Expert Review 01

Introduction to QF-PCR

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About the author

Kathy Mann, PhD, is a Principal Clinical Scientist within the Cytogenetics Department, GSTS Pathology, based at Guy's Hospital, London UK. With colleagues she developed and established the first QF-PCR service for the rapid detection of prenatal aneuploidy in the UK National Health Service (NHS) in 2000, a service which now processes >4,000 samples/year. The laboratory has since published more than 15 peer-reviewed papers related to the field of QF-PCR including new service developments such as stand-alone testing, which have subsequently been adopted by other laboratories, and their recent report detailing the results of the more than 40,000 prenatal samples. Other publications detail QF-PCR assays and best practice and describe phenomena such as mosaicism, maternal cell contamination, CNV and somatic microsatellite mutations. Kathy co-authored the first draft of the UK QF-PCR Best Practice Guidelines (2005) and has edited subsequent versions including the 2012 Guidelines. She has been an assessor for the Molecular Rapid Aneuploidy (MRA) UK NEQAS since its inception in 2004 and she was a speaker and joint organiser of the International QF-PCR Workshops from 2003-2008.

Introduction

Rapid diagnosis of aneuploidy using quantitative fluorescence-PCR (QF-PCR) has improved prenatal care for many tens of thousands of women in the past decade. Pregnancies identified at being at increased risk of chromosome abnormality by prenatal screening programmes are given a rapid and accurate result. The most common chromosome abnormalities seen at prenatal diagnosis are trisomy for chromosomes 21, 18 and 13 and monosomy X, which result in the live born phenotypes of Down, Edwards, Patau and Turner syndrome, respectively, and triploidy, all of which can be detected by QF-PCR analysis.

The application of short tandem repeat (STR) analysis for the detection of aneuploidy was first demonstrated by Elaine Mansfield in 1993 (Mansfield, 1993) who described trisomy 21, trisomy 18 and triple X genotypes in DNA prepared from amniotic fluid. Development and validation through the 1990s resulted in the first QF-PCR-based prenatal diagnostic services which were described in 2001 (Cirigliano, 2001; Levett, 2001; Mann, 2001). Due to the technique's impressive rapidity, economy of scale, minimal labour requirements and assay costs, QF-PCR has replaced Fluorescence in Situ Hybridisation (FISH) analysis of interphase nuclei for the prenatal diagnosis of aneuploidy in the majority of European countries and some South American and Australasian laboratories. Numerous studies have been published detailing successful QF-PCR services and the analysis of more than 100,000 prenatal samples (Levett, 2001; Putzova, 2008; Cirigliano, 2009; Badenas 2010; Holgado, 2011; Mann, 2012).

Principle of QF-PCR

QF-PCR analysis exploits the variability of microsatellite sequences (STRs), that vary in length at any given locus to distinguish between chromosome homologues. The detection and quantitation of STR alleles enables relative chromosome quantitation (see Figure 1).

One of the advantages of QF-PCR over other approaches for rapid aneuploidy detection is its ability to identify features associated with prenatal samples such as mosaicism and maternal cell contamination. Analysis of the sample genotype is able to identify if more than one cell line is present which may indicate sample mosaicism or maternal cell contamination or could represent twin, chimeric or external sources of DNA; mosaicism and maternal cell contamination give characteristic allele patterns and can usually be confidently categorised (see Expert Review 02: Detecting mosaicism with QF-PCR and Expert Review 03: QF-PCR and maternal cell contamination). The genotype can also be used for sample identification by comparison with a second aliquot of the original sample or a maternal or paternal genotype.

