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Prenatal Screening

Incorporated by Reference Document:

American College of Medical Genetics and Genomic (ACMG)

Standards and Guidelines for Clinical Genetics Laboratories

American College of Medical Genetics and Genomics (ACMG)

STANDARDS AND GUIDELINES FOR CLINICAL GENETICS LABORATORIES

2018 Edition, Revised January 2018

(For a general overview of these Standards and Guidelines, including purpose and disclaimer, see **Section A**)

E: CLINICAL CYTOGENETICS

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E1 Cell Culture, See <u>D1</u>.

E2 Records

E2.1 Retention of Case Materials
In addition to the general guideline (C3.6) for duration of **retention of case materials**, the following are specific to cytogenetics.

E2.1.1 **Slides** used for diagnostic tests have a limited lifespan. If stained with a "permanent" banding method (G-, C- or R-banded, NOR), slides should be kept at least 3 years or in compliance with state regulations. Retention time of those with fluorochrome stained chromosomes and cytogenomic array slides should be retained as defined by laboratory policy or procedure.

E2.1.2 Each laboratory should establish a policy to assure that any **residual original patient specimens** and/or cell cultures are retained until release of the final report.

E2.1.3 **Processed patient specimens** and/or cell pellets should be retained until two weeks after the final report has been signed. Long-term retention time of those with abnormal results is at the discretion of the laboratory director.

E2.1.4 **Images** for chromosome analysis and FISH images for non-neoplastic disorders, should be retained for at least 20 years, while FISH images for neoplastic disorders are to be retained for at least 10 years.

E2.1.5 For **cytogenomic microarray data**, see section <u>E10</u> for the **retention of files and documentation.**

E3 Procedural Guidelines

E3.1 General Analytical Standards
E3.1.1 Terminology
Chromosome counts are defined as the number of centric chromosomes per metaphase cell. During the establishment of the modal number for a study, all aneuploid metaphase cells should be characterized for specific gain/loss.

Analyzed cells are defined as banded metaphase cells in which the individual chromosomes are evaluated in their entirety, either at the microscope or from intact digitized images or photographic

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prints of intact cells.

Karyogrammed cells are defined as the cutout and paired chromosomes from photograph(s) or computer-generated image(s) from a single cell following the format in An International System for Human Cytogenomic Nomenclature 2016 (ISCN 2016) [McGowan-Jordan, Simons and Schmid, 2016].

Scored cells refer to cells evaluated for the presence or absence of a specific cytogenetic feature, usually indicated by either a particular clinical history or by the finding of one or two abnormal cells during the course of a study. Numbers of cells to be scored in most situations are left to the discretion of the laboratory director, unless otherwise specified in the guidelines.

Clone is defined as a cell population derived from a single progenitor cell. Clonal origin is inferred by the presence of at least two cells containing the same extra chromosome(s) or structural chromosome abnormality or by the presence of at least three cells that have lost the same chromosome [Second International Workshop on Chromosomes in Leukemia, 1980].

For the purpose of constitutional studies, the use of the terms **cell line** and **clone** are interchangeable.

Mosaicism is the presence of two or more cytogenetically distinguishable cell lines.

Pseudomosaicism refers to the presence of an abnormal cell(s) in cultured cells that arise from an in vitro culture artifact and do/does not represent the true karyotype.

Uniparental disomy is defined as a condition in which both homologous chromosomes are derived from a single parent.

E3.1.2 **Slide number** and **microscope stage coordinates** should be recorded for all metaphases analyzed or counted. If additional cells are evaluated in questions of mosaicism, slide number should be recorded for all cells that are scored and slide coordinates should be recorded for all abnormal metaphases or suspected abnormal metaphases.

E3.1.3 All laboratories must be able to perform studies using G- and/or R-banding, in addition to special stains and/or FISH, to characterize heteromorphisms or variants, when indicated and at the discretion of the laboratory director.

E3.1.4 Current ISCN must be used to describe all karyotypes.

E3.1.5 A number of different objective methods have been described for the calculation of band stage of resolution. One or more objective and reproducible method(s) must be used to assess **banding level of**

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resolution and must be formally described in the laboratory standard operating procedures/protocol manual. [Kao et al., 1990]. Specific standards for resolution should be appropriate to the case and type of tissue studied. The 550-band stage should be the goal of all peripheral blood studies. A minimum of 400 bands should be reached for 90% of analyses from amniotic fluid and chorionic villi cells.

E3.1.6 Minimum standards established for **the numbers of cells to count and/or analyze and karyotype** during the "routine" component of a cytogenetic study are described in specific subsections appropriate to a specific tissue type, culture method and/or reason for referral. The numbers of cells to study in individual situations is dependent on the specific abnormality observed, the tissue being examined, whether the analysis involves prenatal diagnosis, etc. General recommendations are noted in the following subsections (see Table 1).

E3.1.6.1 Each laboratory should establish guidelines for procedures (e.g., numbers of cells to score) to follow for each general type of abnormality (hypodiploidy, hyperdiploidy and structural abnormality) with the recognition that uniformity among laboratories is not required.

