QF-PCR: application, overview and review of the literature

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ABSTRACT
Quantitative fluorescent polymerase chain reaction has been in diagnostic use in the UK for over 10 years and has proved to be a cost-effective, robust and accurate rapid prenatal test for common aneuploidies. Specific advantages include detection of triploidy, mosaicism and maternal cell contamination. Its application at our centre is described, with developments including stand-alone testing and improvements in strategies for the preparation and testing of chorionic villus biopsies. © 2012 John Wiley & Sons, Ltd.

BACKGROUND
In the 1950s, the correct human chromosome complement was finally established,1 followed 3 years later by the discovery of the aetiology of Down syndrome.2 Prenatal diagnosis of trisomy 21 could then be offered as a diagnostic test for women perceived to be at increased risk of a pregnancy with Down syndrome; women at increased risk were initially identified based on maternal age, and later by increasingly sophisticated screening approaches.3,4 The culture and karyotyping of cells from amniotic fluid (AF), and later from chorionic villus (CV) samples, to identify foetuses with trisomy 21 and other chromosome abnormalities has been carried out worldwide since the late 1960s or early 1970s.

Karyotype analysis of cultured prenatal samples has a reporting time of around 2 weeks because of the need to grow sufficient numbers of cells and for the analysis and interpretation of the resulting banded chromosome preparations. Where women were identified with a particularly high risk (such as ultrasound markers highly indicative of Down syndrome or a serum screen risk of, for example, greater than 1 in 5), the importance of rapid confirmation or exclusion of Down syndrome was realised, to allow decisions to be made about pregnancy management. The availability of fluorescence in situ hybridisation (FISH) technology led in the mid 1990s to the introduction of rapid testing of uncultured cells to identify chromosome 21 copy number in these cases.5 The use of additional probes for chromosomes 13, 18 and the sex chromosomes allowed the identification not only of Down syndrome pregnancies, but also those with Patau syndrome (trisomy 13), Edwards syndrome (trisomy 18) and sex chromosome aneuploidy, within 24–48 h. Because FISH is labour-intensive and the fluorescent probes are expensive, publicly funded centres using this technology, such as those in the UK, could only apply it to a limited number of samples. However, once the service was introduced, there was increasing demand from obstetricians and patients for rapid testing to be available more widely.

Introduction of QF-PCR
Quantitative fluorescent polymerase chain reaction (QF-PCR) can be applied to the detection of chromosome copy number by amplification of repeat sequences at polymorphic loci. These repeat sequences are amplified by PCR, and the labelled products are separated by gel electrophoresis. An allelic pattern of two equal peaks within the same chromosomal region is diagnostic of two copies of the target region, whereas three peaks within the same chromosomal region or two peaks with a ratio of 2:1 are indicative of trisomy for the target region (see Figure 1). This diagnostic approach was first suggested in 1993,6 and prospective studies were carried out in the mid-1990s,7 followed by the development of a QF-PCR-based test for sex chromosome imbalance.8,9 These studies indicated that this methodology has significant advantages over FISH, being more robust, less labour-intensive and more suitable for large sample numbers. However, it was not until 2001 that the first report of clinical application of this test appeared.10 QF-PCR was introduced into the UK National Health Service (NHS) as a validated diagnostic test in 200011 and has since been introduced into other UK Genetics Centres, as well as being offered privately in the UK12 and in Europe.13–15 Primer pairs for the polymorphic loci (markers) are multiplexed together to give a rapid, efficient and inexpensive diagnostic test for trisomy 13, 18, 21, sex chromosome aneuploidies and triploidy; commercial kits are now available from a number of different companies. Best practice guidelines have been developed by a collaboration between the Association of Clinical Cytogenetics (ACC) (http://www.cytogenetics.org.uk/) and the Clinical Molecular Genetics Society (CMGS) (http://www.cmgs.org), and a 2012 version has been ratified by both councils. In
The quality and size of the original CV biopsy. For very small samples, it may be difficult to interpret the villus structure, and cleaning may be problematic.

addition, a UK Molecular Rapid Aneuploidy External Quality Assessment Scheme has been in place since 2004. DNA extraction is generally from uncultured AF or CV samples; sample preparation, extraction protocols, and strategies for follow-up karyotyping vary between testing centres.

