n=87) from normal controls and a variety of aneuploid samples and a prospectively collected series of amniotic fluid samples (n=91).

Our results can be summarised as follows:

- 59% of samples had ChromoQuant results that were concordant with the sample karyotype and which could have been reported either following initial analysis or after additional analysis with extra markers to ensure that there were two informative markers for each chromosome.
- 35% of samples produced ambiguous results that would have required follow-up studies due to skewed allele ratios for individual markers or because of lack of informativity for chromosome X. Single marker assays are not available for ChromoQuant version 1 and therefore these ambiguous results could not be resolved. Additional markers for X and Y were not available for use with ChromoQuant version 1.
- 6% of samples produced results that were discordant with the reported karyotype (or we were unable to verify the karyotype). 70% of these discordant results were due to limitations of the QF-PCR technique e.g. low level mosaicism and maternal cell contamination and 30% were specific to ChromoQuant.
- Of the 35% of samples which showed ambiguous results 90% of these problems were associated with markers C (Chromosome 18), O and P (Chromosome 21).

ChromoQuant™ version 1 is technically easy to use, CE marked and therefore IVD compliant. However, interpretation of results is not straightforward and the kit is unable to cope with variable sample quality, it often produces ambiguous results which cannot be resolved due to the lack of single marker assays and the kit has a high failure rate for some markers. Many of these problems have been addressed in a revised (ChromoQuant version 2) version of the kit (see accompanying report for details).

## 1. INTRODUCTION

Invasive prenatal diagnosis is offered routinely to pregnant women who have been identified as having an increased risk of foetal chromosome abnormalities. Pregnancies at high risk are identified by serum or ultrasound screening, advanced maternal age or because one parent is known to carry a chromosome abnormality. Invasive sampling takes place at either 10-12 weeks (chorionic villus sampling) or 15-20 weeks (amniocentesis) and diagnosis has traditionally been based on karyotype analysis which can detect both numerical and structural chromosome abnormalities. The most commonly detected abnormalities are trisomies for chromosome 21 (Down syndrome), chromosome 18 (Edwards syndrome), chromosome 13 (Patau syndrome) and sex chromosome aneuploidy (leading to syndromes such as Turner (monosomy X) and Klinefelter (XXY)). Karyotype analysis of chorionic villus (CV) and amniotic fluid (AF) samples requires cell culture to obtain cells at the metaphase stage and skilled analysis of resulting banded chromosome preparations is also essential. Currently the UK average reporting times for full karyotype analysis are 13.5 days for AF (5.5% abnormality detection rate) with a range of 7.2 – 18.9 days and 14.8 days for CV (16.9% abnormality detection rate) with a range of 7.9 – 23.6 days (UK NEQAS 2002/2003). In an effort to improve pregnancy management and alleviate maternal anxiety rapid aneuploidy detection techniques are now being implemented into routine prenatal diagnosis e.g. interphase FISH, quantitative fluorescent PCR (QF PCR), multiplex ligation dependent amplification (MLPA). These tests are usually capable of delivering results within 1-3 days and are viewed as a prelude to, rather than a replacement of, full karyotype analysis.

Rapid prenatal aneuploidy tests need to fulfil certain criteria: the assay must be accurate and no false positive results should be obtained as this could result in the termination of a healthy pregnancy. The test should be robust enough to cope with variable sample quality, provide unambiguous results and have a low failure rate. Ambiguous results have the potential to increase maternal anxiety and can cause delays in reporting while additional investigations are carried out. The test should be adaptable to cope with high sample throughput and test costs should be low since rapid tests are often performed in addition to karyotyping. Ideally, the test should be able to detect maternal cell contamination (MCC), mosaicism and triploidy (Mann *et al.*, 2004).

QF PCR analysis of short tandem repeats (STR) is being used successfully in many UK and European laboratories for the rapid diagnosis of prenatal aneuploidy (e.g. Verma *et al.*, 1998; Pertl *et al.*, 1999; Schmidt *et al.*, 2000; Cirigliano *et al.*, 2001; Levett *et al.*, 2001; Mann *et al.*, 2001). Chromosome specific polymorphic repeat sequences, which vary in length between individuals, are amplified using fluorescent primers. The PCR amplicons are analysed using an automated genetic analyser capable of 2bp resolution and the representative amount of each allele is quantified by calculating the ratio of the peak height or area using appropriate software.

ChromoQuant™ is currently (September 2005) the first and only prenatal diagnostic test kit available worldwide which is CE marked and therefore compliant with the *In Vitro* Medical Devices Directive (98/79/EC). This QF PCR based kit is produced and marketed by Cybergene<sup>AB</sup>. The kit is CE marked for the prenatal diagnosis of trisomy 13, 18, 21 and sex chromosome aneuploidy. NGRL (Wessex) has performed a technology assessment of the kit by analysing retrospectively collected DNA samples (n=87) from normal controls and a variety of aneuploid samples and a prospectively collected series of amniotic fluid samples (n=91). All samples were anonymised.

## 2. MATERIALS AND METHODS

### 2.1 ChromoQuant™ (version 1) kit composition

ChromoQuant™ (version 1) is supplied with pre-aliquoted primer mixes frozen in a 12 x 8- strip format, PCR strip caps, 1X enzyme dilution buffer and 1X QF PCR Buffer. Taq polymerase needs to be supplied by the user and Cybergene<sup>AB</sup> recommend the use of Promega Taq (#1661 or 1665). Samples are tested using a 2 tube multiplex where 17 tetra-nucleotide STRs are analysed in total; four for each of chromosomes 13 & 18 (tube 1) and chromosome 21 (tube 2) and five for the sex chromosomes (distributed between tubes 1 and 2). Each kit will test 48 samples.

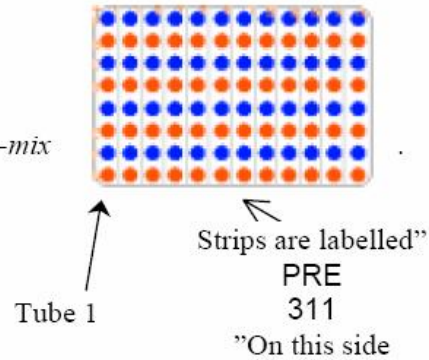
## Kit Composition

- Twelve (12) 8-tube strip.

Each PCR tube contain a ready-to-use primer-mix

Primer-mix Solution1 – *chromosome 13/18.*

Primer-mix Solution2 - *chromosome 21.*



Reagents are stable for 1 year at -18°C and unused primer mix tubes and the QF PCR buffer can be stored in the dark at -18°C. The enzyme dilution buffer can be stored at 4- 8°C.

## 2.2 DNA purification and preparation

Retrospectively collected DNA samples (normal controls, n=37; trisomy 18, n=16; trisomy 13, n=10; trisomy 21, n=21; sex chromosome aneuploidy, n=7) and DNA samples from 0.75 – 1ml prospectively collected amniotic fluid samples (n=91) were tested using ChromoQuant™ (version 1). Reagents for extraction of DNA from amniotic fluid samples are not supplied with the kit. For this evaluation, DNA was extracted from amniotic fluid using either InstaGene Matrix (BIORAD) or the EZ1 DNA tissue kit (QIAGEN) in conjunction with the BioRobot EZ1 Workstation (QIAGEN). No chorionic villus samples were evaluated.

The kit recommends the use of 100ng DNA at a concentration of 10 – 20ng/μl. For this evaluation the retrospectively collected DNA samples were quantified and diluted to 10ng/μl and the amniotic fluid DNA samples were tested using 10μl of the InstaGene DNA solution (not quantified).

## 2.3 Multiplex PCR amplification

DNA (100ng) was added to the PCR master mix (25μl final reaction volume) and amplified using the PCR conditions specified in the ChromoQuant protocol:

94°C	3 min	} 26 cycles
94°C	30 sec	
57°C	1 min	
71°C	2 min	
71°C	5 min	
60°C	1 hour	
4°C	HOLD	

Samples were tested using a 2 tube multiplex where 17 tetra-nucleotide STRs were analysed in total; four for each of chromosomes 13 & 18 (tube 1) and chromosome 21 (tube 2) and five for the sex chromosomes (distributed between tubes 1 and 2, figure 1). A positive and a negative control were included in each PCR run.



a) Tube 1

Marker	Location	Chromosome	Size bp	Colour	Dye
A	Xp22.1-22.31	X/Y (AMEL)	X:104-106 Y:112-114	Green	HEX
B	18pter-18p11.22	18	135-185	Green	HEX
C	18q22.3-q23	18	155-205	Blue	6-FAM
D	Xq26.1	X	260-304	Blue	6-FAM
E	13q11-q21.1	13	230-326	Green	HEX
F	18q22.1-q22.2	18	330-405	Green	HEX
G	13q14.3-q22	13	380-445	Blue	6-FAM
H	13q31-q32	13	420-475	Yellow	NED
I	13q12.1-13q14.1	13	425-470	Green	HEX
J	18q12.2-q12.3	18	450-500	Blue	6-FAM

b) Tube 2

Marker	Location	Chromosome	Size bp	Colour	Dye
Q	Xq26	X	93-119	Blue	6-FAM
R	Yp11.3	Y	206	Blue	6-FAM
K	(PAR 2) Xq/Yq	X/Y	190-250	Green	HEX
L	21q21	21	220-285	Blue	6-FAM
N	21q22.2-21qter	21	260-305	Green	HEX
O	21q22.1	21	435-475	Blue	6-FAM
P	21q22.1	21	445-500	Green	HEX

**Figure 1:** STR Marker information supplied in ChromoQuant version 1. STR marker names, cytogenetic locations, amplified size ranges and fluorescent label for a) tube 1 (analysis of chromosomes 13 and 18) and b) tube 2 (analysis of chromosome 21)

## 2.4 Electrophoresis of amplified products

Instructions supplied with ChromoQuant™ recommend that PCR amplicons are desalted prior to analysis. However, laboratories that currently run a QF PCR service do not desalt samples and UK Clinical Molecular Genetics Society (CMGS) best practice guidelines advise that no post-PCR clean up is required. Desalting of samples requires post-PCR tube transfers which are undesirable and may increase the risk of PCR contamination. We evaluated the effect of desalting by testing 32 samples with ChromoQuant version 1 and analysing them before and after desalting. The results from the samples analysed directly after PCR and those analysed after desalting using Multiscreen PCR µ96 plates (Millipore) were 100% concordant and all allele ratios were accurately preserved. We therefore suggest that desalting of the samples is not required and the results presented in this report are from samples which were not desalted prior to analysis.

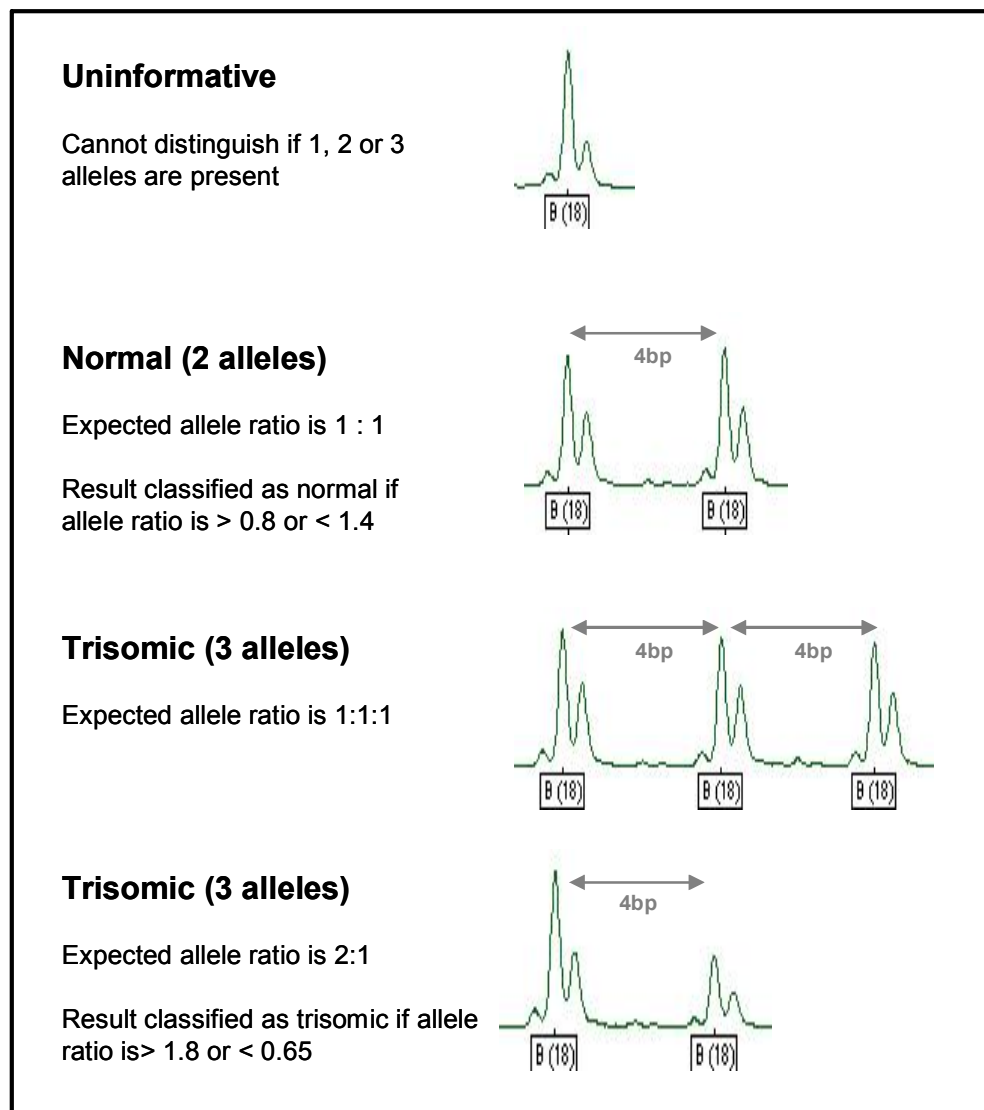
ChromoQuant™ is compatible for use with Applied Biosystems® Genetic Analysers that support dye set D and with the Amersham Biosciences MegaBACE™ 500 using filter set 2. These analysers support the use of 6-FAM, HEX, ROX and NED.