Assay design

The incidence of uninformative results for any particular STR marker can be deduced from its heterozygosity value The higher the heterozygosity the more diverse is the STR's allele size range resulting in fewer uninformative results. STR loci with high heterozygosities are therefore chosen for QF-PCR analysis. Although this strategy minimises the number of uninformative results they still occur and therefore QF-PCR assays include several STRs per chromosome. In addition, polymorphisms that result in aberrant allele patterns can, rarely, mimic a trisomy result and therefore more than one informative and consistent marker result per chromosome is recommended for a reportable result (UK Best Practice Guidelines, 2012). Thus, in order to provide enough informative markers for the majority of samples and to streamline the technical process, multiplex PCR reactions have been developed containing tens of markers which are co amplified in

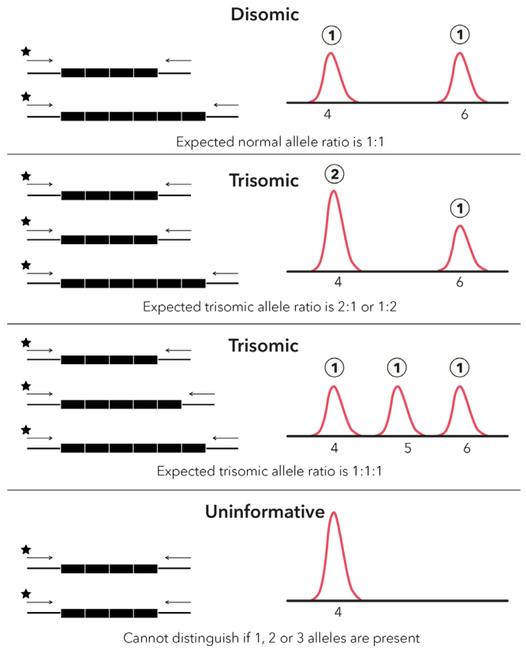

Fig. 1 A set of primers, one of which is labelled with a fluorophore (★), is designed to amplify a specific STR from the chromosome of interest. Following PCR amplification from sample DNA, the amplified STR alleles are size-separated by polymer electrophoresis on a capillary genetic analyser. The amount of fluorescence generated from each allele is recorded as a peak on an electropherotogram. Dedicated software determines allele length (base pairs) with the use of a size standard, and assigns allele peak height and peak area (arbitrary fluorescent units) both of which are proportional to the amount of starting sequence. Thus, if there are two or more different length alleles at any given locus then the sample is hetero-zygous at that locus and is informative for QF-PCR analysis. The number and/or ratio of the allele peaks represent the number of alleles at that locus such that two alleles in a 1:1 ratio represent two chromosome homologues, whilst three allele peaks in a 1:1:1 ratio or two allele peaks in a 1:2 or 2:1 ratio represent three chromosome homologues and thus trisomy. Uninformative results are loci that display a single allele peak and thus no information can be obtained regarding the number of chromosome homologues.

the same assay using identical PCR conditions (Figure 2) (Mann, 2012). However, since robust multiplex assays can be technically difficult to establish and maintain, the availability of commercial QF-PCR assays has proven a welcome development.

The inclusion of di and trinucleotide repeats in QF-PCR assays is generally avoided as PCR amplification of these short repeated sequences generates larger stutter peaks that compromise result interpretation. Therefore, tetranucleotide repeats which generate smaller stutter peaks are most frequently used.

Finally, in order to increase the likelihood of detecting the rare segmental trisomies, marker location should be considered. It is preferable that markers are identified that cover the length of each chromosome and that the short arm of chromosome 18 and the Down syndrome critical region on chromosome 21 are both represented.

Sex chromosome markers

The identification of monosomy X requires a different approach from the detection of trisomy; although polymorphic X chromosome STR markers can confirm the presence of two or more X chromosomes, a single allele peak may represent one or more X chromosomes that have the same allele and is therefore not diagnostic of monosomy X. The use of X-chromosome counting markers, non polymorphic paralogous sequences located on both the X chromosome and an autosome, has resolved this issue and is now recommended practice (UK Best Practice Guidelines, 2012). This strategy exploits a known sequence length difference between the paralogous sequences; both are amplified using a single primer pair, identified according to size differences and, assuming the presence of two autosomes, the number of X chromosome homologues can be deduced (Figure 3). A monosomy X sample is differentiated from a normal male by the presence

