ACGS best practice guidelines for use of Quantitative Fluorescence-PCR for the detection of aneuploidy v4

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/Introduction and scope

QF-PCR analysis of microsatellite markers is widely used for rapid diagnosis of common aneuploidies and triploidy in prenatal, fetal tissue and newborn blood samples; aneuploidy mosaicism, maternal cell contamination and zygosity are also identified. These recommendations should be used in conjunction with other relevant ACGS guidelines (http://www.acgs.uk.com/quality/best-practice-guidelines/). They should be used within local testing and reporting arrangements and assume that patient consent has been obtained. They assume that the analytical process takes place in an appropriate, accredited laboratory setting where routine aspects of good laboratory practice such as sample tracking and record keeping are in place. They incorporate the standards required by the United Kingdom Accreditation Service and Medical Laboratories – Requirements for Quality and Competence (ISO 15189:2012) and by statute (Clinical Governance), whilst considering current practice in the UK. It must be noted that these recommendations are minimum requirements and that professional judgement is of paramount importance for many circumstances. The use of 'shall' or 'must' in this document indicates a requirement and the use of 'should' indicates a recommendation. In addition, the use of 'acceptable' highlights an area where more than one approach is satisfactory and 'unacceptable' indicates areas where the quality of the service may be compromised. Where there appears to be contradiction between available recommendations/guidelines, the most recently published should be taken to apply to all. All diagnostic laboratories shall be accredited to nationally or internationally accepted standards. Laboratories shall

participate in an External Quality Assessment (EQA) for all aspects of their service for which a scheme is available.

1. Test overview

A QF-PCR approach for the detection of trisomy 21 was first described by Elaine Mansfield in 1993 and subsequently validated for the rapid diagnosis of aneuploidy (Verma, 1998; Pertl, 1999; Schmidt, 2000; Cirigliano, 2001; Mann, 2001). The published results from more than 117,000 samples demonstrate that QF-PCR is an accurate and robust approach (Levett, 2001; Putzova, 2008; Cirigliano, 2009; Holgado, 2011; Mann, 2012). The semi-quantitative PCR-based approach requires just a few nanograms of DNA and comparison of homologous alleles provides reliable quantitation, although preferential amplification effects of shorter length alleles must be taken into account. The microsatellite genotype provides information regarding maternal cell contamination (MCC), mosaicism, the timing of a non-disjunction event, sample identity and twin pregnancies which may inform result interpretation and clinical management.

QF-PCR analysis is a diagnostic, targeted test and good quality normal and triallelic abnormal results do not require confirmation by another approach. However, karyotype analysis should be undertaken following identification of aneuploidy to identify structural rearrangements; where these are identified, or where karyotype analysis of the original material is not possible, karyotype analysis of parental samples is indicated to assess recurrence risk. QF-PCR analysis may be used as a standalone test for samples that are not at increased risk of other chromosome abnormalities.

Results are achievable within seven hours. The ACGS website gives target UK reporting times; currently it is recommended that 90% of samples are reported within three calendar days.

1.1 Prenatal samples (chorionic villus (CV), amniotic fluid (AF), fetal blood (FB))

The testing of prenatal samples is challenging due to limited sample quantity and variable sample quality; procedures should be optimised so that results are routinely obtained for substandard samples. Procedures should also be optimised for the detection of mosaicism and MCC which have clinical and quality implications, respectively. All prenatal samples shall be tested using markers for chromosomes 13, 18 and 21. If the sex chromosomes are not routinely tested, they should be included for samples where ultrasound anomalies are suggestive of sex chromosome abnormality. For samples referred with ultrasound abnormalities but with a normal QF-PCR result, genome-wide testing e.g. array CGH analysis should be undertaken. For samples with an abnormal QF-PCR result, karyotype analysis of cultured cells shall be carried out to determine recurrence risk. A targeted karyotype analysis is acceptable. The processing of a number of prenatal samples requires stringent quality control measures to minimize the risk of sample mix-up; sample identity must be confirmed prior to reporting abnormal results.