For this evaluation, amplicons were analysed using an Applied Biosystems 3100 Genetic Analyser. A standard run module was used which allows analysis of fragments from 90 – 500 nucleotides using the ABI GeneScan – 500 [ROX] size standard.

## 2.5 Data analysis and interpretation

Data were analysed with Genotyper 3.7 (Applied Biosystems) using macros written by NGRL (Wessex) to analyse the markers in tubes 1 and 2 (section 2.3). Genotyper 3.7 was used to determine the size and peak height (and area) of amplicons present within the designated size range for each marker. The ratio of the peak heights (as recommended in the ChromoQuant protocol) was calculated using the macro and results were then exported into Excel. Data were analysed according to the CMGS best practice guidelines and the ChromoQuant protocol. Allele dosage ratios between 0.8 –

1.4 were defined as normal (a ratio of 1.5 was considered acceptable if the alleles were separated by more than 24bp), ratios of  $>1.8$  or  $<0.65$  were considered to be indicative of trisomy and the presence of three alleles of equal peak height was also considered to be indicative of trisomy (figure 2). Allele ratios that fell between the normal and abnormal ranges were termed inconclusive. A marker allele ratio was termed inconsistent if the ratio suggested the presence of three alleles when other informative markers for the same chromosome demonstrated normal di-allelic ratios. The presence of a single peak was defined as uninformative and a minimum of two informative markers were considered necessary for confident interpretation. Markers with allele peaks below 100 relative fluorescent units (rfu) or above 6000 rfu were excluded from the analysis.



**Figure 2:** Interpretation of Data. Allele ratios can be uninformative, normal (2 alleles with a 1:1 ratio), trisomic (3 alleles) or trisomic (3 alleles with a 2:1 ratio)

### 3. RESULTS

Retrospectively collected DNA samples (normal controls, n=37; trisomy 18, n=16; trisomy 13, n=10; trisomy 21, n=21; sex chromosome aneuploidy, n=7) were analysed using ChromoQuant™ version 1). DNA had been extracted from a variety of tissues including; lymphoblastoid cell lines, skin, muscle, placenta and villi, cultured amniocytes, fibroblasts, urine, mouthbrush, blood and foetal tissue. DNA samples from prospectively collected amniotic fluid samples (n=91) were also analysed.

#### 3.1 Marker Information

##### 3.1.1 Marker Heterozygosity

The percentage heterozygosity for the autosomal markers was determined from the analysis of 178 samples. Details are shown in table 1.

	Marker	Location	% Heterozygosity
Tube 1	B (18)	18pter-18p11.22	73
	C (18)	18q22.3-q23	60
	E (13)	13q11-q21.1	92
	F (18)	18q22.1-q22.2	87
	G (13)	13q14.3-q22	86
	H (13)	13q31-q32	78
	I (13)	13q12.1-q14.1	74
	J (18)	18q12.2-q12.3	78
Tube 2	L (21)	21q21	84
	N (21)	21q22.2-21qter	74
	O (21)	21q22.1	56
	P (21)	21q22.1	72

Table 1: % Heterozygosity for autosomal markers

Markers C and O showed low heterozygosity (60 and 56% respectively) in our test population. Other markers demonstrated greater than 70% heterozygosity. Marker E (chromosome 13) had the highest % heterozygosity (92%).

##### 3.1.2 Inconsistent and Inconclusive Ratios

Table 2 shows the frequency of inconsistent allele ratios (*i.e.* one marker allele ratio suggested the presence of three alleles when other informative markers for the same chromosome demonstrated normal di-allelic ratios) and inconclusive allele ratios (*i.e.* the allele ratio falls between 0.65 and 0.8 or between 1.4 and 1.8) detected for each autosomal marker.

	Marker	Marker Range (bp)	Retrospectively collected samples (n=87)		Prospectively collected amniotic fluid samples (n=91)	
			Inconsistent Ratio (%)	Inconclusive Ratio (%)	Inconsistent Ratio (%)	Inconclusive Ratio (%)
Tube 1	B (18)	135 – 185	0	0	0	0
	C (18)	155 – 205	6	1	6.5	8
	E (13)	230 – 326	0	0	0	1
	F (18)	330 – 405	2	1	3	1
	G (13)	380 – 445	0	0	1	0
	H (13)	420 – 475	0	1	1	0
	I (13)	425 – 470	0	0	0	1
	J (18)	450 – 500	0	0	3	0
Tube 2	L (21)	220 – 285	1	1	1	0
	N (21)	260 – 305	0	0	1	0
	O (21)	435 – 475	0	4	5	6
	P (21)	445 - 500	5	14	22	20

**Table 2:** Percentage of inconsistent and inconclusive allele ratios obtained for autosomal markers for retrospectively and prospectively collected samples

Markers C, O and P had the highest frequency of inconsistent or inconclusive allele ratios in both sample populations.

### 3.1.3 Individual marker failures

The percentage failure rate for individual markers is shown in table 3. Markers were considered to fall into this category if they failed to amplify, were too weak/strong to analyse or for technical reasons e.g. bleedthrough. Results from total tube failures are not included.

	Marker	Marker Range (bp)	Retrospectively collected samples (n=87)	Prospectively collected amniotic fluid samples (n=91)
			Failure rate (%)	Failure rate (%)
Tube 1	B (18)	135 – 185	0	0
	C (18)	155 – 205	1	1
	E (13)	230 – 326	0	0
	F (18)	330 – 405	2	2
	G (13)	380 – 445	0	0
	H (13)	420 – 475	0	2
	I (13)	425 – 470	6	2
	J (18)	450 – 500	1	6
Tube 2	L (21)	220 – 285	0	0
	N (21)	260 – 305	0	0
	O (21)	435 – 475	6	13
	P (21)	445 - 500	8	25

**Table 3:** Percentage failure rates for autosomal markers (total tube failures excluded)

## 3.2 Retrospectively collected tissue samples

Results for this section are summarized in table 4.

### 3.2.1 ChromoQuant Results concordant with karyotype

The results from ChromoQuant version 1 were concordant with the karyotype for 54 of the 87 samples (62%). 43 of the 54 (80%, 49.4% of samples tested) could be confidently interpreted without any further testing being required. The remaining 11 samples (20%) required additional tests to be carried out for the following reasons:

- **Informativity (n=11)**

- 2 had only one informative marker for Chromosome 13
- 3 had only one informative marker for Chromosome 18
- 6 had only one informative marker for Chromosome 21

When these samples were tested with sets of extra markers supplied with version 1 (section 3.5) there were at least 2 informative markers for each sample.

### 3.2.2 ChromoQuant Result ambiguous but not necessarily discordant with karyotype

27 samples (31%) would have required additional testing to clarify single marker results that either showed inconclusive or inconsistent allele ratios. No single marker assays (for markers in tubes 1 and 2) are available for use with the ChromoQuant kit and therefore these ambiguous results could not be investigated further.

- **Single Marker anomalies (n=23)**

The following number of samples would require re-testing of single markers:

- 4 marker C (inconsistent allele ratio)
- 1 markers C (inconsistent allele ratio) and O (inconclusive allele ratio)
- 1 markers C and P (inconclusive allele ratios)
- 2 marker F (inconsistent and inconclusive allele ratio)
- 1 marker F (inconsistent allele ratio) and marker P (inconclusive allele ratio)
- 1 marker H (inconclusive allele ratio)
- 1 marker L (inconsistent allele ratio)
- 11 marker P (7 inconclusive allele ratios and 3 inconsistent allele ratios)
- 1 markers P and O (inconclusive allele ratios)

- **Informativity (n=4)**

- 4 samples had no informative markers for Chromosome X (3 were 45, X and one case was 46, XX). However, additional markers were unavailable for chromosome X at the time of this evaluation and therefore the absolute concordance of the ChromoQuant version 1 result with the karyotype could not be determined. Although 3 cases had the karyotype 45, X, the number of X markers included in the kit, in our opinion, are not sufficient to be indicative of monosomy X.

### 3.2.3 ChromoQuant Results discordant with karyotype

The results from ChromoQuant version 1 were discordant with the karyotype (or not able to verify karyotype) for 6 of the 87 samples (6.9%). These discordant results can be divided into those cases which would remain discordant if tested using any QF PCR/molecular methodology and those which are specific to ChromoQuant.

#### 3.2.3.1 Discordant results due to limitations of QF PCR technique

- 3 samples were reported as very low level mosaics (46,XY/47, XY, +13) (<5%). In each case, the ChromoQuant result was consistent with a male foetus with no evidence of trisomy 13. QF PCR has been reported to detect mosaicism at a lower level of 15% and therefore, like other molecular techniques, would not be sensitive enough to detect the 5% mosaicism present in these samples (Donaghue *et al.*, 2005).

- 1 sample was reported as having multiple 46, XX metaphases consistent with heavy maternal cell contamination (POC). The foetal karyotype was 47, XY, +13. The ChromoQuant result was consistent with a female foetus with a no evidence of trisomy 13. Again the number of cells exhibiting the foetal karyotype would be too low to be detected using QF PCR or other molecular techniques.

### 3.2.3.2 *Discordant results specific to ChromoQuant*

- 1 sample had the karyotype 47, XXX. ChromoQuant markers D and K were uninformative and marker Q had an inconclusive allele ratio and therefore the X chromosome ratio could not be verified. Additional markers for X and Y were unavailable for this evaluation and the karyotype could not be verified.
- 1 sample had the karyotype 47, XYY. The ChromoQuant result showed an inconclusive allele ratio for amelogenin (marker A) and marker K was uninformative. Hence, sex chromosome ratios could not be verified. Additional markers for X and Y were unavailable at the time of this evaluation and the karyotype could not be verified.

## 3.3 Prospectively collected amniotic fluid samples

Results for this section are summarized in table 4.

### 3.3.1 *ChromoQuant Results concordant with karyotype*

Results for the prospectively collected amniotic fluid samples will be presented with the quantitative analysis of the sex chromosome markers excluded (see section 3.4). The results from ChromoQuant version 1 were concordant with the karyotype for 51 of the 91 samples (56%). 37 of the 51 (72.5 %, 40.6% of all samples tested) could be confidently interpreted without any further testing being required. The remaining 14 samples (27.5%) required additional tests to be carried out for the following reasons:

- **Informativity (n=14)**

- 5 had only one informative marker for Chromosome 18
- 2 had no informative markers for Chromosome 18

- 5 had only one informative marker for Chromosome 21
- 1 had no informative markers for Chromosome 21

- 1 had only one informative marker for Chromosome 18 and one informative marker for Chromosome 21

When these samples were tested with the extra sets of markers supplied with ChromoQuant version 1 (section 3.5) there were at least 2 informative markers present for the chromosome tested. Therefore the samples were reportable.

### 3.3.2 *ChromoQuant Result ambiguous but not necessarily discordant with karyotype*

36 samples (40%) would have required additional testing to clarify single marker results that either showed inconclusive or inconsistent allele ratios. Single marker assays (for markers in tubes 1 and 2) are not available for use with ChromoQuant version 1 and therefore these ambiguous results could not be investigated further. Two samples required extra tests for single marker anomalies and informativity

- **Single Marker anomalies (n= 29)**

- 5 marker C (3 inconclusive allele ratios and 2 inconsistent allele ratio)
- 1 markers C (inconsistent allele ratio) and O (inconclusive allele ratio)
- 1 markers C (inconclusive allele ratio) and J (inconsistent allele ratio)
- 3 markers C and P (2 inconclusive and 1 inconsistent allele ratio).
- 1 marker F (inconsistent allele ratio)
- 14 marker P (6 inconclusive allele ratios and 8 inconsistent allele ratios)
- 2 marker O (1 inconclusive allele ratio and 1 inconsistent allele ratio)

2 markers O (inconclusive allele ratio) and P (inconsistent allele ratio)

- **Informativity (n=4)**

1 sample had only one informative marker for Chromosome 21 and no informative markers for Chromosome X (2 markers were informative for chromosome 21 after testing with extra markers)

2 samples had no informative markers for chromosome X. Additional markers were unavailable for chromosome X at the time of this evaluation and therefore the absolute concordance of the ChromoQuant result with the karyotype could not be determined. This highlights the issue that the number of X markers included in the kit, in our opinion, are not sufficient to be indicative of monosomy X. The 3 cases which had uninformative X markers (3%) all had the karyotype 46,XX.