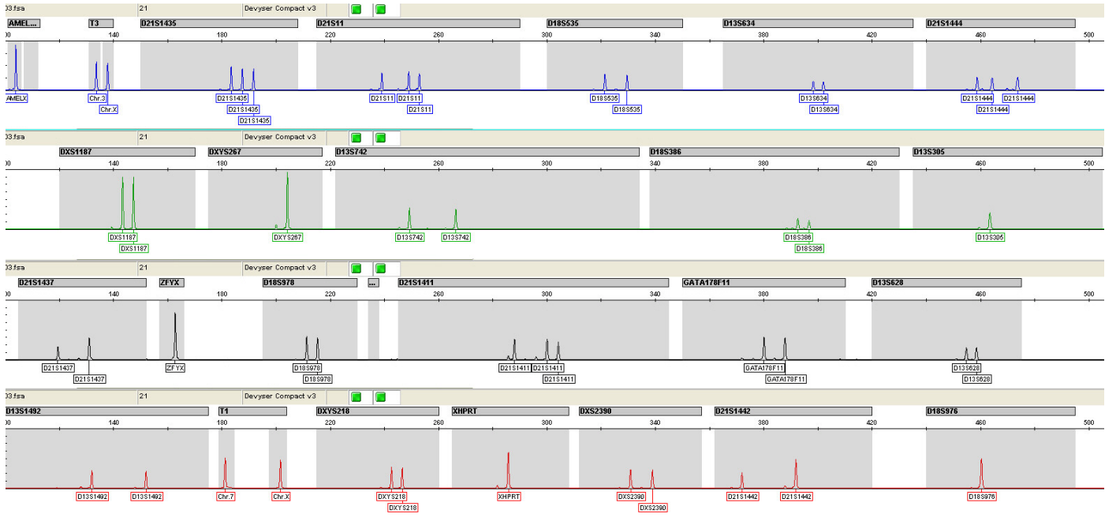

Fig. 2. Electrophoretogram showing a trisomy 21 result; twenty six markers from chromosomes 13, 18, 21 X and Y are amplified together in a single multiplex. Chromosome 21 markers show either 1:2 (D21S1437, D21S1442) or triallelic (D21S1435, D21S11, D21S1444, D21S1411) allele patterns consistent with three copies of chromosome 21.
Chromosome 13 and 18 markers either show two alleles in a 1:1 ratio or are uninformative consistent with normal copy number.

or absence of Y-specific sequences (again, due to the risk of polymorphisms more than one Y sequence is required). The inclusion of X chromosome STR markers and markers that investigate the ratio of X to Y sequences, such as the amelogenin locus (Sullivan, 1993), are required for the identification of XXY (Klinefelter syndrome) and other sex chromosome aneuploidies.

Sample processing

QF-PCR can be applied to any sample type (chorionic villi (CV), amniotic fluid (AF), fetal tissue and fetal and postnatal blood samples) once DNA of sufficient quantity and quality has been prepared. In this regard QF-PCR is a robust assay; as little as 1-3ng of DNA is required and a relatively crude preparation of DNA is sufficient for successful analysis. As the strategy compares alleles amplified using the same primer pair, variation in PCR dynamics caused by differences in DNA quality, salts and/or proteins applies to both alleles and generally does not result in skewed allele ratios. Even partially degraded DNA from fetal tissue samples is usually successful, particularly for the shorter length markers. However, prenatal samples are notoriously variable in terms of both sample quantity and quality; CV samples can range from a single villus to tens of mgs of material whilst AF samples taken at 14-15 weeks gestation may contain few cells and blood staining may be significant and either maternal or fetal in origin. In addition, samples must be shared with other testing pathways; cultures are usually established and material may be required for biochemical assays or DNA-based analyses for monogenic or genomic disorders. Thus, any DNA preparation method must be able to provide DNA of suitable quality and quantity for QF-PCR analysis, should ideally use the same procedure for all sample types, be quick and have few tube transfers to minimise DNA loss and the risk of sample mix-up. For these reasons, many labs use a basic chelex-based prep (Cirigliano, 2001; Mann, 2004) which can be applied to all sample types, takes just 15 minutes, requires small amounts of starting material, involves no tube transfers and can be adjusted for variations in sample quality. This method involves a cell lysis step followed by the addition of a chelex to chelate metal ions that may degrade DNA and/or inhibit the PCR. The amount of chelex can be adjusted depending on the amount of starting material available, and the addition of an initial H2O wash step or repeat chelex extraction can greatly improve the results in blood-stained samples. The robustness of this DNA preparation approach is demonstrated by the fact that fewer than 1/1000 samples fail to produce a genotype (Mann, 2012). Other labs prefer automated extraction although data regarding the success of an automated approach with different sample qualities and quantities is not available.