In the case of a twin pregnancy, twin identifiers shall be given if possible. Twin zygosity can be determined using the QF-PCR genotype and may be useful clinically, particularly in the case of different presentations and/or results. Identical genotypes are only indicative of monozygotic twins, with the likelihood related to the number of markers tested and maternal/paternal relatedness. Sampling of the same twin twice cannot be excluded.

1.2 Products of conception (POC) / fetal tissue (FT) samples

QF-PCR is used as a cost-effective initial screen for the detection of common aneuploidies and triploidy in POC/FT samples to minimise the number of genome-wide tests. Donaghue et al., 2017, reported that using a QF-PCR assay, aneuploidy was identified in 25% of POC/FT samples with a 1.4% failure rate; samples with a normal QF-PCR result then have genome-wide testing. The QF-PCR assays reflect the incidence of the different aneuploidies in POC/FT samples; in addition to testing chromosome 13, 18, 21, X and Y, the inclusion of markers for chromosomes 15, 16 and 22 may be clinically useful. There are several different tissue types that may be received for testing and sample quality is variable particularly from retained POCs/fetuses or following a delay due to post-mortem examination. The QF-PCR assay should be optimised for the testing of these substandard samples. MCC and mosaicism are frequently observed particularly in POCs and procedures should be optimised for these findings.

1.3 Postnatal blood samples

Clinical indications include newborns with suspected mosaic or non-mosaic aneuploidy or ambiguous genitalia; rapid results are particularly important for these referrals to inform clinical management. Blood samples in lithium heparin or EDTA can be used with a chelex-based DNA preparation providing the fastest results.

2. Sample preparation

Sample preparation should be carried out in accordance with good laboratory practice and protocols should minimise the risk of sample mix-up. A DNA preparation method that minimises tube-tube transfers is recommended *e.g.* automation or a chelex-based method.

Precautions to minimise PCR product contamination shall be in place including separation of pre- and post-PCR areas. A water control shall be included for all markers in each PCR set-up to identify any DNA or PCR product contamination. As follow-up testing is often carried out, only a proportion of the sample is usually available for QF-PCR aneuploidy testing.

2.1 CV samples

CV samples should be thoroughly cleaned and maternal decidua removed prior to DNA preparation. In the case of poor quality samples where the origin of the material is in doubt, genotype comparison with a maternal sample is recommended to exclude the possibility of maternal contamination. CV DNA should be prepared from a heterogenous cell population in order to minimise the risk of misdiagnosis from confined placental mosaicism (CPM) (Waters, 2006; Waters, 2007). It is recommended that a number of villi should contribute to the DNA pool and that single villi are not analysed in isolation. It is recommended that the same pool of chopped or digested villi is used for all further/additional tests e.g. DNA preparation and culture set-up, to minimise discrepant results. Dissociated samples are likely to represent both cytotrophoblast and mesodermal tissue (Mann, 2007). If the sample is small, or of poor quality, then processing a single villus is acceptable, however, it should be noted that mosaicism may not be detected.

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2.2 AF samples

Generally a minimum of 1.0 ml (or 1/10 of the sample) is used for QF-PCR analysis and will depend on sample size, gestation and other planned tests. Blood-staining in an AF sample should be noted as these samples may exhibit two genotypes consistent with MCC. Blood may be fetal or maternal in origin and therefore blood-staining does not necessarily preclude a QF-PCR result.

2.3 FB samples

Fetal origin should be confirmed; either an Apt test (Ogur, 1997) or comparison with the maternal genotype is recommended.

