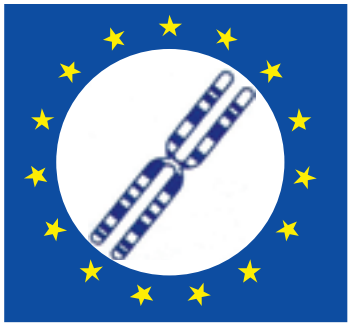
Specific Constitutional Cytogenetic Guidelines



A common European framework for quality assessment for constitutional, acquired and molecular cytogenetic investigations.

E.C.A. Permanent Working Group for Cytogenetics and Society

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SPECIFIC CONSTITUTIONAL GUIDELINES

These guidelines supplement the General Cytogenetic guidelines and give more specific guidance on prenatal and postnatal cytogenetics including some molecular genetics based techniques.

The use of 'must' in this document indicates a requirement and the use of 'should' or 'may' a recommendation.

1. CONSTITUTIONAL ANALYSIS

1.1 STANDARD CYTOGENETICS

It is recommended that prenatal and non-prenatal cultures are incubated separately to minimise the risk of microbial cross-contamination.

1.2 Prenatal samples

Laboratories performing prenatal analyses must possess at least two incubators for splitting of prenatal specimens. To minimise the risk of contamination, or culture loss due to incubator failure, duplicate cultures must be handled separately, kept in separate incubators and if possible, running on different electrical circuits. Batch testing of culture media and added reagents must be undertaken to ensure prenatal growth of cells is not inhibited. Prenatal cultures should be maintained with two different cell culture media, or with different batches of the same cell culture media and other reagents.

In prenatal diagnosis the presence of maternal contamination must be noted and recorded accordingly on the internal records. The possibility of maternal cell contamination, pseudomosaicism, true mosaicism and *in vitro* aberrations must be recognised and the system of culture and analysis used, designed to detect and differentiate these problems.

Harvesting or subculturing of all cell cultures from one individual sample at the same time must be avoided. If possible, back up cultures should be kept until the final report is written.

Facilities should be available for freezing viable cells, *e.g.* for unresolved cases of abnormal fetal pathology.

1.2.1 Chorionic villi (CV) cultures

Before a CV sample is cultured it must be dissected and maternal decidua separated from the villus to reduce the chance of maternal cell contamination. It should be clear from the referral form whether the sample has been dissected or not, prior to its arrival in the laboratory. If an initial cytogenetic diagnosis is made on short-term preparations, a long term culture should be available for confirmation, in order to minimise problems of interpretation (Eucromic 1997; ACC Collaborative study, 1994; ACC Prenatal Diagnosis Best Practice Guidelines 2009). Analysis solely on short-term incubation preparations (direct preparations) is not recommended (Eucromic 1997; ACC Collaborative study, 1994; ACC Prenatal Diagnosis Best Practice Guidelines 2009). The laboratory's choice of direct or culture methods should depend on the success rate. Laboratories should be aware that there is a false positive and false negative with CV samples.

If the sample is of an inadequate size for both short and long term cultures, analysis from a long term culture is recommended and the use of QF-PCR techniques to identify the most common aneuploidies should be considered.

1.2.2 Fetal blood cultures

The fetal blood sample should be checked to ensure it is not mixed with maternal blood, and to confirm it originates only from the fetus. Several haematological methods are available to check the fetal origin such as Alkaline phosphatase, Kleihauer or Coulter counter sizing. If an amniotic fluid sample is taken at the same time as a fetal blood sample, both fetal blood and amniotic fluid samples should be cultured and analysed unless there is a valid reason not to do so *e.g.* abnormal fetal blood result and pregnancy terminated.

1.3 Postnatal samples

1.3.1 Products of conception/ follow up specimens

Follow up of abnormal cases may form a part of internal quality control. However, if fetal morphology does not confirm the laboratory findings, fetal tissue samples should, where possible, be analysed. Confirmation by cytogenetic analysis or other molecular techniques (*e.g.* QF-PCR, MLPA, BoB, FISH or array) may be done to confirm analysis of an aneuploid fetus. Chromosome analysis must be done where the unbalanced structural rearrangement is not detectable by molecular techniques.