Following the introduction of rapid aneuploidy testing, the benefit of a rapid result to women who had been told of an increased risk of common aneuploidy was apparent, as a normal result alleviated their anxiety, whereas an abnormal result allowed earlier decisions on pregnancy management. The targeting of rapid testing to only those with a particularly high risk was no longer necessary with the introduction of QF-PCR instead of FISH, as the higher throughput and lower extra peaks, requires experienced analysis of QF-PCR traces.

Incidence of discrepant results is therefore likely to vary between centres. For instance, a recent paper by Holgado et al.20 reported a 1/815 incidence of completely discrepant results in CV samples, whereas the incidence at our centre is <1/10000.23

ASSAYS

Large multiplexes have evolved as an efficient way to test multiple loci. Outside of the UK, assays are designed to test for both sex chromosome aneuploidy and the viable trisomies, and thus, autosomal and sex chromosome markers are multiplexed together. However, in the majority of UK NHS laboratories, most samples are tested for the autosomal trisomies and triploidy, whereas a separate sex chromosome assay is applied in a minority of cases, specifically those referred with an indication of monosomy X; this is because testing, which reveals incidental findings of debatable clinical significance such as triple X and XYY in prenatal samples, is generally not considered to be justified. For centres using PCR as a stand-alone test for samples referred for increased trisomy risk only, the targeted nature of this testing means that inclusion of sex chromosome markers is not required. However, where karyotyping is carried out, any abnormality detected will generally be reported to the referring clinician. Thus, our centre tests all prenatal samples with a single multiplex PCR assay containing 17 microsatellite markers for chromosomes 13, 18 and 21 (see Figure 2); separate chromosome-specific assays with additional markers are used for inconclusive and uninformative results and for

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample type</th>
<th>Number tested</th>
</tr>
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<tbody>
<tr>
<td>Levett et al.19</td>
<td>AF</td>
<td>5000</td>
</tr>
<tr>
<td>Ogilvie et al.37</td>
<td>AF + CVS</td>
<td>9080</td>
</tr>
<tr>
<td>Puzova et al.42</td>
<td>AF + CVS</td>
<td>6349</td>
</tr>
<tr>
<td>Cirigliano et al.28</td>
<td>AF + CVS</td>
<td>43 000</td>
</tr>
<tr>
<td>Hills et al.61</td>
<td>AF + CVS</td>
<td>9737</td>
</tr>
<tr>
<td>Holgado et al.20</td>
<td>CVS</td>
<td>22 825</td>
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All the above studies describe cohorts of samples with a range of standard referral indications and provide the results of follow-up karyotyping for all samples, with the exception of Hills et al., which reports on two sets of samples, one with QF-PCR as stand-alone and one with follow-up karyotyping.

AF, amniotic fluid; CVS, chorionic villus sampling; QF-PCR, quantitative fluorescent polymerase chain reaction.

Table 1 Publications describing results from large cohorts of prenatal samples tested by QF-PCR

Figure 1 A diagrammatic illustration of four results from a single QF-PCR marker. A trisomy result is either triallelic indicating meiotic generation of the abnormal cell line or biallelic consistent with either meiotic or mitotic generation of the abnormal cell line.
confirmation of abnormality. A separate sex chromosome assay is applied to approximately 20% of referrals including those with a nuchal translucency of 4 mm or greater. Sex chromosome assays are now recommended to include an X/autosome paralagous marker, which allows the relative number of X chromosome sequences to be calculated by comparison to autosome sequence copy number. TAF9L (3p24.2/Xq21.1) is now widely used and provides a more confident diagnosis of monosomy X as well as distinguishing between triple X and monosomy X/XX mosaicism. Additional sex chromosome markers are required for sex determination and identification of other sex chromosome abnormalities.

ESTABLISHING A RAPID DIAGNOSTIC SERVICE

Quantitative fluorescent polymerase chain reaction aneuploidy testing lends itself to a cost-effective rapid diagnostic service with next-working-day reporting; however, this requires investment, which may not be justified for smaller sample numbers. In our centre, 97% of samples are reported the working day following sample receipt; this requires two daily QF-PCR runs to confirm sample identity of abnormal results and resolve inconclusive/uninformative results. In addition, back-up equipment and staff are required to maintain reporting times, and molecular genetic and cytogenetic expertise jointly contribute to a correct interpretation of results and, hence, a high-quality service. QF-PCR consumable costs/sample are estimated to be approximately £5 including DNA preparation, PCR, analysis, repeat testing and controls. Contact time/sample is calculated at 14 min laboratory technician time, including kit preparation and equipment maintenance, and 18 min scientist time, including analysis and report writing. These figures are based on testing approximately 5000 samples/year.