1 sample had only one informative marker for Chromosome 18 (2 markers were informative after testing with extra markers)

- **Reaction failures (n=5)**

2 samples required tube 1 to be repeated

3 samples required tube 2 to be repeated

We were unable to obtain results for these samples on two separate occasions.

### **3.3.3 ChromoQuant Results discordant with karyotype**

The results from the ChromoQuant kit were discordant with the reported karyotype (or not able to verify karyotype) for 4 of the 91 samples (4%). These discordant results can be divided into those cases which would remain discordant if tested using any QF PCR/molecular methodology and those which are specific to ChromoQuant.

#### *3.3.3.1 Discordant results due to limitations of QF PCR technique*

- 1 sample had maternal cell contamination (figure 3) and had been noted as being blood stained. In the absence of a maternal blood sample it was not possible to verify the foetal karyotype (46, XX). Although the foetal karyotype could not be verified, one of the benefits of QF PCR is the ability of the technique to detect MCC and therefore this should not be considered as a negative finding.
- 1 sample had the karyotype 45,X/ 47,XXX but results from the ChromoQuant kit showed normal allele ratios for informative X markers.
- 1 sample had the karyotype 45,X/46,X,r(X) and the results from the ChromoQuant kit showed no informative X markers. Additional markers for X and Y were unavailable at the time of this evaluation.

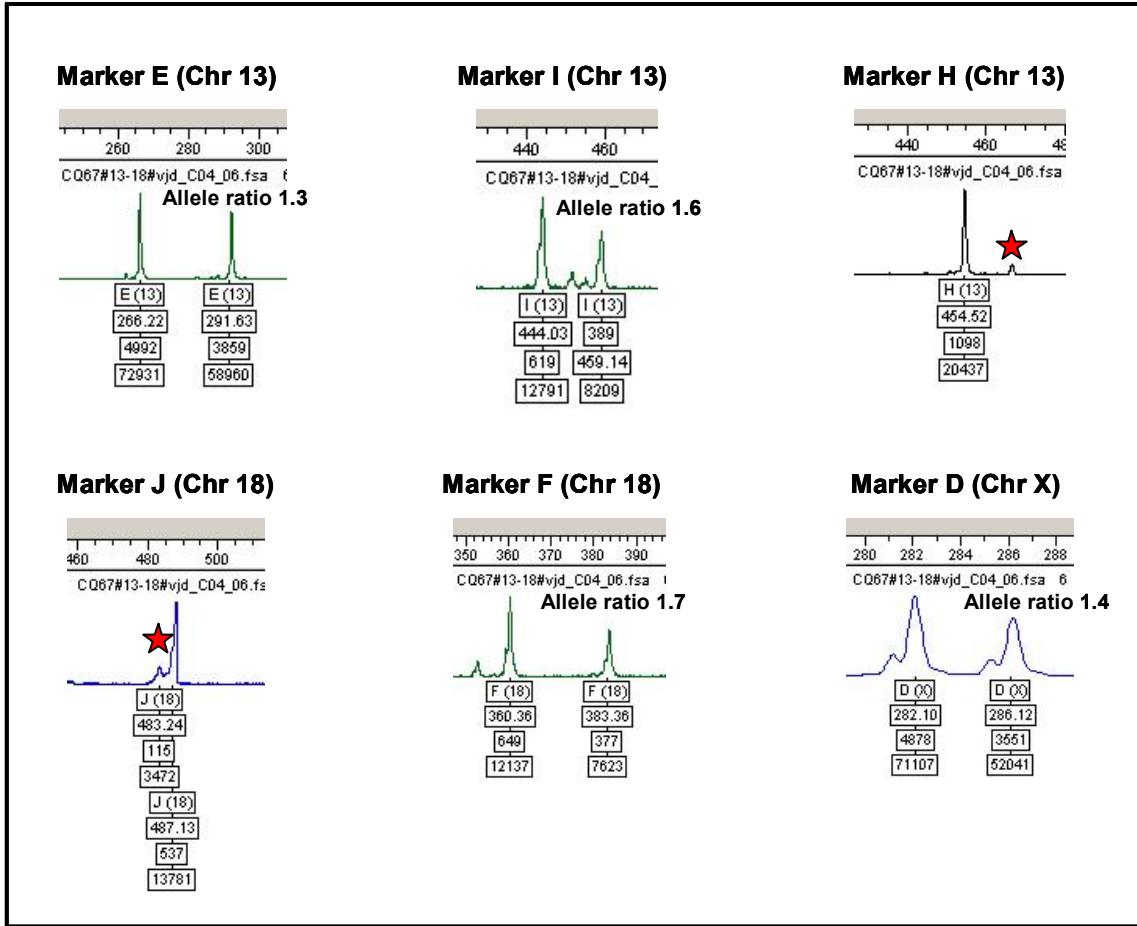
#### *3.3.3.2 Discordant results specific to ChromoQuant*

- 1 sample had the karyotype 47,XX,+21. However, the result from the ChromoQuant kit was ambiguous with markers L and N showing allele ratios indicative of trisomy 21, marker O showed a normal allele ratio and marker P failed to amplify. On retesting the sample with three extra Chromosome 21 markers (section 3.5) two were found to have allele ratios indicative of trisomy 21 but one marker had normal allele ratios. It was not possible to provide an unambiguous result for this sample in the absence of single marker assays.

Result	Retrospectively collected samples (n=87)	Prospectively collected amniotic fluid samples (n=91)
<b>ChromoQuant Result concordant with karyotype:</b>	<b>54 (62%)</b>	<b>51 (56%)</b>
i) no additional tests required	43 (80%)	37 (72.5%)
ii) extra markers tested for informativity	11 (20%)	14 (27.5%)
<b>ChromoQuant Result ambiguous:</b>	<b>27 (31%)</b>	<b>36 (40%)</b>
i) single marker repeats required:	23 (85%)	29 (80.5%)
<i>those involving markers C, P or O</i>	<i>19 (83%)</i>	<i>28 (96.5%)</i>
ii) informativity (extra X markers)	4 (15%)	4 (11%)
iii) whole tube amplification failures	0	5 (14%)
<b>ChromoQuant Result discordant with karyotype:</b>	<b>6 (7%)</b>	<b>4 (4.4%)</b>
i) Due to limitations of QF PCR technique	<b>4 (67%)</b>	<b>3 (75%)</b>
ii) ChromoQuant specific	<b>2 (33%)</b>	<b>1 (25%)</b>

**Table 4:** Summary of concordance of ChromoQuant result with sample karyotype





**Figure 3:** Genotyper traces showing evidence of a second genotype consistent with maternal cell contamination of sample CQ67. Highly and slightly skewed allele ratios (examples above) were obtained for all informative markers. Low level (likely to be the maternal contribution) peaks were also observed for some non-informative signals (red stars).

### 3.4 Inclusion of sex chromosome markers

Some centres consider that it is only appropriate to test for sex chromosome aneuploidy in a subset of referrals. Such decisions are usually made at a local level after consultation with clinical colleagues. The ChromoQuant kit could not be used by laboratories where clinicians do not wish to test for sex chromosome imbalances unless agreements were made to specifically exclude sex chromosome markers from the data analysis.

CMGS best practice guidelines state that:

“It is important to be aware that the QF-PCR sex chromosome assay (Donaghue *et al.*, 2003) is a highly stringent screen for monosomy X but not a diagnostic test. A result consistent with monosomy X, where all polymorphic markers have only a single allele peak and no Y sequences are present, may represent a normal female homozygous for all markers tested. Therefore it is **recommended** that the result is either confirmed using another technique, or that it is reported as being consistent with monosomy X with the caveat that there remains a possibility that a normal female could give the same genotype.”

ChromoQuant version 1 only includes the amelogenin marker, a Y specific marker and 3 polymorphic X and Y chromosome markers. In our prospective study of amniotic fluid samples 3% of samples were uninformative for the three polymorphic markers (in the absence of the Y chromosome amelogenin marker and marker R (Yp11.3)) but had normal female karyotypes. In the absence of additional X markers for further investigation all such cases would require confirmation by FISH or karyotype analysis before reporting. In contrast, Donaghue *et al.*, 2003 have described a QF PCR test for sex chromosome imbalance which can be used independently of an autosomal aneuploidy test and the likelihood of a sample being monosomy X (where all X markers are uninformative and no Y markers amplified) has been given a Bayesian probability of 907:1 when using seven X and Y polymorphic markers. Therefore, we do not consider that there are sufficient numbers of sex chromosome markers in ChromoQuant version 1 to thoroughly evaluate sex chromosome imbalance. We also encountered problems with inconclusive and inconsistent allele ratios particularly with marker K. Again in the absence of single marker assays and extra markers it would be difficult to obtain unambiguous results for many samples. Details of marker informativity and numbers of cases where markers gave ambiguous results are shown in table 5.

Marker	Retrospectively collected samples (n=87)	Prospectively collected amniotic fluid samples (n=91)
<b>A ( Xp22.1-22.31)</b> (XY, XXY and XYY)	40/41 (98%) expected allele ratios 1/41 (46,XY) allele ratio 0.59	100% expected allele ratios
<b>D (Xq26.1)</b> (XX, XXX and XXY)	34/48 (71%) expected allele ratios 14/48 (29%) were uninformative	34/48 (71%) expected allele ratios 2/48 (4%) inconclusive allele ratio 12/48 (25%) were uninformative
<b>Q (Xq26)</b> (XX, XXX and XXY)	31/48 (65%) expected allele ratios 17/48 (35%) were uninformative	25/48 (52%) expected allele ratios 1/48 (2%) inconclusive allele ratio 3/48 (6%) failed to amplify 19/48 (40%) were uninformative
<b>K (PAR2 Xq/Yq)</b>	73/87 (84%) expected allele ratios 14/87 (16%) were uninformative	45/91 (49%) expected allele ratios 20/91 (22%) were uninformative 14/91 (15%) inconclusive allele ratios 8/91 (9%) inconsistent allele ratios 4/91 (4%) failed to amplify

**Table 5:** Summary of details of performance of sex chromosome markers

### 3.5 Extra markers

The extra markers supplied with ChromoQuant version 1 performed well (table 6). No inconsistent or inconclusive allele ratio results were obtained and the 27 samples that were tested all had at least two informative markers for each chromosome when the results were combined with the original test data. Additional markers for Chromosomes X and Y were not supplied with ChromoQuant version 1.

Marker	Location	Size Range (bp)	Label
<b>Chromosome 13</b> D13S252 D13S762	13q12.1 13q31-q32	330 – 360 270 – 320	6-FAM HEX
<b>Chromosome 18</b> D18S1002 D18S976 D18S878	18q11-q11 18pter-18qter 18pter-18qter	280 – 370 171 – 198 164 – 188	6-FAM 6-FAM HEX
<b>Chromosome 21</b> D21S1444 D21S1435 IFNAR	21pter-21qter 21q21.2 21q22.1	232 – 260 170 – 210 445 – 500	6-FAM HEX HEX