E3.1.6.2 The laboratory's scoring guidelines should be based on current knowledge of the potential clinical significance of particular chromosome abnormalities and non-modal cells.

E3.1.6.3 Fewer cells than indicated under analytical standards may be studied in circumstances in which screening for a specific abnormality is the indication for the study (e.g., checking for a known familial abnormality) or when an abnormality is detected but no more cells are available (see E3.2).

E3.1.7 Analyses should be performed and/or evaluated by at least two qualified individuals.

E3.2 Abbreviated, Focused or Limited Chromosome Studies
E3.2.1 General Considerations
It is acknowledged that there are specific clinical circumstances for which an abbreviated or limited cytogenetic study may be appropriate. For example, in the tissue confirmation of an abnormal prenatal chromosome result or in peripheral blood chromosome studies on extended family members to exclude an identified chromosome rearrangement, limited analyses may be suitable.

E3.2.2 Analytical Standards
The laboratory should have established written criteria for which focused or abbreviated studies are permissible. Criteria should specifically address the rationale for such studies, the clinical reason for referral, the tissue type, and the minimum number of cells counted, analyzed and karyotyped under such circumstances.

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E3.3 Maternal Cell Contamination (MCC): General Considerations
E3.3.1 Amniotic Fluid

E3.3.1.1 The overall frequency of MCC is approximately 0.5% of genetic amniocenteses [Hsu, 1992]. Factors that increase the chance of MCC include the gauge of needle used for the amniocentesis procedure [Ledbetter, 1993], the length of time in culture and the presence of blood in the sample.

It has also been documented that cultures initiated from the first 1-2 ml of amniotic fluid drawn at amniocentesis are at an increased risk for maternal cell contamination [Ledbetter, 1993]. It is recommended that the first few milliliters of fluid be labeled appropriately and kept separate from the remaining sample to minimize inclusion of maternal cells. The initial aliquot should be used for cytogenetic analysis only if absolutely necessary.

E3.3.1.2 Chorionic Villi Sampling (CVS)

The risk for MCC in CVS is significantly higher than for amniocentesis samples (1-2%) [Ledbetter et al., 1992]. A CVS specimen must be viewed under a dissecting microscope to allow for the gross identification and cleaning of villi from maternal decidua, blood vessels, membrane and other materials. It is recommended that sterile instruments (e.g., probes, scissors, forceps) be used to tease apart the sample to isolate the fetal chorionic villi from maternal decidua. It may be helpful to have two laboratory technologists clean or check the dissected tissue prior to initiating cultures.

E3.3.1.3 Products of Conception (POC)

Due to the manner in which abortus tissue and placenta samples are obtained and handled, there is a substantial risk of MCC, particularly in early fetal loss specimens. It is recommended that appropriate measures be taken to specifically identify fetal tissues and to dissect and culture only these tissues, as described above for prenatal CVS. Consultation with the referring physician may be warranted to determine the origin of the sample and/or the appropriateness of chromosome studies, particularly in cases for which the dissection of tissue appears to yield only maternal decidua.

E3.3.2 Analysis of Cultures with Known or Suspected MCC

Cultures with known or suspected MCC based on the condition of the specimen at receipt, or apparent maternal cells morphologically in culture, require variation in the normal analysis procedure. If XX cells are found in an otherwise XY study, the most likely explanation is MCC. Since the true fetal cells are probably represented by the XY complement, the full analysis and cell counts should be performed on these cells whenever possible. Counting and analyzing several cells with an XX constitution is recommended for documentation purposes. For prenatal testing, further studies may be warranted to exclude chimerism. Ultrasound examination to check the gender of the fetus, second amniocentesis or confirmatory amniocentesis after CVS and/or heteromorphism studies (molecular) between a maternal sample and the fetal sample may be required in the investigation.

If cell cultures initiated in the cytogenetics laboratory are to be used for molecular or biochemical testing,

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any serious concerns about MCC in those cultures must be conveyed to the molecular or biochemical testing laboratory. In addition, if direct prenatal samples are sent out for testing, it is recommended that back-up cultures be grown and maintained until the molecular or biochemical testing is complete and reported.

E3.3.3 *MCC Reporting and Quality Assurance*

Reporting of MCC is case-dependent and is at the discretion of the laboratory director. Consultation with the referring physician is recommended, when appropriate. Any significant observation of MCC in a prenatal diagnosis sample should be interpreted in consultation with the physician who performed the procedure. For samples with a significant risk for MCC that produce a normal female karyotype, a disclaimer should be added to the report suggesting that analysis of maternal cells due to MCC cannot be excluded.

Any time that MCC is suspected or confirmed, the laboratory director must ensure that an attempt to determine the cause is documented as part of the laboratory's quality assurance program. Additionally, it is recommended that the ratio of XX:XY cases be monitored as a quality control check for CVS and POC cases. Monitoring the male cases for evidence of female cells is also important for quality control of MCC. FISH with probes for X/Y or molecular methods may be used on cell suspensions prior to culturing to screen for or to estimate the amount of MCC.