SAMPLES WITHOUT RESULTS

The incidence of samples where no QF-PCR result is possible depends on many factors including sample quality, assay design and robustness, and analysis criteria. Failure to obtain a genotype is reported in <1/1000 prenatal samples, an impressive figure given the range of sample quantities and qualities. Although uninformativity is often described as a disadvantage of a QF-PCR approach, <1/2000 samples are uninformative when a multiplex containing five markers for each of chromosomes 13 and 21, and six markers for chromosome 18 are used. The most frequent reason that samples do not receive a result is significant maternal cell contamination (MCC) of AF samples. In our data set 2.1% of AF samples exhibit significant MCC; determining copy number in these samples is not recommended (QF-PCR BPG 2012), and therefore, no result is available. Because of the rapid turn-around time for PCR results, samples can be set up and cultured if required, without compromise of culture due to the age of the sample; full karyotype analysis of cultured cells is usually successful in these cases.

MATERNAL CELL CONTAMINATION, MOSAICISM AND RARE EVENTS

Use of microsatellite markers to determine sequence copy number also provides a genotype of the tested sample. This has the benefit of identifying other cells present in the sample, which may represent MCC, mosaicism, chimerism, a twin genotype or external contamination. The identification of MCC in both uncultured AF and CV samples is essential to minimise...

Figure 2: A Genotyper profile of a trisomy 18 sample amplified in a single QF-PCR multiplex and analysed on a 3100 genetic analyser. Size in base pairs is shown on the horizontal axis, fluorescent units on the vertical axis. Peaks are labelled with marker name. Heterozygous markers for chromosomes 13 and 21 exhibit two allele peaks with peak areas in a 1:1 ratio. Markers represent trisomy 18 as either three allele peaks in a 1:1:1 ratio (D18S535, D18S391, D18S386, D18S819 and D18S976) or 2:1 biallelic ratios (D18S978 and D18S390). Circles identify the informative markers. This result is consistent with a trisomy 18 conception.
misdiagnosis. MCC gives a characteristic pattern for markers on every chromosome and can be distinguished from mosaicism in most cases.\(^{29}\) MCC is most frequently found in bloodstained AF samples\(^{29}\) although it is notable that in some cases, the contaminating blood is foetal in origin (unpublished data). Where there is low-level MCC, a QF-PCR result can be issued; this occurs in approximately 6% of AFs\(^{29}\) whereas significant MCC, where a result is not available, occurs in 2.1% of AFs\(^{27}\) In cases of significant MCC genotyping of maternal blood samples is unhelpful, as the foetal genotype cannot be interpreted in the presence of significant levels of a second genotype. Some centres require maternal blood samples with every prenatal sample, whereas others request them only when there is a single female genotype in a heavily bloodstained fluid or for interpretation of unusual results. For CV samples the incidence of MCC is dependent on the quality of the original CV biopsy and subsequent dissection; in our testing consortium, significant MCC occurs in approximately 0.25% of CV biopsies. If a second genotype is identified in a female CV sample, then both the QF-PCR and karyotype result should be interpreted with care, as the majority cell line may be maternal in origin. A maternal blood sample may be required for confident interpretation of results, as for AF samples.

Trisomy cell lines may be generated by meiotic or mitotic nondisjunction events. Abnormal QF-PCR results that exhibit at least one marker with three different length alleles represent meiotic errors and a trisomy conception (see Figure 2). Those that show only 2:1 and 1:2 biallelic ratios represent either meiotic or mitotic errors and may therefore represent a normal conception with post-zygotic error giving rise to mosaicism. In a UK audit,\(^{30}\) 7.4%, 17% and 10.3% of trisomy 13, 18 and 21 results, respectively, had no triablecric results. However, the number of markers used will affect the proportion of abnormal samples with only biallelic results. A mitotic origin of the abnormal cell line in CVS may indicate confined placental mosaicism; reports detailing such results should therefore recommend waiting for the full karyotype result in the absence of ultrasound abnormalities.