3. QF-PCR assays

A number of commercial assays and published primer sets for the development of in-house assays are available. Verification is required for either approach. Batch testing should be carried out to ensure consistent assay quality. Assays should be designed so that markers cover the length of the tested chromosome. A minimum of four markers should be included for each autosome and the X chromosome although additional markers will minimise uninformative results. A minimum of two Y specific sequences shall be included, to determine sex chromosome status and should include *AMELOGENIN* (*AMEL*) (Sullivan, 1993) or similar to determine the ratio of X to Y sequences. In addition, the use of at least one X chromosome counting marker shall be used to determine the number of X chromosomes in the absence of a Y chromosome; e.g. the paralagous *TAF9* sequences (Deutsch, 2004) present on 3p24.2 and Xq21.1 are amplified using the same pair of primers and, due to sequence length differences, can be compared to assess X chromosome copy number.

For in-house assay development, basic principles of multiplex design should be followed including avoidance of homology to repetitive DNA, CNV, SNPs and primer-dimer interactions by sequence comparison. Tri/tetra/penta/hexanucleotide repeat markers should be used as these have fewer stutter peaks. All markers should have high heterozygosity within the population being tested. New markers that have not been previously reported for PCR aneuploidy diagnosis should be validated by testing a minimum of 100 chromosomes to ensure they are not located in a population CNV or affected by a SNP. Aneuploid samples should be included in all validations to demonstrate new markers are located on the expected chromosome.

3.1 PCR conditions

A H_2O control shall be included in each PCR set-up to identify any DNA or PCR product contamination. It is recommended to use DNA controls (trisomy or normal) with each PCR run in order to monitor variation between runs, but these are not necessary to validate the dosage results; the dynamics of an individual PCR will vary due to differences in DNA concentration/contaminants and therefore the quality of each result should be assessed independently.

To ensure the reaction remains in the semi-quantitative phase, 24-26 PCR cycles should be carried out as standard practice. It is acceptable to use a higher number of PCR cycles following validation and providing both trisomy biallelic and normal control markers show correct allele dosage ratios. The annealing temperature should be set as low as is practical to minimise the effect of primer site polymorphisms.

4. Analysis standards

Alleles must be clearly separated. Either peak height or peak area ratios may be used. Both the electrophoretogram and allele peak ratios must be analysed in order to identify low level cell lines. To ensure the quality of the data, both minimum and maximum peak heights should be established to minimise the risk of base line artefacts and fluorescence saturation, respectively, causing erroneous results. In line with current practice, it is recommended that allele peak ratios are calculated by dividing the peak height or area of the shorter length allele by that of the longer length allele. Preferential amplification of the shorter length allele is a feature of QF-PCR and the normal and abnormal biallelic ratio ranges reflect this. Although there is a requirement for testing laboratories to validate/verify the biallelic ratio range, normal ratios are generally between 0.8 and 1.4 (up to 1.5 for widely spaced alleles), whilst those representing trisomy are between 0.45 and 0.65 or between 1.8 and 2.4. Allele ratio values that are not within these normal or abnormal ranges are classed as inconclusive. Individual inconclusive marker results can usually be resolved by re-testing the sample.

For non-polymorphic markers that are not subject to preferential amplification, such as sex chromosome sequences *TAF9L* and *AMEL*, a narrower allele range may be appropriate. It is recommended that allele ratios that indicate the different sex chromosome aneuploidies are determined.

It is acceptable to fail individual markers if valid technical reasons such as electrophoretic spikes or dye 'bleed-through' are present on the electrophoretogram. If dinucleotide repeat markers are used, care must be taken with analysis of stutter patterns. Dinucleotide repeat markers with alleles separated by 2 bp should not be analysed, as stutter peaks are incorporated into the shorter length allele and may misrepresent allele ratios. For alleles separated by more than 2 bp, significant stutter peaks areas may be included in ratio calculations.

It is unacceptable to interpret and report a single abnormal marker result (all other markers uninformative) as likely to represent whole chromosome aneuploidy, as the single marker result may represent a rare CNV or other polymorphism (see below).

Genotype comparison of all samples within a single set-up is recommended to identify identical genotypes caused by sample processing/pipetting errors.