E4 Prenatal Diagnosis: General Considerations

Amniotic fluid contains single cells sloughed off of the amnion, fetal skin, lung, bladder, and digestive tract. A random sample of cells from the amniotic fluid is drawn and plated. These cells form true distinct colonies. In **chorionic villus sampling**, villi are usually retrieved from one or two sites and are likely to not be completely disaggregated.

Prenatal cytogenetic diagnosis can be performed on various tissues, each requiring different methods of culture and analysis.

A minimum of two cultures should be analyzed on each case whenever possible. FISH analysis for the chromosome of interest can be done on uncultured amniotic fluid cells in addition to chromosome analysis of cultured cells. The Benn and Hsu (2004) guidelines of workup should be followed for potential mosaicism.

If XX and XY cells are observed: Analyze 15 male colonies if available. If a mixture of XX and XY cells is present, it may be helpful to consult with the referring physician about evidence of a twin pregnancy.

Laboratories should have a protocol stating when to reflex to additional studies, such as increased counts,

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and/or FISH, and/or a recommendation for high-resolution ultrasound, uniparental disomy, microarray, and study of a second tissue. While CVS may appear as colonies, they should be analyzed as any other disaggregated tissue.

Uniparental disomy testing should be considered if numeric mosaicism or structural abnormality of chromosomes 6, 7, 11, 14, or 15 is detected because these chromosomes are known to carry imprinted genes and uniparental disomy is associated with congenital abnormalities [Shaffer et al., 2001].

It is the laboratory director's responsibility to monitor quality and to ensure that analytical practices are consistent with the guidelines presented below. **(Also see C4)**

E4.1 Amniotic Fluid, Chorionic Villi and Percutaneous Umbilical Blood Sampling (PUBS)
E4.1.1 At least **two independent cell cultures** must be initiated and grown in separate incubators with independent electric circuits or emergency power systems, backup gas sources and emergency alarms.

E4.1.2 With the exception of PUBS, there must be a plan for maintaining **back-up cell culture(s)** pending the need for additional studies.

E4.1.3 If studies of parental chromosomes are necessary to help interpret a fetal chromosome abnormality or heteromorphism, the same laboratory should perform these studies, if possible and reasonable.

E4.1.4 The number of **test failures** (defined as failure to obtain final results from an adequate submitted specimen) should not exceed 1 per 100 consecutive samples (1%).

E4.1.5 Efforts must be made to determine the **cause of all test failures**. These records and records of corrective actions taken must be available for external review and kept for at least 2 years.

E4.1.6 With the exception of PUBS, at least 90% of final results must be completed and reported (verbal or written) within 14 calendar days from receipt of specimen, unless additional studies are necessary.

E4.1.7 Laboratories consistently failing to meet these standards should consider splitting or sending samples to another laboratory until the problems are resolved.

E4.1.8 Laboratories should have specific requirements for the acceptance and rejection of specimens that include the volume and quality of the specimen received.

E4.1.9 Where there is suspicion that MCC may be present (see E3.3), the laboratory director may want to consider analysis of additional cultures, increased colony counts, or molecular genetic analyses (PCR or QF-PCR) to rule out any confounding diagnosis.

E4.2 Amniotic Fluid

E4.2.1 Amniotic Fluid: Processing Standards

E4.2.1.1 If little or no cell pellet is apparent in the sample, the laboratory should consider the use of a method (e.g., assays for pH, protein, glucose, etc.) that will help to distinguish amniotic from other fluids.

E4.2.1.2 Notification of inadequate or poor cell culture growth should be made within 10 days of the amniocentesis procedure.

E4.2.1.3 A laboratory planning to establish amniotic fluid cytogenetic testing must arrange to split and successfully analyze at least 50 consecutive specimens with a laboratory performing such studies by established standards.

E4.2.1.4 The laboratory should investigate significantly increased chromosome instability in one or several concurrent patient samples.

E4.2.1.5 Sample quality and culture failures should be monitored.

E4.2.2 Amniotic Fluid: Analytical Standards (see also E3.1.6)

E4.2.2.1 **Analysis of in situ cultures** is the preferred method, since it is more reliable for evaluating mosaicism.

Count: a minimum of 15 cells from at least 15 colonies, distributed as equally as possible between at least 2 or more independently established cultures. Single metaphase colonies should only be used when multi-metaphase colonies are unavailable. Document any numerical/structural aberrations observed.

Analyze: 5 cells, each from a different colony, preferably from 2 independently established cultures. Band resolution should be appropriate to the reason for testing.

Karyotype: 2 cells. These cells can be from the 5 analyzed cells. If more than 1 abnormal cell line (as defined in Section E3.1.1) is found, karyotype at least 1 cell representative of each cell line.

If both abnormal and normal cells are observed in a colony, the colony is generally considered normal if the same abnormality was NOT seen in other colonies.

E4.2.2.2 Analysis from a combination of mixed in situ and subcultured cells
When it is impossible to complete the analysis by in situ only, subculturing may be necessary.

Count: Count as many colonies as possible and then increase the count to a total of 20 cells.

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Analyze: 5 cells, distributed between 2 independently established cultures.