**Table 6:** Extra marker information for Chromosomes 13, 18 and 21.

### 3.6 Marker specific problems

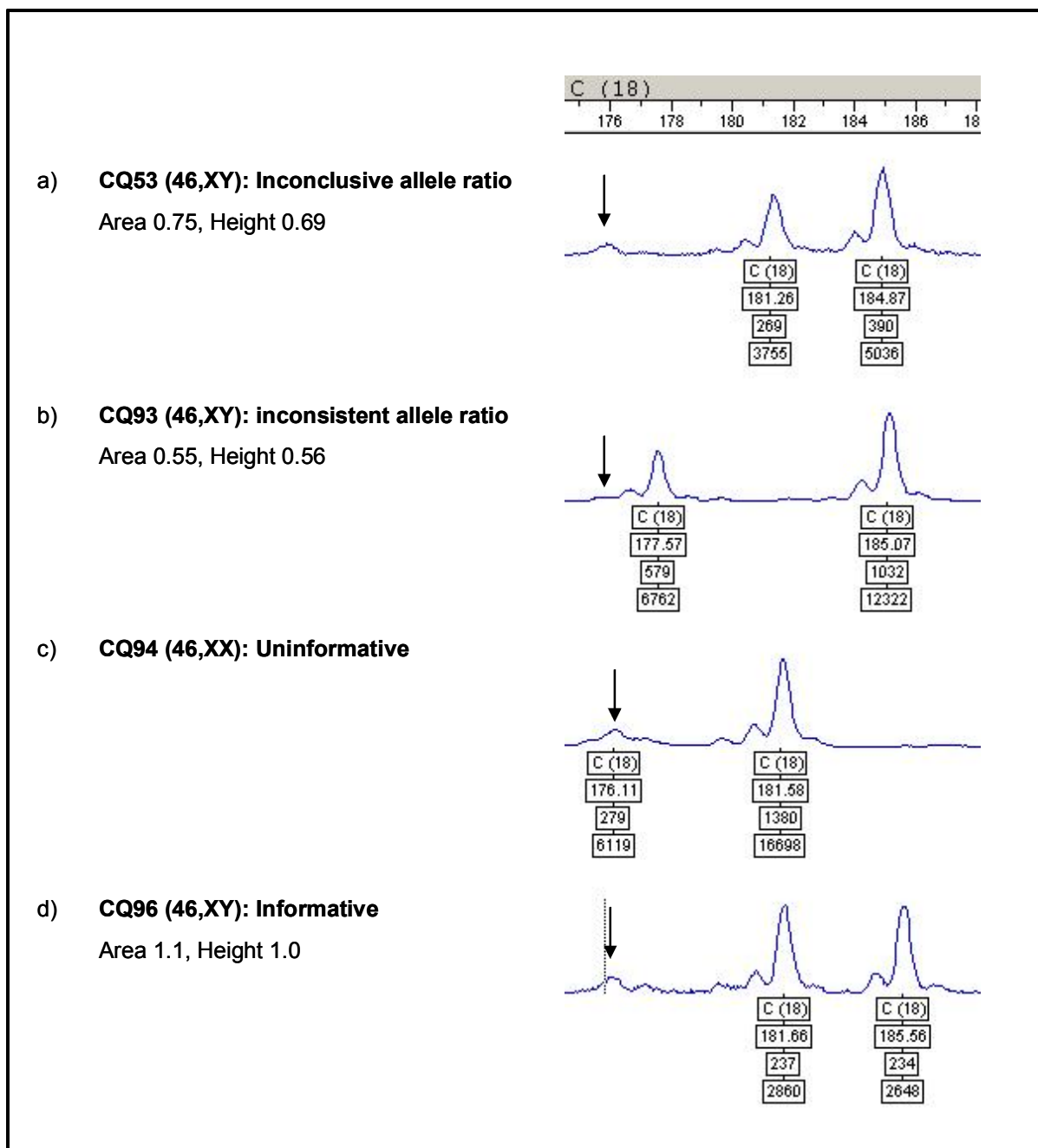
During this evaluation several markers gave consistently problematic results that prevented unambiguous interpretation of the QF PCR result. The most problematic markers were C, O and P which often showed inconclusive allele ratios. In many cases the allele ratio was so skewed that the result appeared to be indicative of a tri allelic result (and hence was considered to be an inconsistent allele ratio if other markers for that chromosome showed normal allele ratios). 83% and 96.5% of single marker repeats for the retrospective and prospective samples respectively involved these three markers. We assume that this is a technical problem with the kit and is not occurring due to the presence of polymorphisms at the primer binding sites or sub microscopic duplications since the frequency of occurrence of these ambiguous results is so high. The sex chromosome marker K (PAR2 Xq/Yq) also showed inconclusive or inconsistent allele ratios in many cases, particularly in the amniotic fluid samples.

#### 3.6.1 Marker C (Chr 18 – tube 1)

Marker C consistently produced inconsistent and inconclusive allele ratios (7% of retrospectively collected samples and 14% of prospectively collected samples). The presence of a non-specific blue fluorescent signal at 176bp (amniotic fluid samples only) also made analysis problematic as the peak falls within the range of analysis. Representative Genotyper traces showing examples of the non specific signal, inconclusive, inconsistent, informative and uninformative allele ratios are shown in figure 4. The marker was also only informative in 64% of cases.

#### 3.6.2 Marker O (Chr 21 – tube 2)

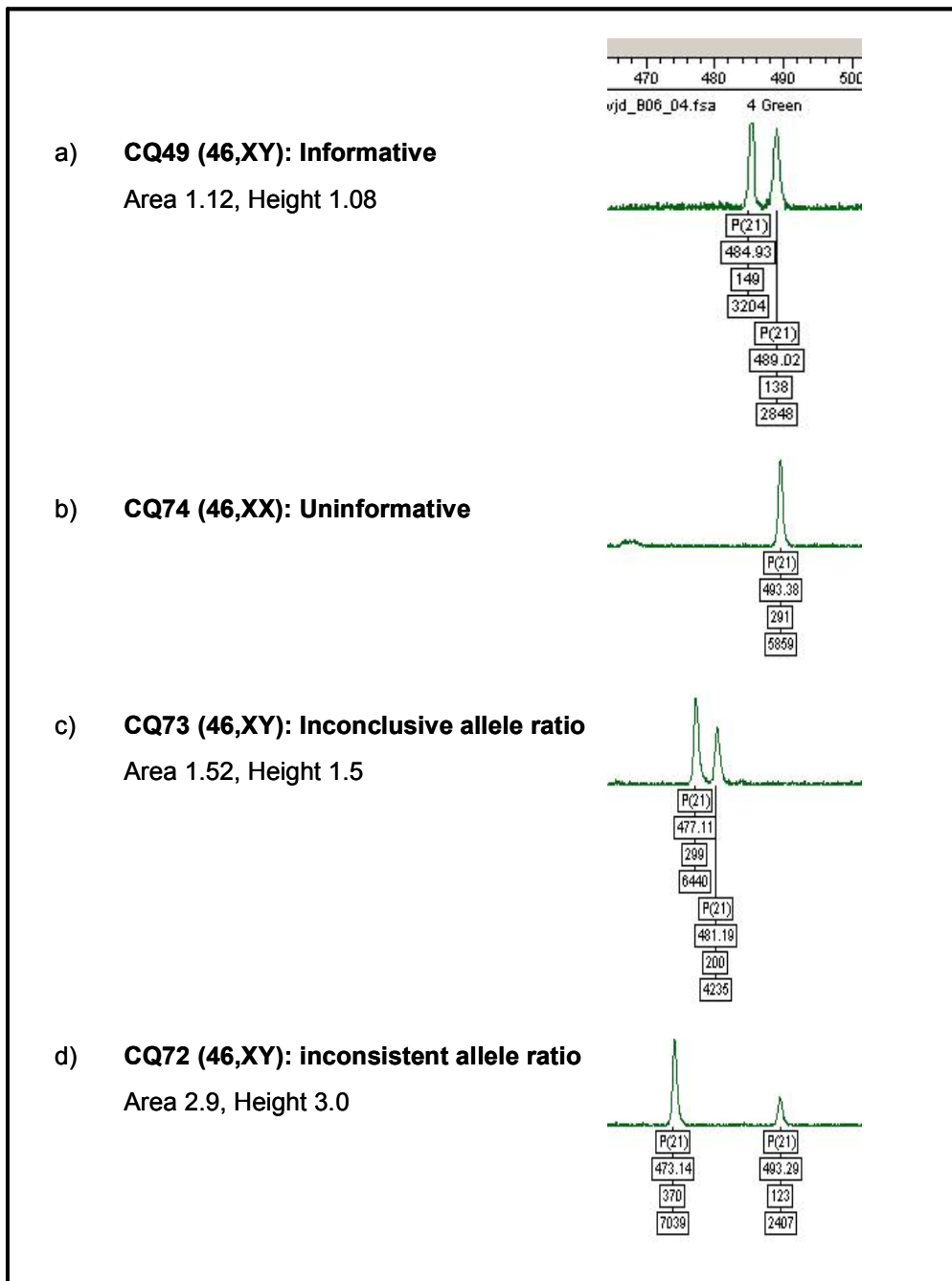
Marker O gave a high number of inconsistent and inconclusive allele ratios (4% of retrospectively collected samples and 11% of prospectively collected samples). The marker also failed to amplify in 6% of retrospectively collected samples and 13% of prospectively collected samples. The marker size range is 435-475bp and with poor quality DNA the product often failed to amplify. The marker was also only informative in 60% of cases.



**Figure 4:** Marker C. Genotyper traces showing examples of a) inconclusive allele ratio, b) inconsistent allele ratio, c) uninformative sample and d) an informative samples with normal allele ratio. The arrow indicated the non-specific peak at 176bp which can interfere with analysis.

### 3.6.3 Marker P (Chr 21- tube 2)

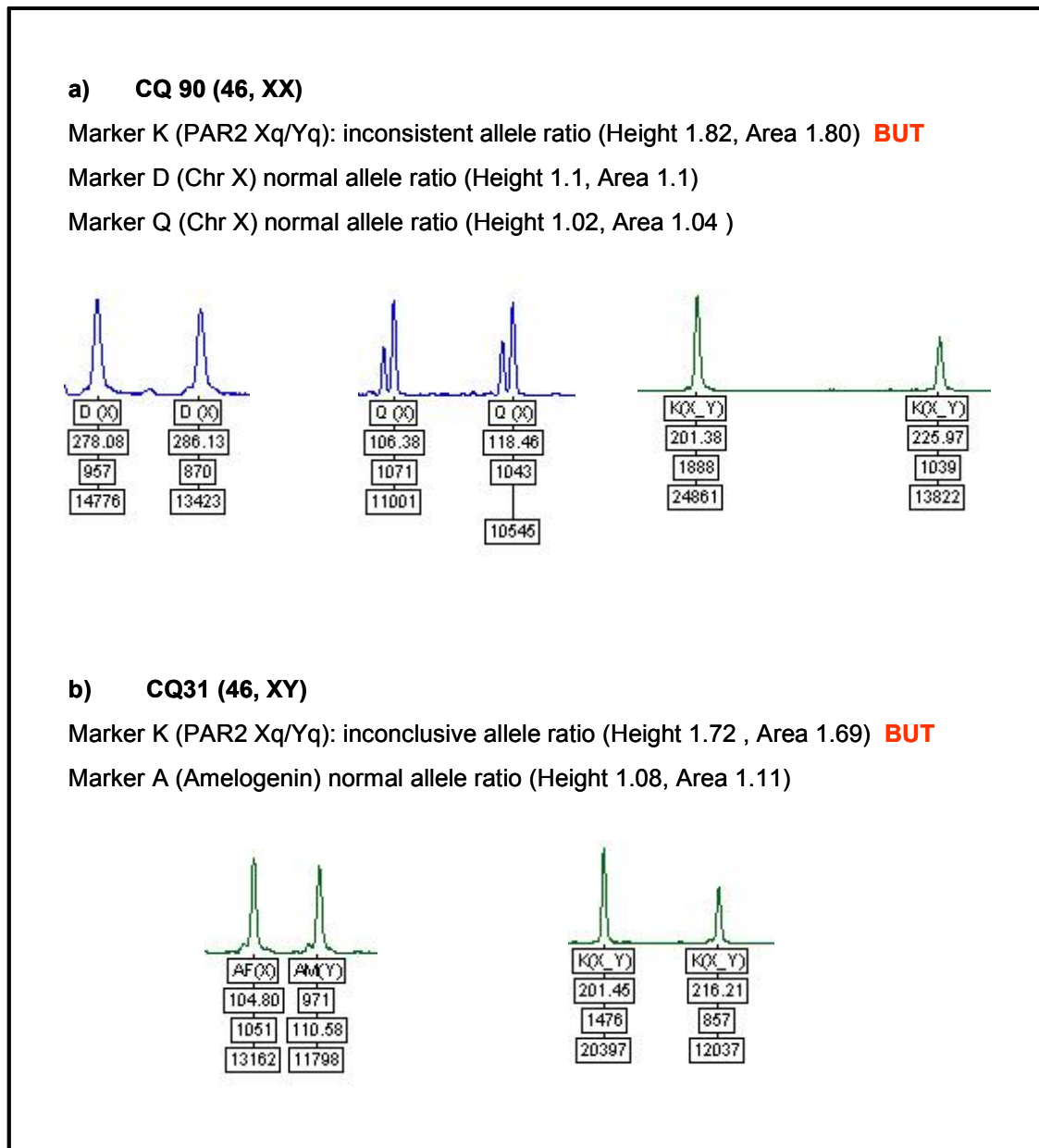
Marker P produced the highest number of inconclusive and inconsistent allele ratio results (19% of retrospectively collected samples and 42% of prospectively collected samples). The marker also failed to amplify in 8% of retrospectively collected samples and 25% of prospectively collected samples. As for marker O, the size range is 445-500bp which is the largest molecular weight amplicon in tube 2 and the failure rate increased with the poorer quality DNA from the amniotic fluid samples. Figure 5 shows representative Genotyper traces for samples with inconclusive, inconsistent, informative and uninformative allele ratios.



**Figure 5:** Marker P. Genotyper traces showing examples of a) an informative sample with normal allele ratio b) uninformative sample, c) inconclusive allele ratio and d) inconsistent allele ratio.