2. KARYOTYPING/CHROMOSOME ANALYSIS

2.1 Banding

Numerical and structural abnormalities have to be excluded at a banding level appropriate to the referral reason. Specific standards for resolution should be appropriate to the case and the type of tissue studied. A banding quality of <300 bphs (QAS 2) is the minimum level of resolution for studies to establish common aneuploidies (see General Guidelines section 4.4.4). A 550 bphs (QAS 6) level should be the minimum standard for referrals of intellectual disability, birth defects, dysmorphic children or couples with recurrent pregnancy loss. (see General Guidelines section 4.4.4).

2.2 Metaphase analysis

Metaphase analysis must involve a comparison of every set of homologues (including X and Y), band by band. If one of the homologue pair is involved in an

overlap with another chromosome the pair of homologues should be independently scored to ensure there is no structural rearrangement (See General Guidelines, Section 4.4.5 for more information).

2.3 Chromosome instability syndromes

The rarity of chromosome instability syndromes and the interpretational problems associated with chromosome breakage syndromes requires that inexperienced laboratories refer such cases to laboratories with proven expertise. Classic breakage syndrome disorders include Ataxia telangiectasia, Bloom syndrome, Fanconi anaemia, Nijmegen syndrome, Roberts, Werner, ICF and mosaic variegated aneuploidy (MVA). Other syndromes involving defective DNA replication/repair (*e.g.* Cockayne syndrome and Xeroderma pigmentosum) are not amenable to cytogenetic methods of confirmation.

For breakage syndrome referrals, clastogen studies must only be undertaken with appropriate negative matched control samples and, if available, positive matched control samples. All control and test samples should be collected, processed, cultured and harvested in parallel. Controls should be appropriately matched (*e.g.* sex, age etc.). The patient and control samples should be analysed blind. Sufficient numbers of metaphases must be examined in order to ensure that any chromosomal damage detected is significant.

- **Bloom syndrome**
  As some Bloom syndrome patients have a population of cells with a normal SCE frequency, examination of 20 harlequin banded metaphases is recommended. The laboratory should have an internal record of the SCE frequencies found when the same methods are applied to a range of normal control samples.

- **Fanconi anaemia**
  Sufficient cells must be examined to exclude the possibility of somatic mutation, which is common in Fanconi anaemia. Analysis of at least 50 but preferably 100 metaphases is recommended. The efficacy of the clastogen used should be checked against either an untreated control or SCE levels in treated samples. The mean breakage per aberrant cell and the mean breakage per normal cell should be calculated.

- **Ataxia telangiectasia and Nijmegen syndrome**
  The aberration frequency in irradiated cultures should be calculated by scoring 50 to 100 metaphases. As some ataxia telangiectasia patients display an intermediate response to irradiation, screening of 50 banded metaphases for rearrangements, involving the T-cell antigen receptor loci on chromosomes 7 and 14, should also be carried out.

2.4 Other rare syndromes detected by cytogenetic analysis

Despite recent advances in the understanding of the molecular basis of some disorders, cytogenetic studies are often the first step in making a diagnosis. Sufficient numbers of metaphases must be examined in order to ensure that any chromosomal damage detected is significant.

- **Roberts syndrome**
  50 block (Leishman/Giemsa stained) or C-banded metaphases should be scored for centromere puffing and tramline chromosomes. 50 banded metaphases should be counted, for evidence of aneuploidy.

- **ICF syndrome**
  50 banded metaphases should be scored for anomalies of the heterochromatic regions of chromosomes 1, 9 and 16 and for multi-branched configurations.