E4.2.2.3 Suspension Harvest Technique
Situations in which suspension harvest technique is appropriate include: suboptimal sample or when the primary concern is growing cells for other testing methodologies.
Count: a minimum of 20 cells, distributed as equally as possible among independently established cultures. Document any numerical/structural aberrations observed (see E4.2.2.1 for analysis and karyogram guidelines).

E4.3 Chorionic Villus Sample (CVS)
E4.3.1 Chorionic Villus Sample (CVS): Processing Standards
In cases of multiple gestations particularly in those of in vitro fertilization, one should be aware that a deceased co-twin with remaining viable placental material may be the source of a chromosome abnormality.

Confined placental mosaicism (CPM) is defined as at least two cell lines from a single fertilized egg seen in chorionic villi analysis that are only present in the placenta, not in the fetus itself. It is observed in about 1-2 % of CVS [Crane and Cheung, 1988]. Laboratories should have protocols to distinguish CPM from true mosaicism in the fetus. Additional studies may be recommended depending on the chromosome involved and the type of abnormality. These may include analysis of additional cultures and in some cases analysis of amniotic fluid cultures or fetal cord blood. In situ culture strategy is appropriate for CVS or any other tissues that grow in a monolayer, but the analysis protocols may differ from amniotic fluid.

The significance of mosaicism in CVS may differ based on the distribution of the abnormal cells in the direct and cultured preparations, as well as the chromosomes involved. When mosaicism is documented, in general, amniocentesis is recommended, since the amniotic fluid cells are more likely to represent the fetus.

E4.3.1.1 When direct (uncultured) preparations are used clinically, a cell culture technique (defined as longer than 48 hours) must also be used.

E4.3.1.2 Final written reports should include a summary of the analysis results of the cultured cells and direct preparation, if performed.

E4.3.1.3 A laboratory planning to establish CVS cytogenetics should already be testing amniotic fluid cells by established standards and methods. Prior to independent CVS analysis, the laboratory must split and confirm at least 25 samples (with an adequate volume) with a laboratory already performing CVS cytogenetics by established standards and methods. Note: During this period, samples that are too small to split should be sent to a qualified reference laboratory for culturing and analysis.

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E4.3.2: Chorionic Villi: Analytical Standards (see also E3.1.6)

E4.3.2.1 **Direct (Uncultured) Preparations**: should not be exclusively used in obtaining final results. (See Section E4.3.2.3 below.) Interphase FISH or molecular screening for sex chromosomes and common aneuploidies should be used to generate alternative preliminary results.

E4.3.2.2 Cultured Preparations

Count: a minimum of 20 cells distributed as equally as possible between at least 2 independently established cultures. Document any numerical/structural aberrations observed (see E 4.2.2 for analysis and karyogram guidelines).

E4.3.2.3 Combination of Direct Preparation and Culture Technique

Count: a minimum of 20 cells, at least 10 of which come from cultured preparations. Document any numerical/structural aberrations observed.

Analyze: 5 cells, preferably at least 4 cells from cultured preparations. Resolution should be appropriate to the reason for testing (see E 4.2.2 for karyogram guidelines).

E4.3.2.4 If mosaicism is documented in a CVS sample, cytogenetic studies of amniotic fluid are recommended.

E4.4 Fetal Blood: Percutaneous Blood Sampling (PUBS)

E4.4.1 Fetal Blood: Processing Standards

E4.4.1.1 Final results of PUBS should not be released until the sample has been confirmed to be fetal in origin.

E4.4.1.2 A minimum of 2 cultures should be established, if adequate specimen is submitted.

E4.4.1.3 Processing after 48 and 72 hours in culture is recommended.

E4.4.1.4 Final reports (verbal or written) should be available within 7 calendar days.

E4.4.2 Fetal Blood: Analytical Standards (see also E3.1.6)

Count: a minimum of 20 cells (see E 4.2.2 for analysis and karyogram guidelines).

E4.5 Diagnostic Cytogenetic Testing Following Positive Noninvasive Prenatal Screening (NIPS) Results [See Genet Med 2017;19(8):845-850 OR Appendix 1]

E5 Peripheral Blood and Solid Tissue Constitutional Chromosome Study

E5.1 Peripheral Blood (Stimulated Lymphocytes): Routine Studies
E5.1.1 Peripheral Blood: Processing Standards
E5.1.1.1 At least 2 cultures should be established for each specimen.

E5.1.1.2 At least 90% of all routine peripheral blood analyses must have final written reports completed within 28 calendar days (21 calendar days is recommended) from receipt of the specimen. Clinical indications may dictate more rapid turn-around time. Specialized stains and studies may take longer.

E5.1.1.3 Test failures should not exceed 2% per year.

E5.1.1.4 The 550-band stage should be the goal of all constitutional studies to rule out a structural abnormality, particularly in cases of intellectual disability, birth defects, dysmorphology, or couples with recurrent pregnancy loss.

E5.1.2 Peripheral Blood: Analytical Standards (see also E3.1.6)
E5.1.2.1
Count: a minimum of 20 cells, documenting any numerical/structural abnormalities observed.

Analyze: 5 cells. Resolution should be appropriate to the reason for testing.

Karyotype: 2 cells. If more than 1 clone (as defined in Section E3.1.1) is found, karyotype 1 cell representative of each clone.