### 3.6.4 Marker K (Xq/Yq – tube 2)

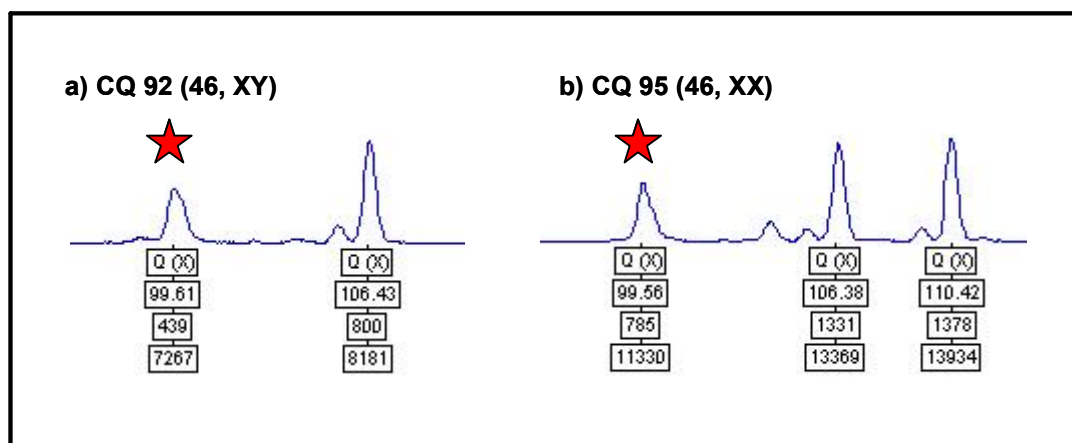
Marker K showed amplification anomalies when using DNA from the amniotic fluid samples. Inconclusive allele ratios were observed in 14 cases. Seven of these cases were male and showed normal amelogenin marker allele ratios. Four of the female samples showed normal allele ratios for one other X marker. One sample was a Turner mosaic and the remaining 2 samples did not have another informative sex chromosome marker. Inconsistent allele ratios for marker K were observed in 8 cases of which 6 were male and 2 were female. The male samples had normal amelogenin marker allele ratios, one female sample had normal allele ratios for 2 informative X chromosome markers and the remaining female sample was uninformative for other sex chromosome markers (figure 6).



**Figure 6:** Marker K. Examples of samples with a) normal female and b) normal male karyotype where marker K had inconsistent or skewed allele ratios but other sex chromosome markers showed normal allele ratios.

### 3.6.5 Marker Q (Chr X – tube 2)

There was a non-specific signal at 99.6bp for marker Q which made interpretation problematic as the peak falls within the range of analysis. Representative Genotyper traces showing examples of the non specific signal for samples which were informative and uninformative are shown in figure 7. This peak only appeared in DNA samples prepared using the InstaGene matrix.



**Figure 7:** Marker Q. Example of Genotyper traces showing the presence of the non-specific signal at 99.6bp (red star) in a) normal male and b) normal female samples.

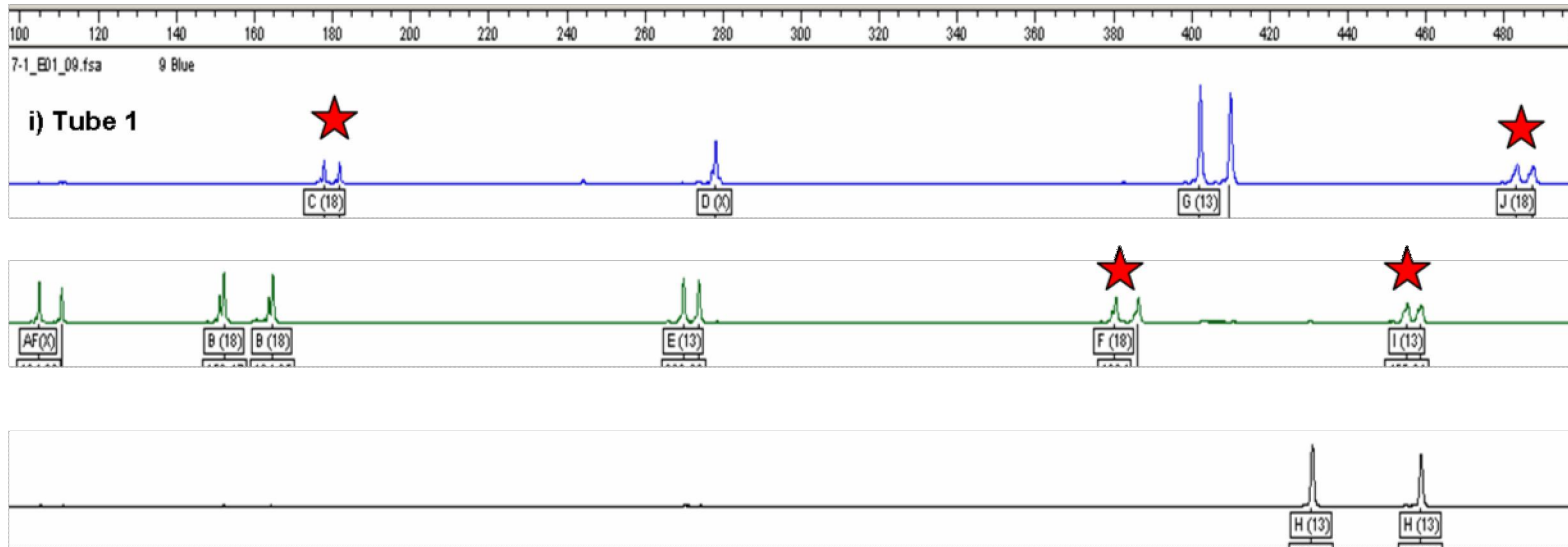
### 3.7 Unequal amplification of markers

In general, when running multiplex PCR, the lower molecular weight amplicons amplify more efficiently than higher molecular weight products. By altering primer concentrations for the various amplicons it is usually possible to ensure that amplicons of different sizes can be amplified with equal efficiency in the multiplex. During our evaluation we found that the ChromoQuant markers in tube 1 and 2 amplified with different efficiencies. For tube 1 (chromosomes 13 and 18) the retrospectively collected high quality DNA samples (from cultured cells and tissue samples) produced reliable amplification of all markers but in general marker G amplified more efficiently and markers C, F, I and J were less efficiently amplified (figure 8a). When lower quality DNA samples from amniotic fluid were analysed the higher molecular weight products (markers F, G, H, I and J) did not amplify very efficiently in relation to the smaller sized products (figure 8b). For markers G and H this did not affect the analysis because the amplicons were still strong enough to be analysed. However for markers C, F, I and J the amplicons often failed to amplify or were too weak to be reliably analysed (table 3). Unequal amplification of markers was also found in tube 2 (chromosome 21). Using the high quality DNA from the retrospectively collected DNA samples the markers generally amplified to a level that enabled analysis. However, in general, the higher molecular weight products from markers O and P (>430bp) were much less efficiently amplified than other markers in the multiplex (figure 8a). These markers were much more prone to fail to amplify in both the retrospective and prospective samples (table 3). When the DNA samples from the amniotic fluids were analysed the failure rate for these markers increased presumably due to poor DNA quality (figure 8b, table 3). This effect was far greater for markers O and P in tube 2.

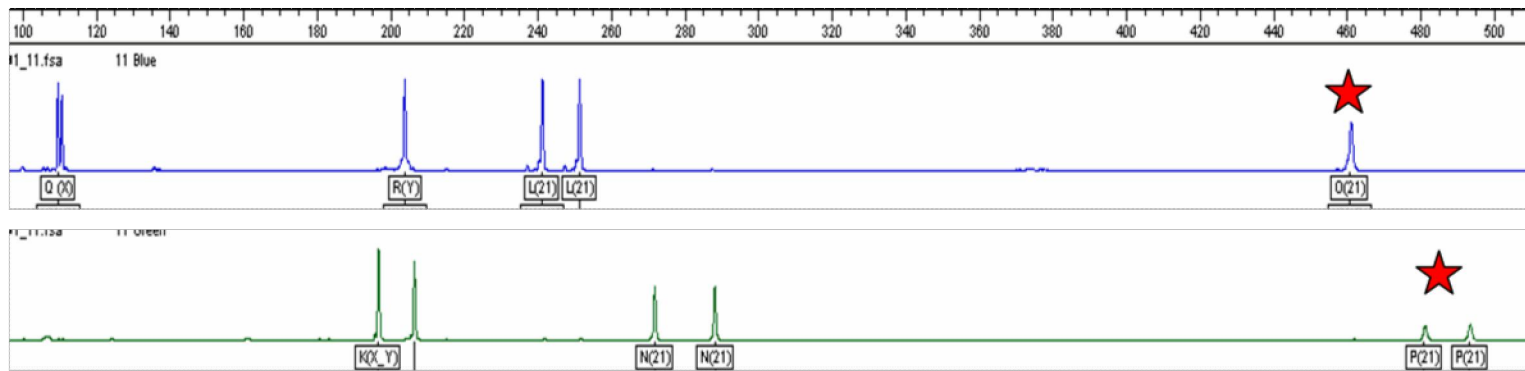
The markers that were amplifying with lower efficiency were also those that often produced inconsistent and inconclusive allele ratios (table 2). It is possible that primer concentration is playing a role in the skewing of the amplification of the alleles for these markers. Unfortunately we were unable to investigate this further since primers for individual markers are not available for this kit.

**Figure 8:** (over page) Examples of Genotyper traces from a) high quality and b) low quality DNA. The markers marked with red stars in tubes 1: C (Blue), J (Blue), F (Green), and I (Green) and tube 2: O (Blue) and P (Green) amplified with lower efficiency than other markers in the multiplex for high and low quality DNA samples. However with low quality DNA this often resulted in these markers failing to amplify or being too weak to analyse. These markers also tended to produce inconclusive or inconsistent allele ratios.