It is possible to detect mosaicism in the tested sample at a level of approximately 15% if the abnormal cell line exhibits a triablelic QF-PCR result or 20% if only biallelic ratios are observed. Donaghue \textit{et al.}\(^{26}\) reported that 0.1% of AF and 0.6% of CV samples exhibited mosaicism in either uncultured or cultured cells or both. Cell lines present in AF are very likely to represent the true foetal genotype. However, QF-PCR detection of mosaicism in uncultured CV samples may be due to confined placental mosaicism, and analysis of cultured mesenchyme and/or a follow-up AF sample is recommended in these cases. Sample preparation should ensure the extraction of DNA from the mesenchyme as well as trophoderm layers of the villi\(^{22}\); however, an abnormal cell line detected in these preparation may nevertheless be confined to the trophoderm.

The QF-PCR genotype provides information not available from other testing methods. For instance, twin pregnancies can be identified as either dizygotic or probable monozygotic, which can be clinically useful, and indeed may indicate if one twin has been sampled twice. In addition, molar pregnancies and chimaeras are detected; in our tested population, we have identified three chimaeras, all of which had a cell line shown to be of uniparental origin in addition to a normal biparental cell line.

\textbf{MIXED RESULTS}

The presence of both normal and abnormal QF-PCR marker results on the same chromosome may represent clinically significant partial chromosome imbalance or a polymorphism with no clinical effect. The location and number of abnormal results may inform the interpretation of a partial chromosome imbalance (i.e. detectable on G-banded chromosome analysis), found to occur in approximately 0.03% of samples\(^{27}\); such imbalance can be confirmed by karyotype/FISH studies. However, single marker abnormal results may represent copy number variants\(^{27}\) primer site polymorphisms\(^{24}\) or somatic microsatellite mutations\(^{27}\) and should be interpreted with care. Parental samples may be required for confident interpretation, and therefore, it is important to minimise the incidence of these polymorphisms by careful marker selection and validation.

\textbf{PRODUCTS OF CONCEPTION}

A QF-PCR approach can be used to diagnose aneuploidies in foetal tissue samples, either to confirm prenatal aneuploidy or combined with multiplex ligation-dependent probe amplification (MLPA) as an alternative test to karyotype analysis.\(^{31}\) The MLPA test uses detects copy number abnormalities of the subtelomere regions of every chromosome and will therefore detect whole chromosome aneuploidy, terminal deletions or duplications, and derivative chromosomes from balanced rearrangements.\(^{31}\) Triploidy is not identified by MLPA in female samples and can be difficult to detect in male samples. In addition, mosaicism for an abnormal cell line may not be identified. QF-PCR is therefore required in addition to MLPA in order to diagnose triploidy and detect mosaicism, and can also be designed to identify other trisomies in addition to the common prenatal aneuploidies. QF-PCR is applied first and detects abnormalities in approximately 16% of POC samples,\(^{32}\) which do not therefore need to proceed to MLPA testing. Follow-up karyotype analysis of parental samples is required to assess the recurrence risk of some abnormalities. Although it is estimated that a QF-PCR/MLPA approach does not detect 2.7% of nonmosaic imbalance identified by karyotype analysis,\(^{31}\) the diagnostic success rate is much higher at 95% compared with 70% success rate for culture and karyotype analyses, the approach is cost-effective, and reporting times are lower than for culture and karyotyping.\(^{31}\) This is a timely development as sample numbers are predicted to rise following the Royal College of Obestetricians and Gynaecologists (RCOG) recommendation that POCs should be tested instead of parental bloods for the investigation of recurrent miscarriages (http://www.rcog.org.uk/womens-health/clinical-guidance/investigation-and-treatment-couples-recurrent-miscarriage-green-top-). This recommendation has been made following data indicating that karyotyping of couples suffering from recurrent miscarriages is an inefficient diagnostic testing strategy.\(^{32}\) Testing of a POC instead of parental bloods gives direct information on the cause of that miscarriage and is therefore more useful than the indirect
exclusion of a balanced rearrangement in the parents. In addition, some balanced translocations are benign and not causative of a couple’s miscarriages, which therefore need to be further investigated.