3 MOSAICISM

3.1 Mosaicism in postnatal samples

In cases where mosaicism may be expected to be present (*e.g.* sex chromosomes abnormalities or chromosome breakage syndromes), the number of cells counted and scored should be sufficient to rule out mosaicism or clonality. Extended analysis includes analysing a minimum of 30 cells when clinically relevant mosaicism is suspected (giving appropriate confidence limits using Hook's tables for lymphocyte cultures, Hook 1977). However, the laboratory should consider the common occurrence of age related sex chromosome losses and/or gains before reporting sex chromosome mosaicism (Guttenbach *et al.,* 1995; Gardner, Sutherland & Shaffer, 2012; Russell *et al.,* 2007). Laboratories should also be aware that the level of mosaicism may vary between tissues and when pseudomosaicism is likely. Loss of one X chromosome in older females is well documented (Russell *et al.,* 2007). Also normal individuals may have very low level mosaicism for chromosome 7 and 14 rearrangement due to the culture conditions, not a breakage syndrome.

FISH analysis may be the most appropriate method of confirming suspected mosaicism if a suitable probe is available. In some instances more than one tissue type should be investigated *e.g.* Pallister-Killian syndrome or Trisomy 8 mosaicism.

It is not possible to reliably exclude mosaicism from any analysis. When low level mosaicism is detected and the clinical significance is uncertain, the result should be discussed with the referring clinician.

A minimum of two metaphases must be fully analysed with an independent check. Additional cells may be counted depending on laboratory policy. An extended analysis and/or cell count is warranted when mosaicism is clinically indicated or suspected. The laboratory should have a written protocol for the analysis criteria (See General Guidelines section 4.4).

3.2 Mosaicism in prenatal samples

Two or three cultures should be set up for each sample. Analysis of a second or third culture is essential in cases of suspected mosaicism or pseudo-mosaicism *e.g.* Trisomy 2 or where the abnormality is not consistent with continued fetal development (see Hsu *et al*., 1996, 1997, Gardner, Sutherland & Shaffer, 2012). In general, if the same abnormality is present in two independent cultures, mosaicism is confirmed.

For *in situ* preparations, analysing cells from one cell culture may be sufficient provided they are not all from the same colony. However, it is recommended that at least two independent cultures are established to be able to rule out pseudomosaicism. When sufficient colonies are available, no more than two cells should be counted and analysed from a single colony (except when excluding a single cell anomaly). If colonies are insufficient for this to be achieved, a comment should be made in the report. It is unreasonable to expect all cases of true fetal chromosome mosaicism or small structural rearrangements to be detected by a routine level of analysis.

A written procedure for delineating different types of mosaicism (Level I, II & III) should be drawn up for guidance within the laboratory. In an amniotic fluid culture, detection of a mosaicism must be followed up by extensive examination of cells from an independent culture, or from independent colonies. Failure to confirm the abnormal cell line provides reassurance of a normal pregnancy but, depending on chromosomes involved and the nature of the abnormality, supplementary investigations may be appropriate (see Hsu *et al*., 1996, 1997; Gardner, Sutherland & Shaffer, 2012). To facilitate the elucidation of mosaicism and *in vitro* abnormalities, the independent colony method is recommended. Individual cases can require careful assessment and discussion and the number of cells counted and analysed may exceed the minimum (Gardner, Sutherland & Shaffer, 2012).

In CV samples, the significance of mosaicism may depend on the distribution of the abnormality amongst different cell types in direct and cultured preparations, and the chromosome(s) involved (Eucromic 1997; ACC Collaborative study, 1994; ACC Prenatal Diagnosis Best Practice Guidelines 2009). The laboratory should have a written protocol on how to proceed when the referral reason (*e.g.* ultrasound anomalies) is not consistent with the cytogenetic findings.

The possibility of fetal uniparental disomy in some cases cannot be ignored, and additional tests may be required to resolve uncertainty. UPD studies should be considered where there is mosaicism or confirmed placental mosaicism involving chromosomes 7, 11, 14 & 15 and in homologous and non-homologous Robertsonian translocations involving 14 & 15, and marker chromosomes of chromosome origin 7, 11, 14 & 15 (Kotzot 2002; Robinson *et al*., 1996; ACC Prenatal Diagnostic Best Practice Guidelines, 2009).

3.3 Maternal cell contamination

If MCC is level III or greater than 10% in one culture, further investigations may be required (*e.g.* QF-PCR) before reporting. QF-PCR comparison with maternal alleles will distinguish a chimera from MCC.