a) High Quality DNA



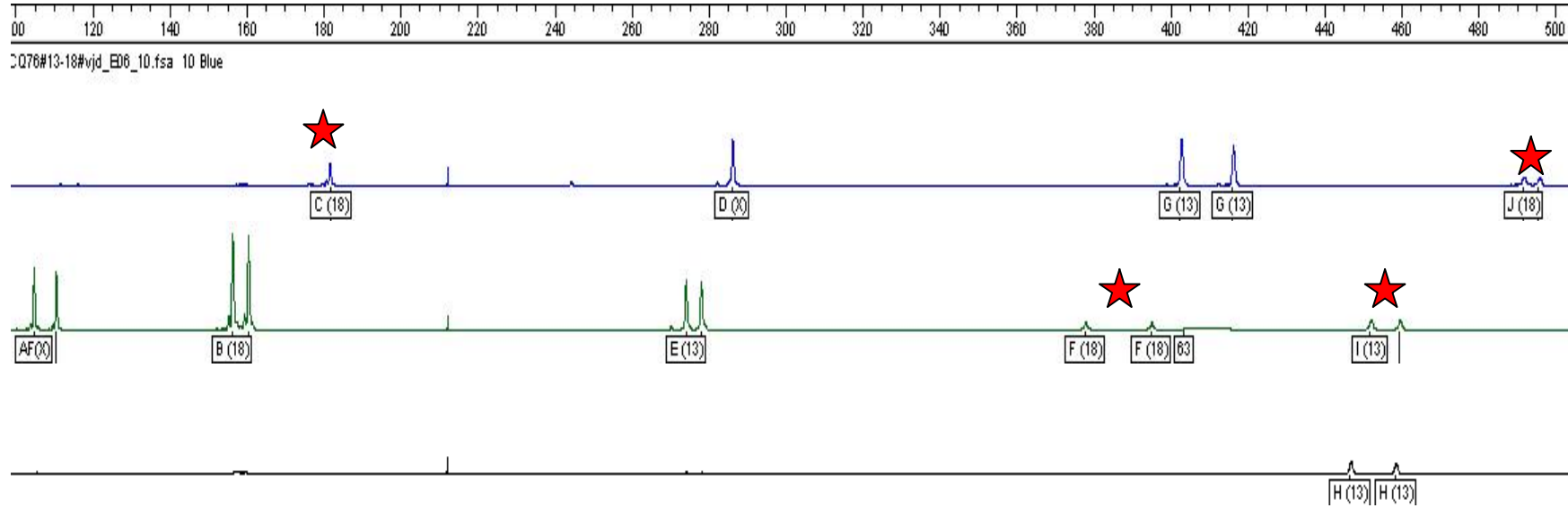
**ii) Tube 2**



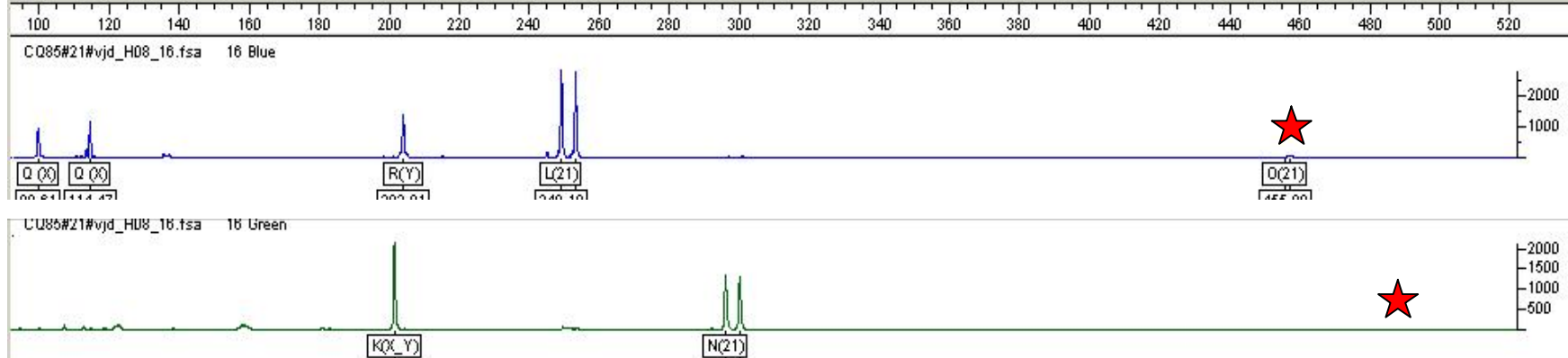


## b) Low Quality DNA

### i) Tube 1

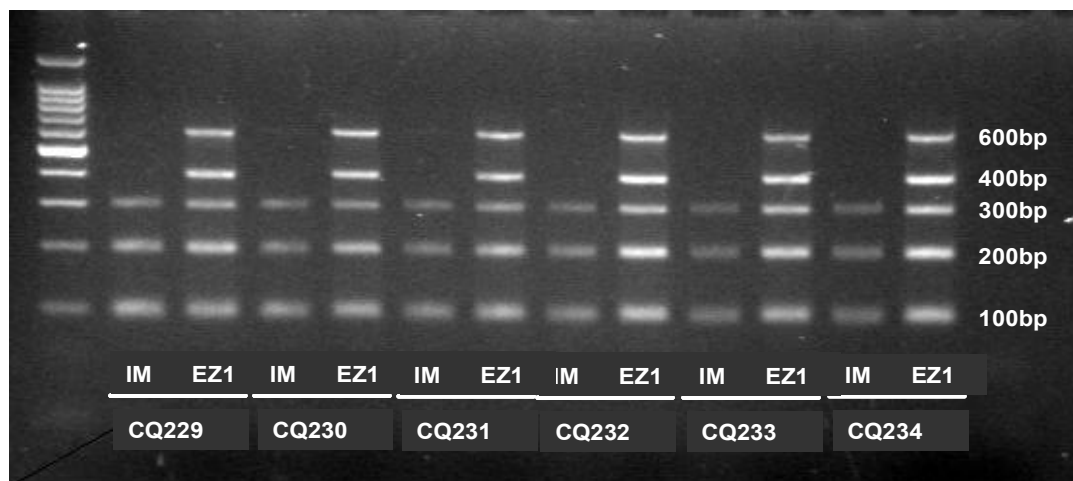


### ii) Tube 2



### 3.8 Effects of DNA extraction method

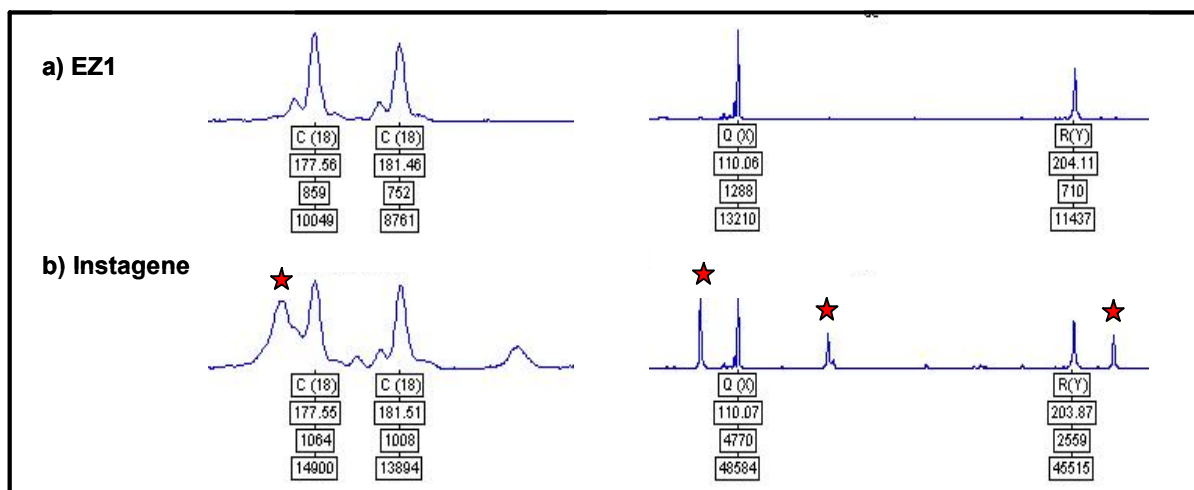
There was an obvious difference in the quality of the results obtained from the retrospectively collected samples and the amniotic fluid samples. We investigated whether the InstaGene method of DNA extraction was affecting amplification efficiency and the generation of non-specific signals when using ChromoQuant version 1. We extracted DNA from 6 amniotic fluid samples using the InstaGene matrix and the BioRobot EZ1 Workstation (QIAGEN). The duplicated samples were analysed using a control gene PCR primer set which assesses the integrity and amplifiability of DNA (van Dongen *et al.*, 2003). The control gene PCR set allows amplification of products of 100, 200, 300, 400 and 600bp with equal intensity in high quality DNA samples. The results are shown in figure 9.



**Figure 9:** Agarose gel showing results of control gene PCR. DNA extracted from 6 amniotic fluid samples (CQ229 – CQ234) using InstaGene matrix (IM) or the BioRobot EZ1 Workstation (EZ1) were amplified using the control gene PCR. IM samples amplified to 300bp whereas EZ1 samples amplified to 600bp.

The DNA extracted using the BioRobot EZ1 Workstation (QIAGEN) amplified to 600bp but the DNA extracted using the InstaGene matrix only amplified products up to 300bp. Therefore it appears that the EZ1 DNA extracted from the amniotic fluid samples may inhibit PCR less than that extracted using the InstaGene matrix.

Although the EZ1 DNA amplified more efficiently in the control gene PCR, there did not appear to be any difference in the amplification efficiency between the two sample preparations when using ChromoQuant version 1. However, the non-specific signals for markers C (Chr 18 - tube 1) and Q (Chr X – tube 2) were not found when using the EZ1 DNA samples. In general, less background and fewer non-specific peaks were detected when using the EZ1 DNA (figure 10).



**Figure 10:** Genotyper traces from sample CQ230 analysed using a) EZ1 DNA or b) InstaGene matrix DNA. Red stars indicate the non-specific peaks that are present using the IM DNA but are absent from the EZ1 samples.

### 3.9 General Comments on ChromoQuant protocol

An 8 page protocol is supplied with ChromoQuant version 1. In general this was easy to follow but there were some ambiguous instructions which we felt could be clarified. The amplification protocol is over complex with no PCR master mix being prepared in advance. This requires that 2µl of diluted Taq polymerase is added to each tube which could lead to pipetting error, possible contamination and variation in the overall composition of individual PCR reactions. The interpretation section of the protocol is very limited and no advice is given about what additional follow-up studies should be undertaken when an ambiguous result is obtained. No clear advice is available for interpretation of sex chromosome aneuploidy which is more difficult to interpret as the markers are distributed between the two tubes. There is also no troubleshooting section or recommended electrophoresis conditions.

## 4. CONCLUSIONS

ChromoQuant™ is technically easy to use, CE marked and therefore IVD compliant. However, version 1 does not currently fulfil the typical requirements of a rapid prenatal test; it is unable to cope with variable sample quality, it often produces ambiguous results which cannot be resolved due to the lack of single marker assays and the kit has a high failure rate for some markers. Only 59% of samples could have been reported using the kit in its current format.

### 4.1 Marker and Primer Information

At present, no information is made available about the markers other than their G band location. Primer sequences are also not available. This can make interpretation of ambiguous results difficult since supporting published evidence for causes of ambiguous results (e.g. submicroscopic duplications) cannot be used unless the molecular locations of the markers are known. The lack of primer sequences means that information about common polymorphisms which may lie under primer binding sites cannot be assessed. There is also a possibility that lack of marker information may cause problems for reporting since labs would be unable to report the exact regions that have been analysed. This could be particularly important for an inconsistent marker result. Marker (but not primer) information is available for ChromoQuant version 2 which was released in August 2005.

### 4.2 Informativity

Markers C (chromosome 18) and O (chromosome 21) had low heterozygosity (60 and 56% respectively). 13.5 % of samples required testing with additional markers for 18 and 21 so that there were at least two informative markers present for these chromosomes. These markers have subsequently been replaced in ChromoQuant version 2.

### 4.3 Single Marker Assays

Individual primer pairs for the markers used in tubes 1 and 2 are currently not available. CMGS best practice guidelines recommend that single marker assays should be performed for markers exhibiting inconclusive allele ratios. Where the skewed ratios are caused by primer site polymorphisms the inconclusive result can usually be resolved by retesting the single marker with a lower annealing temperature in the PCR. In our study severely skewed allele ratios for some markers often resulted in results consistent with the presence of three alleles. It is possible that these too could be resolved using single marker assays. In theory it would be possible to repeat the entire multiplex at a lower temperature but this would almost certainly be prohibitively expensive with 30% of samples in our study requiring such analysis. Single marker assays will not be available for ChromoQuant version 2.

### 4.4 Sex Chromosome Markers

The inclusion of sex chromosome markers in the kit may be problematic for some labs for the reasons discussed in section 3.4. Although ChromoQuant did accurately detect two cases of sex chromosome aneuploidy in the retrospectively collected samples an unambiguous result was not possible for 4 other cases of sex chromosome aneuploidy; 47, XXX

47, XYY  
45,X / 47, XXX  
45,X / 46,Xr(X).

Three cases that had a karyotype 45, X were shown to be indicative of monosomy X but 4 cases with normal female karyotypes were also shown to be indicative of monosomy X. In our opinion there are

insufficient polymorphic sex chromosome markers included in the kit for it to be used as a robust diagnostic screen for monosomy X. We feel that the sex chromosome markers should be made available as a separate PCR and that additional autosomal markers could then be added to tubes 1 and 2 and additional sex chromosome markers could be added to the new reaction. The lack of extra X and Y chromosome markers meant that ambiguous results could not be resolved. Although extra X and Y markers are available in ChromoQuant version 2 there are still sex chromosome markers distributed between tubes 1 and 2.

#### **4.5 Unbalanced Amplification**

The amplification efficiency of markers varied and although for higher quality of DNA this did not represent any problem, for the lower quality DNA samples markers C, F, I, J, O, and P often failed to amplify or amplified with skewed allele ratios. This was particularly a problem for markers C, P and O with 90.4% of skewed allele ratio results and 27% of all marker failures involving these three markers. If the multiplex conditions had been optimized to allow markers to be amplified with the same efficiency these problems may not have been encountered. Markers C, O and P have been replaced in ChromoQuant version 2.

#### **4.6 DNA Extraction**

The method of DNA extraction appeared to have an effect on the quality of the results obtained. When using DNA extracted from amniotic fluid samples we found that, in general, less background and fewer non-specific peaks were detected when using the EZ1 DNA.

#### **4.7 ChromoQuant Version 2**

ChromoQuant version 2 was released in August 2005 and has a number of improvements including the replacement of markers C, P and O. A preliminary report is attached.