E5.1.2.2 Cases being studied for possible sex chromosome abnormalities, in which mosaicism is common, should include the standard 20-cell assessment. If mosaicism is confirmed, the analysis is complete. A minimum of 10 additional metaphase cells should be evaluated when one cell with a sex chromosome loss, gain or rearrangement is observed within the first 20 cells analyzed [Wiktor et al., 2009; Wolff et al., 2010].

E5.2 Peripheral Blood (Stimulated Lymphocytes): Focused High Resolution Analysis
Due to the improved detection rate for subtle chromosome deletions and duplications by genomic microarray analysis, complete high resolution chromosome analysis (resolution at the 850 band level) is no longer recommended as a standard test methodology.

E5.3 Peripheral Blood (Stimulated Lymphocytes): Complete High Resolution Analysis
E5.3.1 Complete High Resolution: Analytical Standards
E5.3.1.1 General processing and analytical standards for routine peripheral blood studies apply. In addition, complete high resolution chromosome analysis should include detailed evaluation of all regions on all chromosome pairs at a level of resolution above the 650-band stage (resolution at the 850 level is

recommended) [see also E5.1.2].

E5.4 Peripheral Blood (Stimulated Lymphocytes): Heritable Fragile Sites (Including Fragile X)
This section initially provided guidelines for the evaluation of patients for fragile X syndrome using the cytogenetic expression of the Xq27.3 (FRAXA) fragile site. Such chromosome testing has been replaced by molecular genetic DNA evaluation of the FMR1 locus, and specific College recommendations have been published to cover such testing (see Section FX, "Technical Standards and Guidelines for Fragile X") [Maddalena et al., 2001; Monaghan, Lyon and Spector, 2013]. For the most part, testing/culturing for fragile sites is no longer performed in the Cytogenetics Laboratory. However, individuals performing chromosome analyses should be aware of their occurrence (e.g. fra(10)(q25), fra(16)(q22), etc.) and are referred to Gardner, Sutherland and Shaffer, 4th edition of Chromosome Abnormalities and Genetic Counseling, for further guidance.

E5.5 Solid Tissues Constitutional Chromosome Study (Skin, Organs, Products of Conception, etc.)
E5.5.1 Solid Tissues Constitutional: Processing Standards
E5.5.1.1 Tissue biopsy specimens and small specimens should be transported in sterile cell culture medium with or without serum. Sterile saline solution may be used if medium is not available. Larger specimens should be transported according to written guidelines in each laboratory.

E5.5.1.2 At least two independent cultures should be established (three are recommended for resolving questions of mosaicism). These can be from explants of tissue grown in flasks or from enzyme-dissociated cells that can be processed in flasks or in situ.

E5.5.1.3 Except for products of conception (POC), test failure rates should not exceed 5% per year, in total. It is suggested that periodic monitoring of POCs be done to assure that the ratio of 46,XX: 46,XY results approximates 1:1.

E5.5.2 Solid Tissues Constitutional: Analytical Standards
See amniotic fluid guidelines (*E4.2.2*) for analytical standards.

E5.6 Bone marrow studies for constitutional disorders. In most laboratories, these studies have been replaced by analysis of short term (overnight) blood cultures and/or FISH analysis performed on interphase nuclei.

E5.7 Chromosome Instability Syndromes: Peripheral Blood Breakage Analyses
E5.7.1 General Standards
The rarity of chromosome instability syndromes requires that inexperienced laboratories should refer cases to reference laboratories with experience in diagnosing such disorders. Additionally, as research leads to the identification and cloning of the putative disease genes, molecular testing is recommended to supplement cytogenetic analysis.

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G-banded or unbanded preparations may be applied, depending upon the particular goal of the study. Unbanded preparations are acceptable only if there is no need to identify abnormalities such as translocations or inversions that will not be visible in unbanded preparations. All abnormalities should be recorded using appropriate ISCN designations.

E5.7.2 *Fanconi Anemia*
Cytogenetic evaluation for Fanconi anemia (FA) should include analysis of crosslinking agent (e.g., mitomycin C [MMC], diepoxybutane [DEB]) induction of breakage in addition to baseline chromosome breakage.

E5.7.2.1 *Fanconi Anemia: Culture Conditions*
Each laboratory should have well-established negative control (non-Fanconi) and positive control (Fanconi) ranges for each culture (with and without mutagen) condition. Each new lot number of crosslinking agent should be appropriately quality controlled for its efficacy and potency for inducing chromosomal breakage. Given variability between drug lots, and the need to routinely prepare fresh stock and working solutions for most of the crosslinking agents, parallel testing of control specimens is recommended, as necessary. When a sufficient amount of blood specimen (and cell count) is available, two drug-treated cultures (e.g., either two different concentrations of either DEB or MMC, or one culture each of MMC and DEB) are recommended.

E5.7.2.2 *Fanconi Anemia: Chromosome Breakage Analysis*
Optimally, 50 metaphase cells (banded or unbanded) should be scored from each culture condition. The average rate of chromosomal aberrations per cell or the distribution of aberrations among cells should be compared to negative and positive control reference ranges. The percentage of cells demonstrating aberrations should be reported to enable identification of those patients who are mosaic for mutant and wild type cells.