4 FISH

See the General Guidelines, Sections 4.5.1 and 4.5.2. In prenatal diagnosis confirmation of rearrangements using FISH may be appropriate to differentiate between an interstitial or a telomeric deletion or more complex rearrangement.

5 ARRAY BASED TECHNIQUES

5.1 Array specific follow up/parental analysis

Dependent on the probability that a constitutional imbalance is causal, follow-up control experiments on the patient and their parents should be performed whenever appropriate. Array follow-up studies do not differentiate between normal or balanced carrier status in the parents. Since both a deletion and/or a duplication may result from the unbalanced segregation of a balanced translocation in one of the parents, it is mandatory to perform additional follow up tests *(e.g.* FISH, chromosome analysis) on the parental bloods to exclude telomeric/insertional translocations or inversions and provide recurrence risk for future pregnancies.

The accuracy of the follow up technique to detect the imbalance must be confirmed on the proband prior to reporting parental samples as normal and hence reporting the change as de novo.

Although this test is validated as a technique it still needs to have an internal verification by the laboratory using at least 100 known abnormalities before being offered as a diagnostic service (>90% sensitivity with 95% confidence limits (Mattock et al., 2010)). Confirmation of the abnormality by an independent technique is advisable in the early stages of introducing this technique diagnostically.

5.1.1 Clinical validity and interpretation

The likelihood for a constitutional chromosomal aberration being a causative mutation depends on different criteria:

- the aberration has previously been reported in an individual with the same phenotype;
- the presence of a known gene in the aberration where copy number is known to be causative;
- there is no common polymorphism known in the current databases;
- whether the aberration is '*de novo*' or inherited;
- the size of the aberration;
- deletion/duplication of imprinted regions of the genome or of genetic and environmental modifiers.

The published literature and public databases such as ENSEMBL, USBC, Database for Genetic Variants,

DECIPHER, ECARUCA, *etc.* should be used when evaluating the clinical significance of a detected imbalance (de Leeuw et al., 2012). By its very nature, this evolving technology will produce significant numbers of results where interpretation remains difficult.

Specific Guidelines are now published on microarrays and should be referred to for more details (see ACC: Professional Guidelines for Clinical Cytogenetics. Constitutional Array CGH Best Practice Guidelines (2009) v1.00, UK; Vermeesch et al., 2012; Vetro et al., 2012).

6 QF-PCR FOR RAPID PRENATAL DIAGNOSIS OF ANEUPLOIDY

This technique is a useful adjunct to prenatal diagnosis and is a more appropriate technique than FISH for aneuploidy testing when dealing with large numbers of prenatal referrals. Internal validation is essential before using this technique (see section 4.1.3 of General Guidelines).

The limitations of QF-PCR in identifying chromosome abnormalities must be clearly known. It is recommended that testing for trisomies 13, 18 and 21 is carried out. The laboratory should have an internal written procedure for reporting sex chromosome abnormalities with lesser clinical relevance *e.g.* XXX, XXY, XYY. If the sex chromosomes are not routinely included in the QF-PCR they must be included when ultrasound anomalies are suggestive of Turner syndrome. QF-PCR analysis provides information only about the probe locus in question. It does not substitute for a complete chromosome analysis.

6.1 Genetic analyser

The genetic analyser used for the analysis of the STR products should be capable of 2 bp allele resolution and peak area/peak height quantification.

6.2 Sample preparation

For amniotic fluid, between 0.5 and 4 ml is recommended for QF-PCR analysis, as larger aliquots may compromise the karyotype analysis. For chorionic villus samples, it is recommended that at least two villi taken from different regions of the CV biopsy should be processed to minimise the risk of misdiagnosis due to confined placental mosaicism.

A DNA preparation that does not require any tube-tube transfers is recommended *e.g.* chelex-based method. Home-made kits should be batch tested using at least a trisomy and a normal DNA sample to ensure consistent assay quality and trisomy diagnosis. An H_2O control must also be included in each PCR set-up to identify any DNA or PCR product contamination.