## 5. REFERENCES

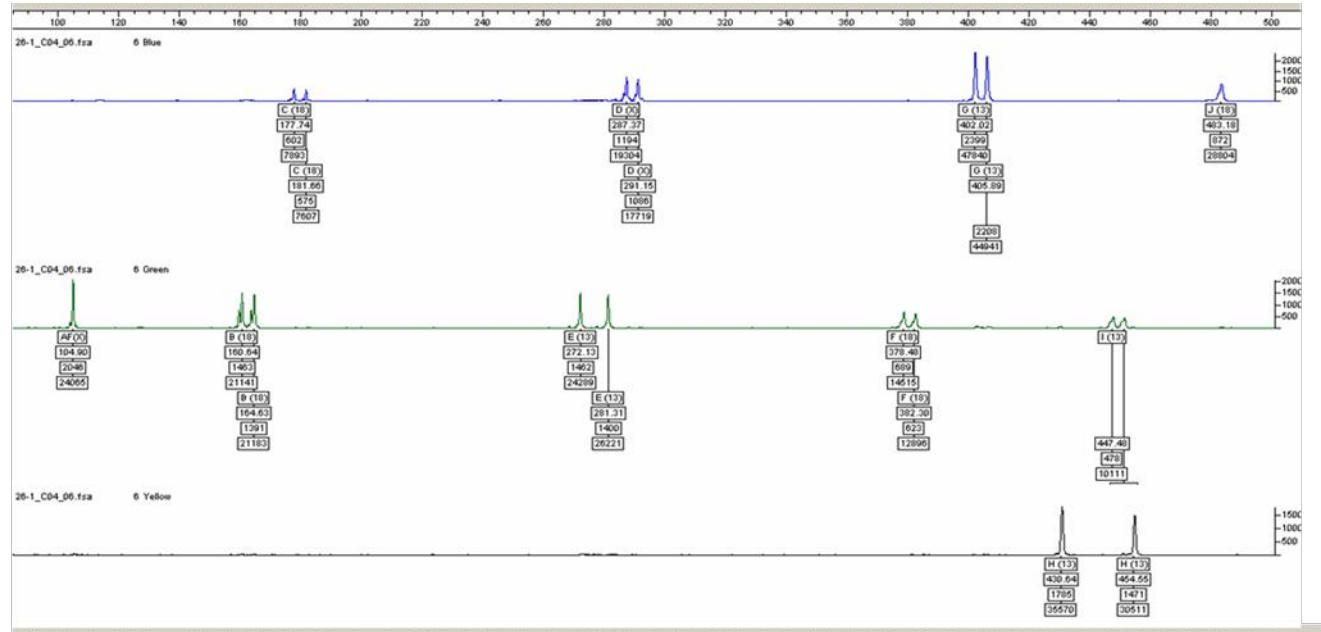
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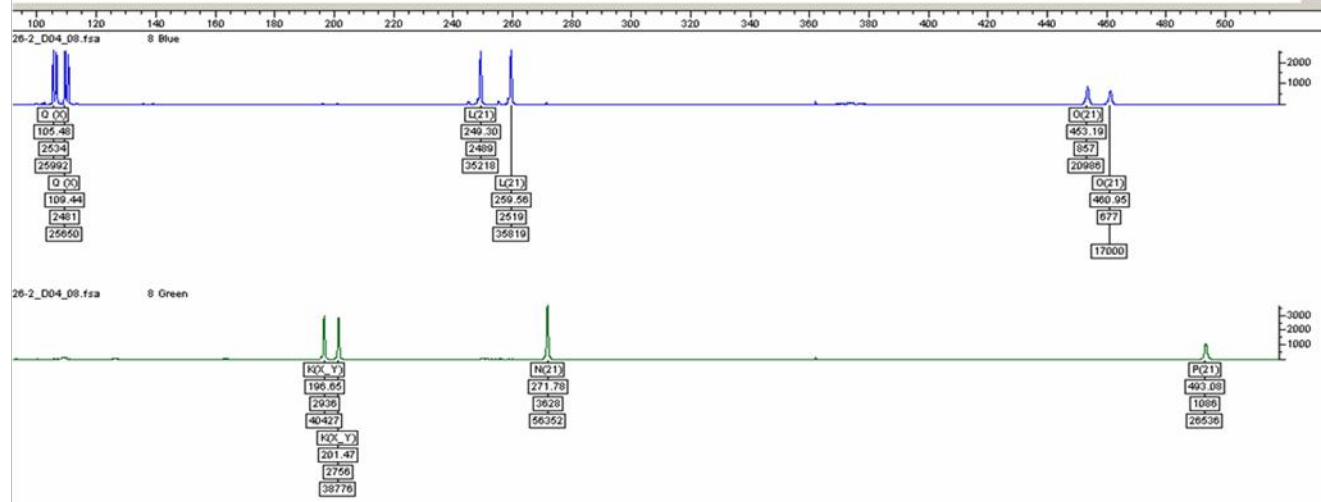
## **6. APPENDIX 1**

### **EXAMPLES OF GENOTYPER TRACES FROM CHROMOQUANT VERSION 1**

**Tube 1**



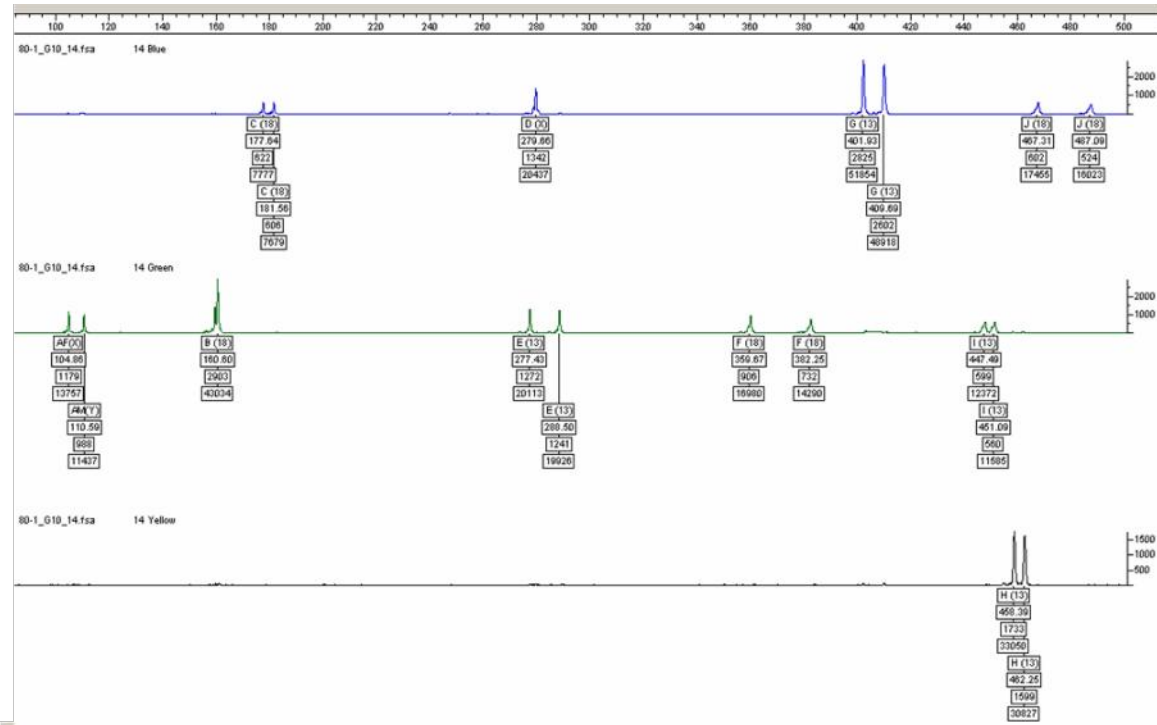
**Tube 2**



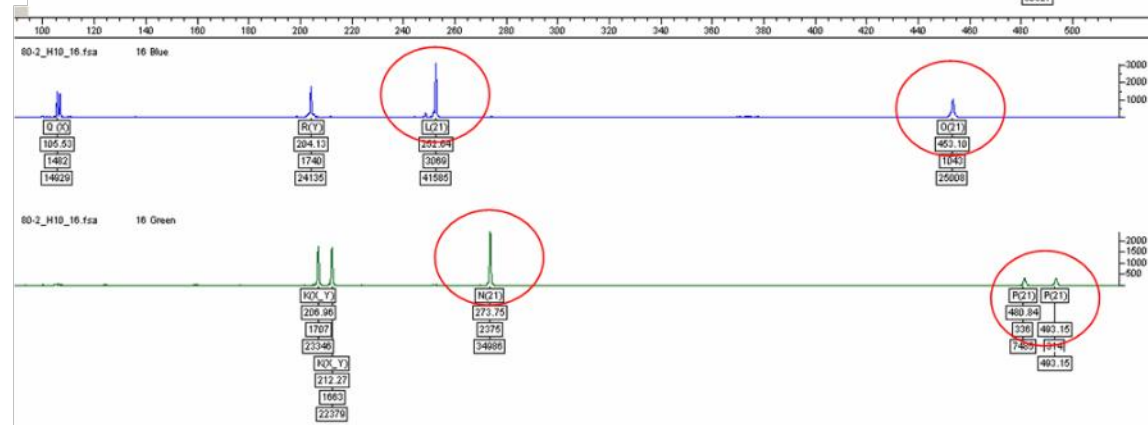
**CQ26:** Karyotype 46, XX. Typical genotyper traces for tubes 1 and 2