E5.7.3 *Bloom Syndrome*
Traditionally, cytogenetic evaluation for Bloom syndrome included assessment of baseline sister chromatid exchange (SCE) rates. As the Bloom syndrome gene BLM has been cloned, molecular evaluation to identify the mutation should be performed. Nearly all affected individuals have mutations of the BLM gene.

E5.7.4 *Ataxia Telangiectasia and Nijmegen Breakage Syndrome*
Evaluation for ataxia telangiectasia (A-T) and Nijemgen Breakage Syndrome (NBS) should include evaluation of sensitivity to radiation. Although such sensitivity can be assessed by cytogenetic methods, it generally is evaluated by survival assays on lymphoblastoid or fibroblast cells.

As the A-T gene (ATM) and the NBS gene (NBN) have been cloned, molecular evaluation should be

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performed for confirming the diagnosis in patients who have positive radiosensitivity assays. Again, nearly all affected individuals with AT or NBS have mutations of the ATM gene detectable by sequence analysis.

E5.7.5 Breakage Studies: Miscellaneous

Cytogenetic evaluation of chromosome breakage may also be undertaken for other reasons, e.g., prior exposure to clastogens. The specific culture methods utilized (e.g., timing of cultures) and the methods of analysis (G-banded vs. unbanded chromosomes) should be appropriate to the referral. The laboratory should have well established positive and negative control ranges for the specific analyses being conducted.

E6 Chromosome Studies for Acquired Abnormalities

E6.1–6.4 of the ACMG technical standards and guidelines: chromosome studies of neoplastic blood and bone marrow–acquired chromosomal abnormalities [See **Genet Med 2016;18(6):635-42** OR **Appendix 2**]

E6.5–6.8 of the ACMG technical standards and guidelines: chromosome studies of lymph node and solid tumor–acquired chromosomal abnormalities [See **Genet Med 2016;18(6):643-8** OR **Appendix 3**]

E7 Sex Chromatin

E7.1 The indirect nature of sex chromatin analysis has rendered the test obsolete. Any patient in whom the question of sex chromosome abnormality is being considered should have complete chromosome analysis.

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E8 Reporting Standards

Final written reports of the results of diagnostic testing should include the following information:

E8.1 **Case identification** includes name (or other first identifier), date of birth of patient, date of collection and/or receipt of specimen, laboratory accession number(s), tissue type and name(s) of physician(s) or authorized person who ordered the test and to whom report is sent.

E8.2 Specific details of the study to be reported should include:
- Indication for study.
- Numbers of cells in which chromosomes were counted, analyzed and karyotyped.
- Cell culture times and conditions and banding methods employed, when they bear on the cytogenetic interpretation.
- Banding method, level of resolution and current ISCN karyotype designation(s) of cells analyzed.
- A statement of additional work done to resolve questions of mosaicism. Correlation with previous studies. When parallel controls are used for comparative purposes in a study, the results of those controls
- Interpretation of results to include: correlation with clinical information, indication of an abnormal result where applicable, recommendations for additional laboratory genetic studies for the patient and/or family, and a discussion of the significance of the findings, when appropriate. When appropriate, recommendations for genetic counseling should be made. The interpretation should be clear to a nongeneticist physician.
- When investigational procedures are employed, the investigational nature of the testing.
- Cautions as to possible inaccuracies and test limitations.
- Individuals qualified as under B3.1 must sign all final reports. Password protected electronic signatures can be used to fulfill this requirement.
- Specifics of any preliminary results given including what the preliminary result was, the date and the person to whom the report was given.
- Date of final report.

E8.3 Laboratory identification includes name, address, and phone number of the laboratory in which the study was performed.

E9 Fluorescence in Situ Hybridization (FISH)

ACMG technical standards and guidelines: Fluorescence in situ hybridization [See Genet Med 2011;13(7):667-675 OR Appendix 4]

E10 Constitutional Cytogenomic Microarray Analysis

ACMG standards and guidelines for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013 [See Genet Med 2013;15(11):901-909 OR Appendix 5]

This also includes:
- *ACMG recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities* [See Genet Med 2011;13(7):676-9 OR Appendix 6]
- *ACMG standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants* [See Genet Med 2011;13(7):680-5 OR Appendix 7]
- *ACMG standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing* [See Genet Med 2013;15(2):150-2 OR Appendix 8]

E11 Cytogenomic Microarray Analysis for Chromosome Abnormalities in Neoplastic Disorders

ACMG standards and guidelines: microarray analysis for chromosome abnormalities in neoplastic disorders [See Genet Med 2013;15(6):484-494 OR Appendix 9]

References

Astbury C (ed). *Clinical Cytogenetics, An Issue of Clinics in Laboratory Medicine.* Volume 31(4). Elsevier Saunders Inc., Philadelphia, 2011.

Benn PA, Hsu LYF. Prenatal Diagnosis of Chromosome Abnormalities through Amniocentesis. In: Milunsky A (ed). *Genetic Disorders and the Fetus: Diagnosis, Prevention and Treatment,* 5th Edition. The Johns Hopkins University Press, Baltimore & London, 2004.