6.3 Analysis

Between 24-26 PCR cycles should be carried out as standard practice and a minimum of 4 markers for each chromosome tested to reduce the number of uninformative results. It is recommended that tri/tetra/penta/hexanucleotide repeat markers are used as these have fewer stutter peaks, although di-nucleotide repeat markers are acceptable if few suitable markers are available within the tested region.

New markers not used previously for QF-PCR aneuploidy diagnosis should be validated by testing a minimum of 100 chromosomes, including aneuploid samples.

It is recommended that both the electrophoretogram and peak measurements, which can be transferred to a spreadsheet for convenience, are analysed. To ensure the quality of the data both minimum and maximum peak heights should be used. It is acceptable to fail individual markers if there are valid technical reasons such as bleedthrough between colours and electrophoretic spikes. It is acceptable to use peak height, peak area or both measurements to calculate allele ratios, although for results obtained from an ABI genetic analyser it is recommended that peak area is used to minimise peak distortion due to widely-spaced alleles.

The area/height of the shorter length allele should be divided by that of the longer length allele and the normal range should not exceed 0.8-1.4.

To interpret a result as abnormal, at least two informative marker results should be consistent with a triallelic genotype (or three informative markers for an abnormal monoallelic result), with all the other markers uninformative. It is unacceptable to interpret a result as abnormal if shown by only one marker. Confirmation of sample identity when a result is abnormal by repeat PCR of the DNA, re-extraction of samples, or maternal blood analysis is recommended.

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

Where maternal cell contamination occurs, if allele ratios are inconclusive and/or the maternal genotype is present at a high level it is recommended that the fetal genotype should not be interpreted.

6.4 Reporting

It is recommended that the report includes a report rider that this test is based on the assumption that fetal material is tested. Limitations of the test should be given e.g. mosaicism and small segment imbalance for chromosomes tested may not be detected. The locations of markers showing a triallelic or an abnormal monoallelic result should be listed to define the trisomic/monosomic region. Abnormal reports should include an interpretative statement such as 'consistent with Down syndrome', 'associated with Down syndrome', 'indicative of Down syndrome' or 'predicted to be affected with Down syndrome'.

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 markers on a normal report, although this should be done in a way that does not 'bury' the result.

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. 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 a quantitative marker [*e.g.* TAF9BP1 on chromosome 3 or TAF9B on the X-chromosome] should be included in the QF-PCR assay. Without this quantitative marker, a monosomy X result must either be confirmed using another technique, or reported as being consistent with monosomy X with the caveat that there remains a possibility that a normal female could give the same genotype.

7 MLPA

MLPA can be used for prenatal aneuploidy detection as well as for pre-and postnatal telomere or microdeletion detection using manufacturers' kits. MLPA can also be used as a confirmatory test following FISH or microarray studies. It is essential the kit name is given in the report and that the limitations of the test are known. MLPA results should be reported using the ISCN nomenclature.

Manufacturer's instructions should be followed including the recommended DNA extraction method. The most appropriate kit should be used given the referral reason. Commercially available CE marked kits should be used in preference to 'in-house' assays.

8 BoBS

BoBS (BACs on Beads) can be used for prenatal aneuploidy detection as well as for pre-and postnatal telomere or microdeletion detection using manufacturers' kits. It is essential the kit name is given in the report and that the limitations of the test are known. BoBS results should be reported using the ISCN nomenclature.

Manufacturer's instructions should be followed including the recommended sample preparation method.

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APPENDIX

A. INDICATIONS FOR CYTOGENETIC ANALYSIS

Whenever a clinician suspects a patients' condition/ disease is due to a chromosomal abnormality, he/she should consider a cytogenetic analysis. Although these conditions are well known to most clinicians referring patients to a cytogenetics laboratory, this list of indications may be helpful to delineate the type of patients eligible, especially if these indications are used in conjunction with the ICD-10 nomenclature of diagnoses. These indications are given as a guideline to enable stakeholders to monitor the referral pattern and the expected workload of a cytogenetics laboratory.