4.1 Normal results

To interpret a result as normal, at least two informative markers consistent with a normal biallelic pattern are required per chromosome, with all other markers uninformative. However, it is acceptable to report a single marker result that has a normal biallelic pattern and all other markers uninformative as consistent with a normal chromosome complement, provided the report states that the result is based on a single marker result and that the result must be confirmed.

4.2 Trisomy results (non-mosaic)

Markers indicating trisomy exhibit three different alleles in a 1:1:1 ratio or biallelic 1:2 or 2:1 ratios (see above). To interpret a result as representing a trisomy, at least two informative markers should be consistent with trisomy with all the other markers uninformative. The presence of one or more triallelic 1:1:1 results is consistent with a meiotic nondisjunction event generating the trisomy cell line. The absence of such a result indicates a possible mitotic non-disjunction event, the likelihood of this is correlated to the number of markers used. The absence of a triallelic 1:1:1 result in CV samples is

associated with a small but increased risk that the abnormal cell line is confined to the placenta. This should be considered when interpreting the result and, if appropriate, such as in the absence of ultrasound anomalies, may be detailed in the report. In these cases, analysis of cultured cells may be recommended prior to pregancy management decisions.

Prior to reporting an abnormal result, the identity of samples with abnormal results shall be confirmed by a repeat test of the original sample or genotype comparison with a maternal blood sample.

4.3 Inconclusive results

Follow-up studies for individual inconclusive results are carried out at the discretion of the laboratory, however, where a majority of markers for any one chromosome are inconclusive, repeat analysis is recommended to exclude mosaicism.

4.4 Mixed abnormal and normal results

As a general rule, it is expected that all informative markers on the same chromosome will show results consistent with a normal, abnormal, mosaic or MCC interpretation. Any discrepant results should be investigated. If both normal and abnormal marker results are observed for a single chromosome, it is recommended that further investigations are carried out, such as lowering the PCR annealing temperature, testing an additional aliquot/cultured cells, parental studies and/or array/karyotype/FISH studies. Such a result may represent a polymorphism of no clinical significance or partial chromosome imbalance.

4.4.1 SNPs
SNPs within the primer sequences may cause reduced allele amplification and give an abnormal or inconclusive result. Lowering the annealing temperature of the PCR reaction usually facilitates primer hybridisation and increases allele amplification. In these cases, it is acceptable to conclude the likely presence of a primer site SNP, however, the marker should not be used to determine copy number status as amplification may not be complete.

4.4.2 Copy number variant (CNV)
Informative markers within a CNV (duplication) will give an abnormal result at that locus. If a single abnormal marker result is flanked by normal markers and has been previously reported in individuals without phenotype or is within an established population CNV, then it is acceptable to not report the single marker result. A collated list of inherited CNV identified by markers used in QF-PCR assays is available from K Mann (kathy.mann@viapath.co.uk). However, if the abnormal marker is the most distal or proximal or has not been reported in a normal individual or as an established population CNV then further studies are recommended prior to interpretation. Parental studies and/or array analysis are recommended to facilitate interpretation.

4.4.3 Somatic microsatellite mutations (SMM)
SMMs (Mann, 2003) are rare events that may confuse result interpretation. They present either as a skewed biallelic ratio or three alleles in an A+B=C pattern. If a single marker gives a characteristic triallelic SMM pattern or inconclusive biallelic ratio, this does not have to be

reported. Analysis of cultured cells may assist interpretation as the proportion of cells with the novel allele is likely to change.

If the aberrant result(s) cannot be shown to be due to polymorphism (primer site SNP, CNV, SMM), they shall be reported and/or further studies undertaken.

4.5 Monosomy X results

To interpret a result as monosomy X, at least one paralogous marker showing a single X chromosome shall be present, the X chromosome polymorphic marker results should be consistent with this interpretation and all (at least two) Y chromosome specific sequences should be absent (present in a control sample). In the absence of paralogous markers or parental genotypes, definitive diagnosis of monosomy or a deletion is not possible.