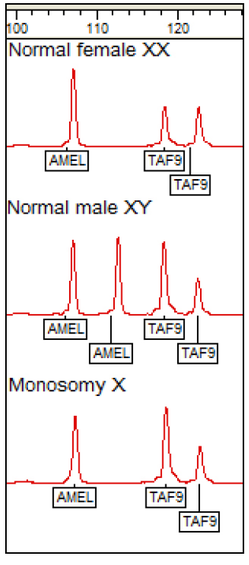

Fig. 3 Electrophoretogram showing the AMEL alleles (106 bp (Xp22.2) and 112 bp (Yp11.2)) and paralagous TAF9 alleles (118 bp (3p24.2) and 123 bp (Xq21.1)) (Deutsch, 2004). The presence or absence of the AMEL Y allele combined with the number of X chromosomes shown by TAF9 identifies monosomy X, although additional sex chromosome markers (not shown) are required to confirm this result and to diagnose other sex chromosome aneuploidies (normal copy number)

Best practice guidelines for QF-PCR

Although assays and markers vary between laboratories, best practice has been established over the past decade with minimal standards described in the current version of the UK Best Practice Guidelines ratified by both the Association of Clinical Cytogeneticists (ACC) and the Clinical Molecular Genetics Society (CMGS) (UK Best Practice Guidelines, 2012).

Clinical practice

Since a QF-PCR approach for rapid aneuploidy was first described as a prenatal service, it has become an integral part of prenatal testing throughout Europe. A UK Molecular Rapid Aneuploidy (MRA) External Quality Assessment (EQA) scheme has been in place since 2004 (Ramsden, 2007) and is open to non-UK laboratories. DNA samples exhibiting normal and abnormal results, mosaicism and maternal cell contamination are distributed and results and reports are evaluated. Issues that arise from the MRA EQA scheme then inform the UK best practice guidelines and minimal standards. Although a QF-PCR approach lends itself to a cost-effective rapid diagnostic service with next working day reporting, this requires investment which may not be justified for smaller sample numbers. Our centre processes approximately 4000 prenatal samples/year and 98% of samples are reported the working day following sample receipt (Mann, 2012); 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 genetics and cytogenetic expertise contribute to a quality service.

In 2006, rapid testing for aneuploidy was recommended as a standalone testing strategy by the UK National Screening Committee (http://www.screening.nhs.uk/) for referrals without ultrasound anomalies, but at increased risk of trisomy 21 due to maternal age, serum markers or a previous trisomy 21 pregnancy. There is no evidence that these pregnancies are at greater risk of having a structural chromosome abnormality than the general population (Ogilvie, 2005); audits show that between 0.07 and 0.1% of pregnancies will have a clinically significant chromosome abnormality that is not detected by this strategy. Therefore in a public health system with limited resources it is difficult to justify the additional testing. Other benefits of this strategy are that parental anxiety is minimised; incidental structural chromosome abnormalities are not identified and women do not have to wait up to 2 weeks for a final report. Following consideration of these issues, a standalone QF-PCR testing strategy was implemented in the London region (Hills, 2010); approximately 25% of prenatal samples were investigated with both QF-PCR and karyotype/aCGH analysis whilst 75%, referred with an increased risk of trisomy 21, did not fulfil the criteria for full chromosome analysis. However, all samples with an abnormal QF-PCR result are karyotyped in order to evaluate the recurrence risk for future pregnancies.