CLINICAL INDICATIONS FOR CYTOGENETIC PRENATAL DIAGNOSIS
(Amniotic fluid, chorionic villi, fetal blood)

- previous livebirth with a chromosome abnormality;
- previous stillbirth with a potentially viable chromosome abnormality;
- parental chromosome rearrangement, chromosome mosaicism or sex chromosome aneuploidy;
- positive maternal serum screening result indicating an increased risk of a chromosomally abnormal fetus;
- increased maternal age;
- abnormal fetal ultrasound;
- resolution of possible fetal mosaicism detected by prior prenatal study;
- risk of chromosome instability syndrome.

CLINICAL INDICATIONS FOR INVESTIGATION OF CONSTITUTIONAL KARYOTYPE
(Peripheral blood, bone marrow, fibroblasts)

Significant family history of:

- chromosome rearrangements;
- mental retardation of possible chromosomal origin where it is not possible to study the affected individual.

Patient with:

- primary or secondary amenorrhea or premature menopause;
- sperm abnormalities - azoospermia or severe oligospermia;
- clinically significant abnormal growth - short stature, excessive growth, microcephaly, macrocephaly;
- ambiguous genitalia;
- abnormal clinical phenotype or dysmorphism;
- multiple congenital abnormalities;
- mental retardation or developmental delay;
- suspected deletion / microdeletion / duplication syndrome;
- X-linked recessive disorder in a female;
- clinical features of a chromosome instability syndrome, including isolated haematologic findings;
- monitoring after bone marrow transplantation;
- tissue from a malformed fetus or stillbirth of unknown etiology;
- products of conception from a third and subsequent consecutive miscarriage(s).

Couple with:

- chromosome abnormality or unusual variant detected at prenatal diagnosis;
- unbalanced chromosome abnormality in the products of conception;
- child with a chromosome abnormality or unusual variant;
- infertility of unknown etiology.

CLINICAL INDICATIONS FOR FISH TESTING OF CONSTITUTIONAL SPECIMENS

Individual with:

- a clinical suspicion of a microdeletion syndrome for which established diagnostic testing is available;
- increased risk for a microdeletion syndrome because of a positive family history;
- clinical features that suggest mosaicism for a specific chromosomal syndrome;
- a bone marrow transplant for follow-up, when the donor is of the opposite sex to the recipient;
- a chromosomal abnormality suspected by standard cytogenetic analysis when FISH testing may prove to be useful in further clarification of the abnormality or in situations where there is an important clinical implication;
- presence of a supernumerary marker chromosome;
- a clinical suspicion of a cryptic subtelomeric rearrangement, including relatives at increased risk for the cryptic subtelomeric rearrangement.

Metaphase FISH

Evaluation of:

- marker chromosome;
- unknown material attached to a chromosome;
- rearranged chromosomes;
- suspected gain or loss of a chromosome segment;
- mosaicism.

Interphase FISH:

Evaluation of:

- numerical abnormalities;
- duplications;
- deletions;
- rearrangements;
- sex chromosome constitution;
- mosaicism;
- gene amplification.

Rapid Prenatal FISH/QF- PCR

- High risk of chromosome abnormality e.g. abnormal ultrasound.
- late gestational age

CLINICAL INDICATIONS FOR INVESTIGATION OF ARRAY based techniques (Peripheral blood, fibroblasts)

Patient with:

- clinically significant abnormal growth - short stature, excessive growth, microcephaly, macrocephaly;
- abnormal clinical phenotype or dysmorphism;
- multiple congenital abnormalities;
- mental retardation or developmental delay;
- suspected deletion / microdeletion / duplication syndrome;
- increased risk of microduplication/microdeletion syndrome due to a positive family history;
- X-linked recessive disorder (female patient).

Prenatal

- Two or more pathological ultrasound anomalies (includes IUGR);
- Parent carriers of a chromosomal rearrangement;
- Previous chromosomally abnormal child;
- Delineation of a chromosome abnormality detected prenatally.

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AUSTRALIA

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BELGIUM

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CANADA

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