4.6 Other sex chromosome aneuploidies

The interpretation of XXY, XYY, XXX chromosome complements should comply with the guidelines above. However, sex chromosome polymorphic markers that give results in the abnormal biallelic ranges may represent mosaicism for monosomy X and/or other cell lines. It is important to pay particular attention to the paralagous marker result(s) in these cases. In addition, sex chromosome markers may give ratios above 2.4 or below 0.45, consistent with the presence of more than 3 copies of the sequence and indicating one of the rarer sex chromosome aneuploidies.

4.7 Maternal cell contamination

MCC may compromise prenatal results. Analysis of a QF-PCR genotype is able to identify the presence of a second genotype to a level of at least 10%. If the two genotypes do not exhibit four alleles at any of the markers, it is likely that the result represents MCC. Specifically, a sample with MCC is evident by the presence of triallelic marker results in an A+B=C pattern, where A and B are the fetal and maternal specific alleles and C is the shared fetal-maternal allele. The relative amount of alleles A and B will depend on the level of contamination and unless a maternal sample is tested or the fetal sample is male, it is not possible to assign origin to either genotype. If allele ratios are inconclusive and/or the maternal genotype is present at a high level, the fetal genotype should not be interpreted even if a maternal genotype is available; the contaminating genotype may compromise the quality of the analysis. These results are categorised as 'significant MCC' for the purpose of this text and it is recommended that an 'uninformative' or 'unsuitable sample' report is issued.

If a low level second genotype consistent with MCC is present, such that all allele ratios are within normal/abnormal range and the sample is of reasonable quality, it is acceptable to interpret and report the majority genotype. Maternal blood samples are not routinely required for QF-PCR testing. For most samples, quality is sufficient to exclude gross MCC. Exceptions are detailed below.

Where MCC is identified, consideration should be given to the significance for follow-up tests such as array, karyotype or monogenic tests.

4.7.1 CV samples

CV samples should be expertly dissected to minimise MCC, which is generally avoidable in CV samples of reasonable quality. In the case of poor quality CV samples that are female, a maternal sample may be required to confirm sample origin. For CV samples exhibiting

significant MCC, a second aliquot of dissected villi may provide an interpretable result. Analysis of cultured cells is possible but care should be taken in the interpretation of these results; maternal tissue may predominate in cultured cells and in the case of female fetuses, maternal genotype analysis may be required to exclude MCC.

4.7.2   AF samples

It is acceptable to process and analyse all blood-stained AF samples as the blood may be fetal in origin or any maternal blood cells may be present at a low level and a QF-PCR result may be possible. The presence of blood in itself does not indicate MCC. However, for heavily blood-stained samples, sexing is recommended and if the sample is female, maternal genotype analysis is necessary to establish the origin of the genotype.

Up to 2% of AF samples exhibit significant MCC and are usually blood-stained (Mann, 2012). In these cases, a QF-PCR result from uncultured fluid will not be available and QF-PCR analysis or genome wide testing of cultured cells is recommended. As maternal lymphocytes do not grow up in culture, a fetal result is usually possible.

Up to 10% of AF samples exhibit low level MCC and in the absence of heavy blood-staining can be interpreted and reported (Stojilkovic-Mikic, 2005).

The detection of maternal cell contamination in an AF sample in the absence of blood staining may indicate the presence of solid maternal tissue. This tissue may be a source of maternal cell contamination (or in rare cases, complete maternal cell overgrowth) in cell cultures and care should be taken in the interpretation of results from cultured cells; genotype analysis of the cultured material should be considered to exclude the risk of persistent maternal cell contamination from maternal tissue.