Benn PA. Prenatal Diagnosis of Chromosome Abnormalities through Amniocentesis. In: Milunsky A and Milunsky JM (eds). *Genetic Disorders and the Fetus: Diagnosis, Prevention and Treatment,* 6th Edition. Wiley-Blackwell Press, 2010.

Bruyère H, Wilson RD, Langlois S. Risk of mosaicism and uniparental disomy associated with the prenatal diagnosis of a non-homologous Robertsonian translocation carrier. *Fetal Diagn Ther* 2004;19(5):399-403.

Cherry AM, Akkari YM, Barr KM, Kearney HM, Rose NC, South ST, Tepperberg JH, Meck JM. Diagnostic cytogenetic testing following positive noninvasive prenatal screening results: a clinical laboratory practice resource of the American College of Medical Genetics and Genomics (ACMG). *Genet Med* 2017;19(8):845-850.

Cooley LD, Morton CC, Sanger WG, Saxe DF, Mikhail FM. Section E6.5-6.8 of the ACMG technical standards and guidelines: chromosome studies of lymph node and solid tumor-acquired chromosomal abnormalities. *Genet Med* 2016;18(6):643-8.

Crane JP, Cheung SW. An embryogenic model to explain cytogenetic inconsistencies observed in chorionic villus versus fetal tissue. *Prenat Diagn* 1988;8(2):119-29.

De Leon-Luis J, Santolaya-Forgas J, May G, Tonk V, Shelton D, Galan I. Prenatal diagnosis of FRA10A: a case report and literature review. *Am J Med Genet A* 2005;136(1):63-5.

Gardner RJM, Sutherland GR (eds). *Chromosome Abnormalities and Genetic Counseling,* 3rd Edition 2004. Oxford University Press, pp 260-261, 319-330, 331-335.

Gardner RJM, Sutherland GR, Shaffer LG (eds). *Chromosome Abnormalities and Genetic Counseling,* 4th Edition 2012. Oxford University Press, pp 260-268.

Hastings RJ, Cavani S, Bricarelli FD, Patsalis PC, Kristoffersson U; ECA PWG Co-ordinators. Cytogenetic Guidelines and Quality Assurance: a common European framework for quality assessment for constitutional and acquired cytogenetic investigations. *Eur J Hum Genet* 2007;15(5):525-7.

17

Hsu LY. Prenatal diagnosis of chromosomal abnormalities through amniocentesis. In: Milunsky A (ed). *Genetic disorders and the fetus: diagnosis, prevention and treatment*, 3rd Edition. Johns Hopkins University Press, Baltimore, 1992.

Kao YS, Kao GA, Walters CS. Banding resolution of amniotic cell chromosome preparations for prenatal diagnosis. *Am J Clin Pathol* 1990;93(6):765-70.

Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. *Genet Med* 2011;13(7):680-5.

Kearney HM, South ST, Wolff DJ, Lamb A, Hamosh A, Rao KW. American College of Medical Genetics recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities. *Genet Med* 2011;13(7):676-9.

Ledbetter DH. Prenatal cytogenetics: indications, accuracy and future directions. In: Simpson JL and Elias S (eds). *Essentials of prenatal diagnosis*. Churchill Livingstone, New York, 1993.

Ledbetter DH, Zachary JM, Simpson JL, Golbus MS, Pergament E, Jackson L, Mahoney MJ, Desnick RJ, Schulman J, Copeland KL, et al. Cytogenetic results from the U.S. Collaborative Study on CVS. *Prenat Diagn* 1992;12(5):317-45.

Maddalena A, Richards CS, McGinniss MJ, Brothman A, Desnick RJ, Grier RE, Hirsch B, Jacky P, McDowell GA, Popovich B, Watson M, Wolff DJ. Technical standards and guidelines for fragile X: the first of a series of disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. Quality Assurance Subcommittee of the Laboratory Practice Committee. *Genet Med* 2001;3(3):200-5.

Mascarello JT, Hirsch B, Kearney HM, Ketterling RP, Olson SB, Quigley DI, Rao KW, Tepperberg JH, Tsuchiya KD, Wiktor AE. Section E9 of the American College of Medical Genetics technical standards and guidelines: fluorescence in situ hybridization. *Genet Med* 2011;13(7):667-75.

Manning M, Hudgins L. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet Med* 2010;12(11):742-5.

McGowan-Jordan J, Simons A, Schmid M (eds). *ISCN 2016: An International System for Human Cytogenomic Nomenclature.* Karger, Basel, 2016.

Míguez L, Fuster C, Pérez MM, Miró R, Egozcue J. Spontaneous chromosome fragility in chorionic villus

cells. *Early Hum Dev* 1991;26(2):93-9.

Mikhail FM, Heerema NA, Rao KW, Burnside RD, Cherry AM, Cooley LD. Section E6.1-6.4 of the ACMG technical standards and guidelines: chromosome studies of neoplastic blood and bone marrow-acquired chromosomal abnormalities. *Genet Med* 2016;18(6):635-42.