ADVANTAGES OF QF-PCR OVER OTHER RAPID ANEUPLOIDY DIAGNOSTIC APPROACHES

A number of other techniques, detailed in the accompanying reviews in this issue of Prenatal Diagnosis, are available for rapid diagnosis of the viable aneuploides/polyploides. However, it is important to point out the significant strategic and performance differences between the approaches so that an optimum service is available to patients. A disadvantage of the other molecular-based approaches is their inability to identify triploids; for triploid samples, the comparative analysis between chromosomes used by MLPA, BACs-on-beads and array comparative genomic hybridization may appear as either a normal diploid or MCC result. Other clinically important benefits of QF-PCR are an ability to identify other cell lines at levels of 10% (MCC in samples from female foetuses) and 20% (mosaicism). It has proven to be extremely robust with all sample types and is particularly amenable to sample batching, which results in a cost-effective service that can generate results in 6 h from the sample receipt. However, potential drawbacks of QF-PCR are (1) that cost-effectiveness is dependent on sample throughput and (2) that a genetic analyser is needed, which must be available for priority use by the prenatal service, conditions that may be difficult to maintain in small diagnostic laboratories. For these reasons, samples from all four major diagnostic laboratories in London are tested at a single centre.

QF-PCR AS A STAND-ALONE TEST

The implementation of QF-PCR as an initial, rapid test for aneuploides has raised the question of the utility of following this test with full karyotype analysis where there is no indication for non-trisomy chromosome abnormality. This possibility was first suggested in 2001 and has since been widely considered, with a number of retrospective audits published. These audits generally took the form of examining karyotype results for cohorts of prenatal samples, and establishing the number and nature of abnormal results that would not have been detected by QF-PCR alone. Overall, these audits suggest that the prevalence of non-trisomy chromosome abnormalities of clinical significance in women at raised risk of trisomy is around 0.07%, close to the prevalence in the general population. However, with the higher-resolution testing now available, the prevalence of detectable significant non-trisomy abnormalities in the general population will be higher.

Since then, two models for the implementation of QF-PCR as a stand-alone test have been introduced. The first model, implemented by the Karolinska Institute, Stockholm, gives women who have no increased risk of a non-trisomy chromosome abnormality, the choice of a rapid test for trisomies or a full chromosome analysis, but not both. The second model involves testing all prenatal samples by QF-PCR, regardless of referral indication, but lays down criteria for full karyotyping of a subset of samples. This model has been introduced in the UK, in the centres funded by the London Genetic Commissioners. The results of the first 2 years of implementation have been published. In a cohort of over 9000 samples received in this period, 7284 had no indication for karyotype analysis; 25 (0.3%) of these received a normal QF-PCR result but subsequently had an abnormal karyotype detected either prenatally as a privately funded test or postnatally. Of these 25 samples (those without subsequent abnormal ultrasound findings), five had a chromosome abnormality associated with a poor prognosis, representing 0.069% of samples referred for trisomy testing, consistent with the prevalence estimated from the retrospective audits.

THE FUTURE

With the imminent introduction of more sensitive and specific non-invasive screening for Down syndrome, the number of women having invasive testing will decrease. For those with a high risk of Down syndrome, invasive testing to confirm the screening results will be a requirement prior to pregnancy termination, and QF-PCR should be considered as the method of choice for this rapid confirmation. In addition, some women at low risk following non-invasive screening will present later for invasive testing following foetal anomalies detected on ultrasound screening. For these women, a preliminary QF-PCR test to confirm the absence of a trisomy should precede the more expensive whole genome test, whether G-banded chromosome analysis or array comparative genomic hybridization. Thus, QF-PCR will continue to have an essential role in the prenatal diagnosis of the future.

WHAT’S ALREADY KNOWN ABOUT THIS TOPIC?

- QF-PCR has been applied to the rapid prenatal diagnosis of the common aneuploides for more than ten years.
- It has proven to be a reliable, accurate and robust test.

WHAT DOES THIS STUDY ADD?

- This review considers methodology, data, issues and results specific to prenatal samples, and the advantages of QF-PCR over other available options.
- Reviews applications to products of conception.
- Reviews application as a stand-alone test.
REFERENCES