4.8 Mosaicism

Mosaicism for trisomy and normal cell lines can be detected by QF-PCR analysis, evident as extra peaks and skewed allele ratios on a chromosome-specific group of markers. These results may be subtle; skewed allele ratios representing the mosaic chromosome may remain in the normal or abnormal ranges (i.e. ratios may all be borderline inconclusive or the greater area/height peak may be associated with the longer allele). Expert analysis should detect second cell lines when present at levels of at least 20%.

It is recommended that subtle but consistent skewing and/or small extra peaks on a chromosome-specific group of markers should be investigated further. Usually it is possible to distinguish between a mosaic genotype pattern and a pattern representing two genotypes. If the QF-PCR result can be confidently interpreted the mosaic result should be reported, although the clinical significance of a mosaic result shall be carefully considered in the report; specifically, for CV samples CPM should be considered.

Rare completely discrepant QF-PCR and karyotype results due to placental mosaicism in CV samples have been reported (Allen, 2006; Waters 2006; Waters 2007; Holgado, 2011). In most of these cases QF-PCR testing of small or confined regions of the CV sample was carried out and the result may represent predominantly cytotrophoblast material. Testing of dissociated cells representing the whole

CVS is now recommended practice (see Section 3.1). The abnormal cell line in all but one case exhibited only biallelic abnormal results consistent with a mitotic non-disjunction event (see Section 5.2).

5. Reporting standards

The chromosomes tested should be stated and the results should be clearly presented with appropriate clinical interpretation. QF-PCR is a diagnostic test and does not require confirmation. Follow-up tests should be stated.

5.1 Abnormal results

When reporting a trisomy QF-PCR result, the name and location of informative markers should be listed to define the trisomic region. If no results indicating meiotic non-disjunction are observed this may, where appropriate, be detailed in the report, for example, in the absence of ultrasound anomalies. The result does not definitively show whole chromosome aneuploidy; instead, wording such as 'the results are consistent with' or 'indicate' aneuploidy is recommended. The associated syndrome should be named, and the result should be related to the referral reason where appropriate. For results indicating triploidy, markers may be listed. For monosomy X results, the name and location of paralagous markers should be given. The laboratory should have written procedures for reporting other sex chromosome abnormalities e.g. XXX, XXY, XYY.

For reports that detail mosaic results it may be helpful to detail a meiotic conception if this is indicated. If no triallelic results are observed, the increased risk of a mitotic non-disjunction event may be reported. Mosaic findings should be related to the referral indication. For mosaic results found in CV samples, confined placental mosaicism should be discussed. For mosaicism identified in AF, the report should consider that the proportions of the abnormal and normal cell lines in fetal tissues may vary and is unknown.

The results of the other tested chromosomes should be given.

Karyotype analysis is required in order to determine recurrence risks.

5.2 Normal results

It is acceptable to report normal QF-PCR results as 'consistent with a normal diploid complement for chromosomes 13, 18 and 21', 'an apparently normal complement of chromosomes 13, 18 and 21 was detected', 'no evidence of trisomy' or similar statement. It is acceptable to list informative markers on a normal report, although this should be done in a way that does not 'bury' the result.

Where normal QF-PCR results are found in samples referred with a high risk NIPT result, it is recommended that non-concordance of the QF-PCR with the NIPT result is highlighted and a statement that the QF-PCR diagnostic result supersedes the NIPT screening result should be included in the report. In the case of AF samples with no ultrasound anomalies, and where there is no evidence of an abnormal cell line, further analysis is not required. Guidance regarding CVS samples with no ultrasound anomalies awaits additional data and further review. However, in cases where confined placental mosaicism is a possible cause of the discrepant result, ultrasound monitoring should be recommended due to an increased risk of placental insufficiency.

7.0 Limitations

QF-PCR cannot detect any changes that lie outside the target sequence of the markers and will not detect balanced rearrangements. For aneuploidy cases, karyotype analysis is required to characterise the abnormality and determine recurrence risk. Deletions and partial monosomy are not detected. Low level mosaicism may not be detected. It is recommended that relevant limitations are detailed in the report.

8.0 References

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