**Tube 1**

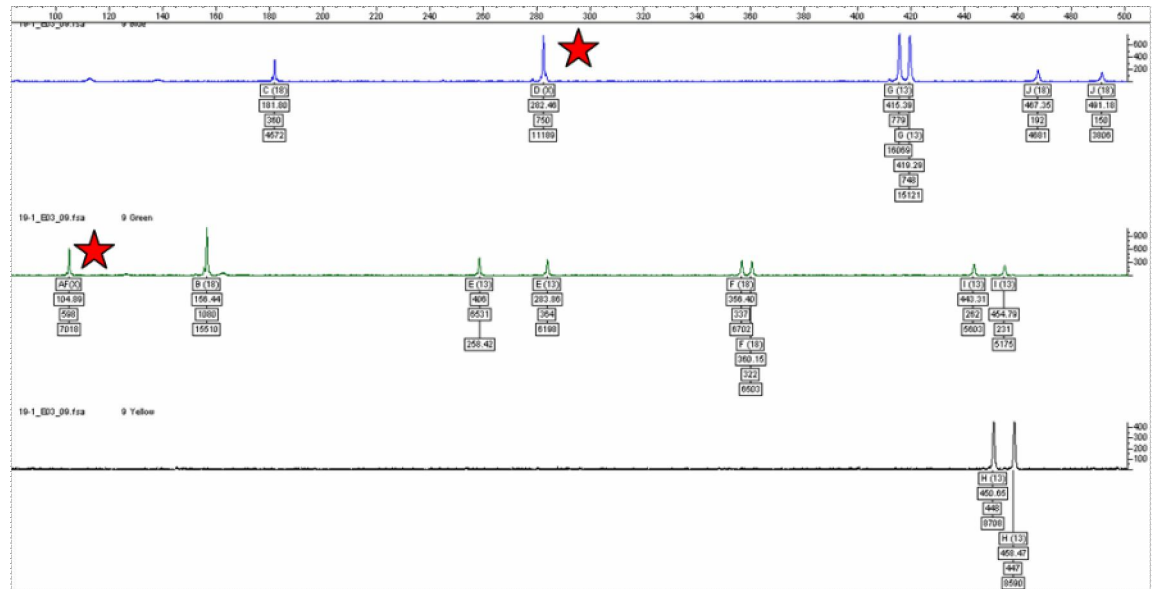


**Tube 2**

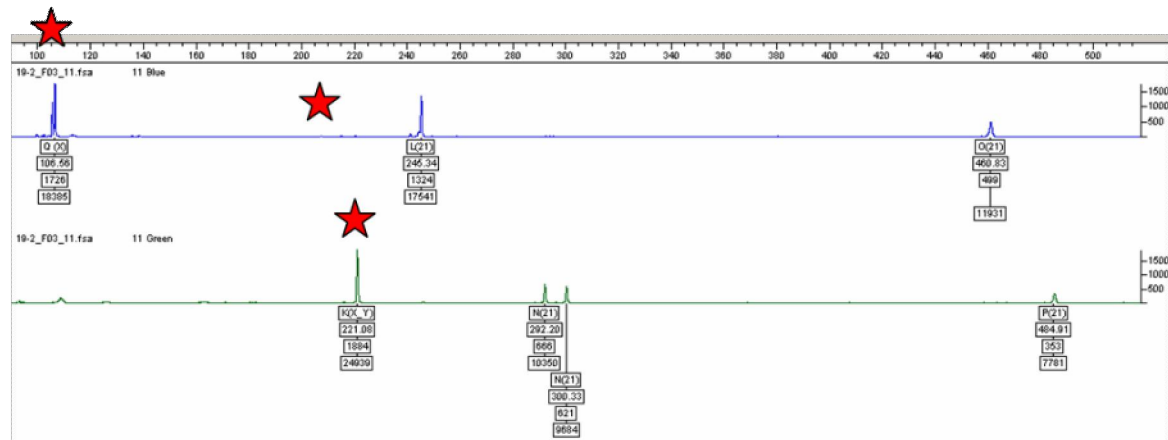


**CQ80:** Karyotype 46, XY. Sample has only one marker informative for chromosome 21 (marker P)

Tube 1

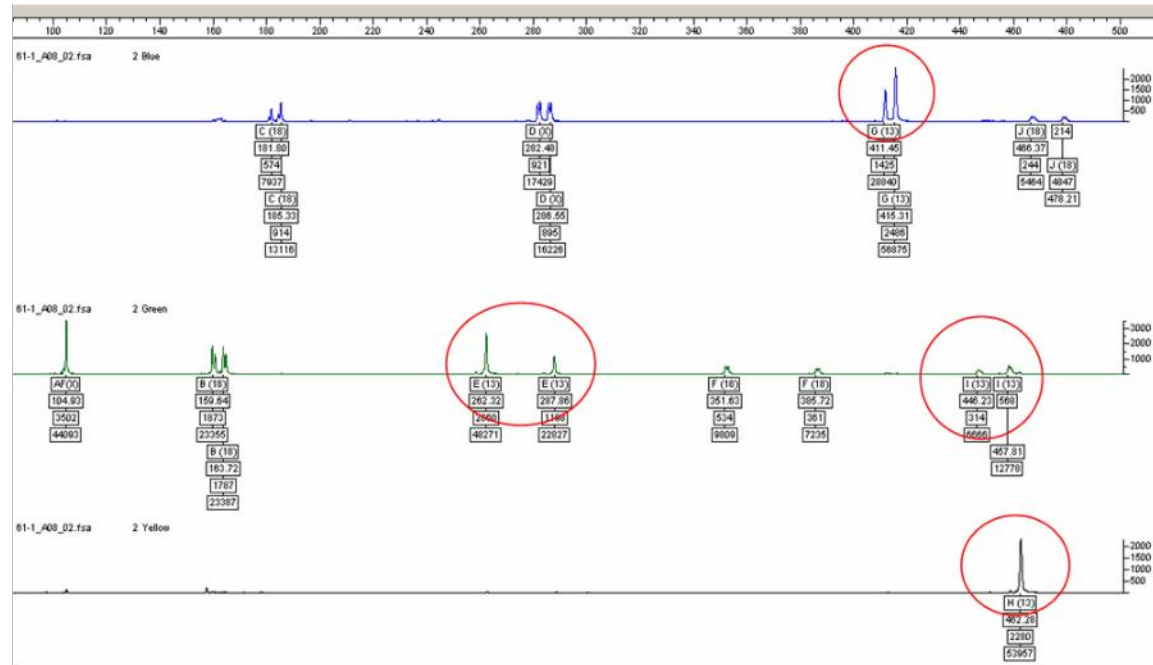


Tube 2

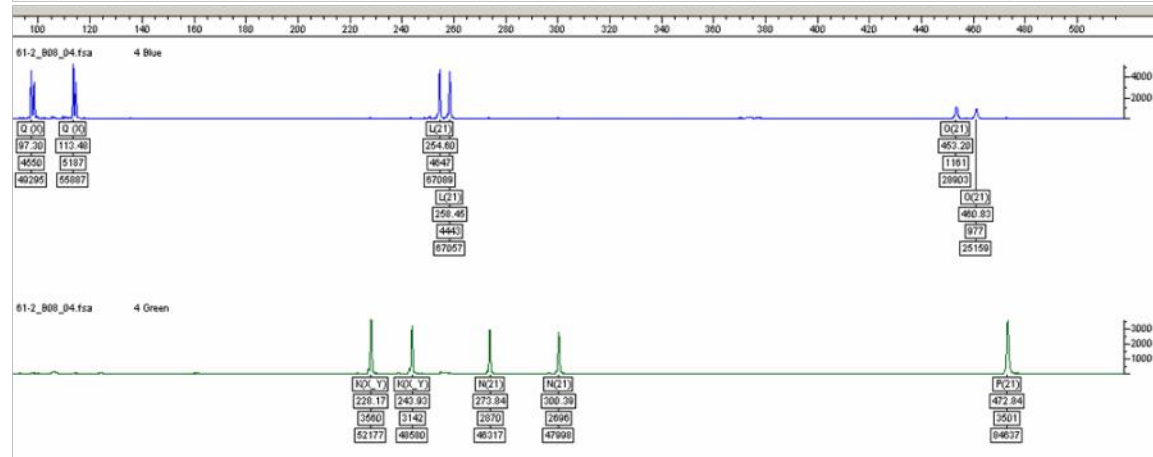


**CQ19:** Karyotype 46, XX. No Y signal present (marker A and R) and all polymorphic X markers uninformative. Sample would require additional testing to exclude the diagnosis of Turner syndrome. Position of sex chromosome markers indicated by red stars.

**Tube 1**

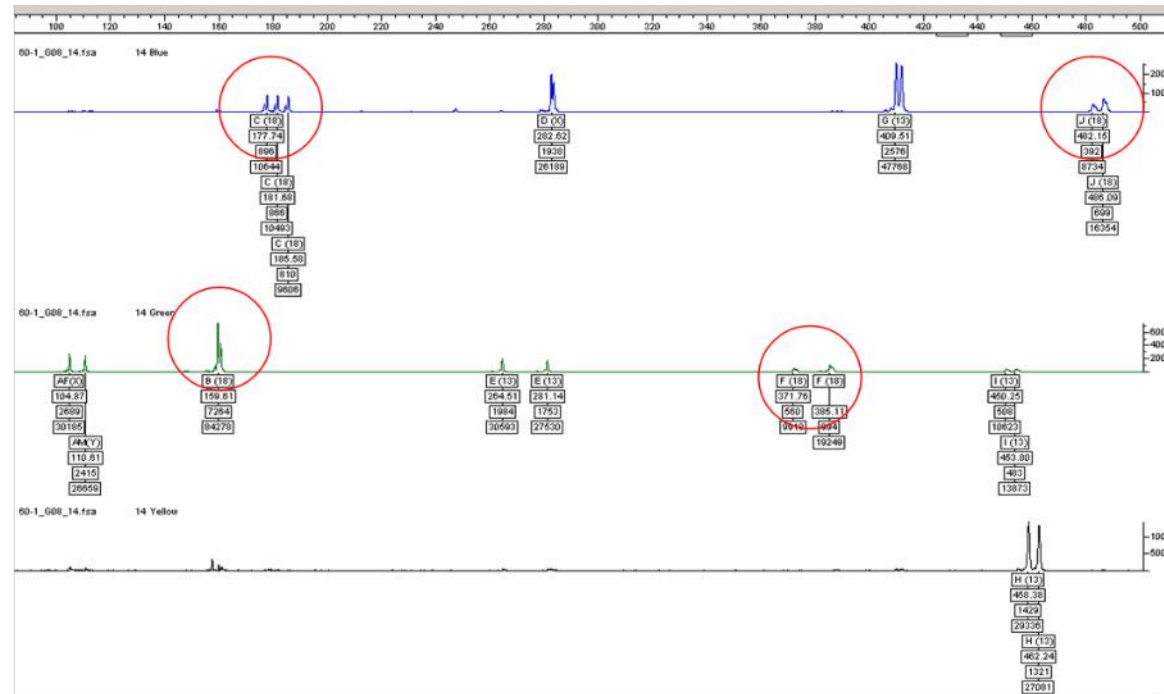


**Tube 2**

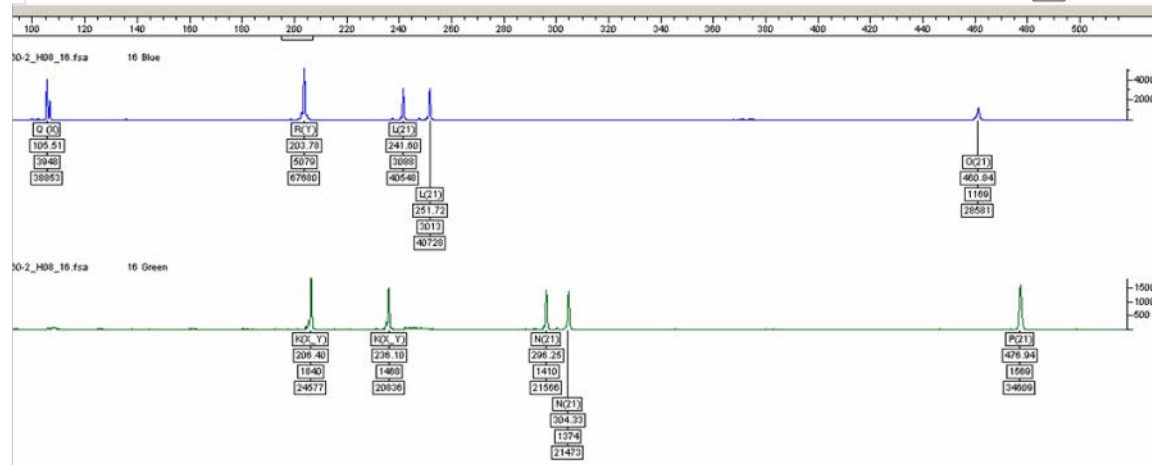


**CQ61: Karyotype 47, XX, +13. Markers for chromosome 13 and ringed in red**

Tube 1

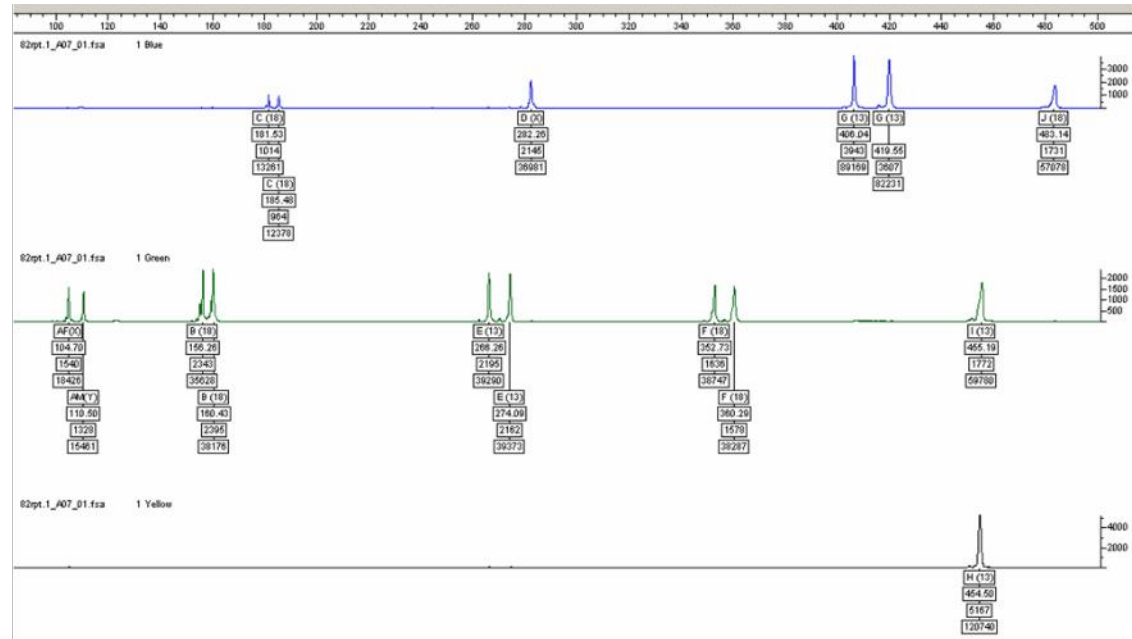


Tube 2

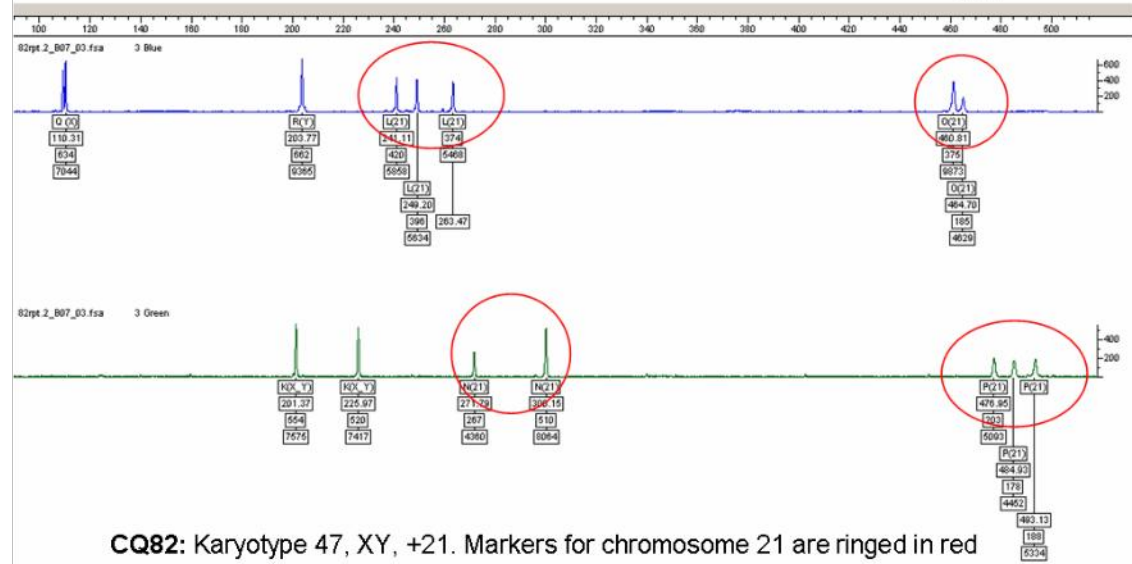


CQ 60: Karyotype 47, XY, +18. Markers for chromosome 18 are ringed in red

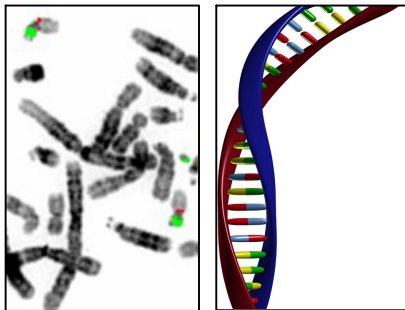
**Tube 1**



**Tube 2**



**CQ82:** Karyotype 47, XY, +21. Markers for chromosome 21 are ringed in red



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