QF-PCR has proven to be a highly effective initial test for products of conception (POCs) and fetal tissue samples; an integrated approach where QF-PCR is combined with aCGH, MLPA or karyotype analysis is a cost-effective strategy as recent data shows that up to 32% of samples have an aneuploidy detectable by an extended QF-PCR assay that also tests chromosomes 15, 16 and 22 (Donaghue, 2013). These abnormal samples have shorter reporting times and are not then subject to the more expensive test. In addition, the QF-PCR failure rate in these notoriously poor quality samples is close to zero (Donaghue, 2009). However, it is important to note that follow-up karyotype analysis of parental samples is required to assess the recurrence risk of some abnormalities.

QF-PCR assays have proven to be useful for the rapid analysis of new born blood samples with referrals that indicate aneuploidy, triploidy or ambiguous genitalia. The same extraction and analysis procedures can be applied as those for prenatal samples. In addition, clarification of structural chromosome abnormalities, particularly those involving the sex chromosomes, may be assisted by QF-PCR analysis.

Data collections and abnormality rates

Several large prenatal QF-PCR data sets have been published: 5,000 AF samples (Levett 2001); 6,349 prenatal samples (Putzova, 2008); 43,000 prenatal samples (Cirigliano, 2009); 22,825 CV samples (Holgado, 2011) and 40,624 prenatal samples (Mann, 2012). These describe the results of a total of 70,236 AF and 41,798 CV samples. Regarding AFs, there have been no reported complete discrepancies between a diagnostic QF-PCR result and the follow-up analysis of cultured amniocytes, fetal tissues or postnatal blood. QF-PCR analysis of CV samples is more complicated due to mosaicism which may be confined to different regions of the placenta. Thus, the incidence of discrepant QF-PCR and karyotype results is likely to be determined by the quality and size of the original CV biopsy, sampling of the biopsy, DNA preparation method and analysis methods and will therefore vary between centres. This is discussed in more detail in the accompanying paper.

Abnormality rates vary between data sets and depend on the referral parameters; populations tested due to maternal age have a lower abnormality rate than those identified through combined or integrated Down syndrome screening programmes. Pregnancies with abnormal ultrasound findings have the highest risk of trisomy. In recent years the screening programme in the UK has evolved from maternal age to serum and ultrasound markers, including nuchal translucency. This is demonstrated by the difference between our QF-PCR abnormality rate reported in 2001 as 6.5% and in 2012-2013 as 14% (CV 19%, AF 9%).

Limitations

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 (Mann, 2004), an impressive figure given the range of sample quantities and qualities. Although uninformative marker results are often described as a disadvantage of a QF-PCR approach, using the larger available multiplexes <1/1600 samples have an uninformative chromosome (Mann, 2012). The most frequent reason that samples do not receive a result is significant maternal cell contamination (MCC) of AF samples, discussed in the accompanying paper. 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 these QF-PCR results which occur in 0.03% of samples (Mann, 2004) and can be investigated by karyotype/FISH studies. However, single marker abnormal results may be caused by primer site polymorphisms, somatic microsatellite mutations (Mann, 2003) or copy number variants (CNVs), and should be interpreted with care (a list of markers known to be within rare CNVs is available from kathy.mann@gsts.com). 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.

"As a cost-effective rapid strategy that can detect mosaicism, QF-PCR will continue to have an essential role in prenatal diagnostic strategy."

The future

The transformation of prenatal screening due to the introduction of free fetal DNA testing for Down syndrome is underway; free fetal DNA in the maternal circulation is analysed using next generation sequencing technologies with specificity and sensitivity rates that greatly surpass current screening programmes (Palomaki, 2011). The number of pregnancies identified as being at high risk of trisomy 21 and requiring an invasive prenatal test will reduce significantly. However, confirmation of the screening result is required and the speed of the diagnostic confirmatory test will be critical, as will the identification of mosaicism. As a cost-effective rapid strategy that can detect mosaicism, QF-PCR will continue to have an essential role in prenatal diagnostic strategy.

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