Monaghan KG, Lyon E, Spector EB. ACMG Standards and Guidelines for fragile X testing: a revision to the disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics and Genomics. *Genet Med* 2013;15(7):575-86.

Rehder CW, David KL, Hirsch B, Toriello HV, Wilson CM, Kearney HM. American College of Medical Genetics and Genomics: standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing. *Genet Med* 2013;15(2):150-2.

Second International Workshop on Chromosomes in Leukemia, General Report. *Cancer Res* 1980;40(12):4826-7.

Shaffer LG, Agan N, Goldberg JD, Ledbetter DH, Longshore JW, Cassidy SB. American College of Medical Genetics statement of diagnostic testing for uniparental disomy. *Genet Med* 2001;3(3):206-11.

Wiktor AE, Bender G, Van Dyke DL. Identification of sex chromosome mosaicism: is analysis of 20 metaphase cells sufficient? *Am J Med Genet A* 2009;149A(2):257-9.

Wolff DJ, Van Dyke DL, Powell CM; Working Group of the ACMG Laboratory Quality Assurance Committee. Laboratory guideline for Turner syndrome. *Genet Med* 2010;12(1):52-5.

Table 1: Chromosome Analysis Rubric

	Count (from at least 2 independent cultures)	Analyze	Karyotype
Chorionic Villi	20 metaphases (minimum 10 from cultured preparations)	5 metaphase cells	2 (1 per additional cell line)
Amniotic Fluid	15 in situ colonies 20 flask harvest 20 in situ and flask harvest	5 metaphase cells	2 (1 per additional cell line)
Blood	20 metaphases	5 metaphase cells	2 (1 per additional cell line)
Products of Conception/skin fibroblasts	20 metaphases	5 metaphase cells	2 (1 per additional cell line)
Bone Marrow/ Leukemic Blood/ Solid Tumor	20 metaphases	20 metaphases	2 (1 per additional side line / 2 per unrelated clone)

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APPENDIX 1
(See following page)

Diagnostic cytogenetic testing following positive noninvasive prenatal screening results: a clinical laboratory practice resource of the American College of Medical Genetics and Genomics (ACMG)

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Noninvasive prenatal screening (NIPS) using cell-free DNA has been rapidly adopted into prenatal care. Since NIPS is a screening test, diagnostic testing is recommended to confirm all cases of screen-positive NIPS results. For cytogenetics laboratories performing confirmatory testing on prenatal diagnostic samples, a standardized testing algorithm is needed to ensure that the appropriate testing takes place. This algorithm includes diagnostic testing by either chorionic villi sampling or amniocentesis samples and encompasses chromosome analysis, fluorescence in situ hybridization, and chromosomal microarray.

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Key Words: cell-free DNA; chromosome analysis; chromosomal microarray (CMA); noninvasive prenatal screening (NIPS); non-invasive prenatal testing (NIPT)

BACKGROUND

This document was generated to support clinical cytogenetics laboratories in the testing and management of positive noninvasive prenatal screening (NIPS) results and is designed to be a rubric that can guide laboratory practice. The American College of Medical Genetics and Genomics (ACMG) revised its position statement on the use of NIPS for fetal aneuploidy in July 2016.[1] This is meant to be a companion to that revised statement.

NIPS, also referred to as cell-free DNA (cfDNA) or noninvasive prenatal testing, has been available as a clinical screening option for pregnant women since 2011.[2] Initially, NIPS was available primarily for the detection of trisomy 21,[2,3] but it rapidly evolved to include the detection of trisomies 13 and 18, sex chromosome identification, and sex chromosome aneuploidies.[4,5] NIPS has better performance as a screening test for trisomy 21 than for trisomies 13 or 18, or for sex chromosome aneuploidies.[6] Recently, select micro-deletion syndromes and smaller copy-number changes, as well as other autosomal aneuploidies, have been added by some laboratories as additional screening options.[7,8] Various factors affect the accuracy of NIPS results, including confined

placental mosaicism (CPM), maternal genomic contribution and technical or statistical issues.[9] Follow-up diagnostic testing is uniformly recommended for all patients with positive NIPS results.[1,10,11] This document establishes a standardized testing algorithm that is essential for the cytogenetics laboratory to ensure that the appropriate diagnostic testing has occurred and that the results are reliable, accurate, and reflective of the fetal karyotype.

DIAGNOSTIC TESTING

Follow-up prenatal diagnostic testing is recommended for all patients with positive NIPS results. This can be accomplished by either chorionic villus sampling (CVS) or amniocentesis. In general, diagnostic testing should be appropriate for the suspected anomaly (i.e., chromosomal microarray (CMA) for smaller copy-number changes). Some laboratories may opt to perform fluorescence in situ hybridization (FISH) for the aneuploidy or copy-number change in question and then reflex to either chromosome analysis or CMA, dependent on the FISH results. While FISH is possible for either type of copy-number change, it may not be as accurate, depending on the exact size of the anomaly or structural rearrangements.

Chromosome analysis on either CVS or amniocentesis demonstrating nonmosaic trisomy or sex chromosome aneuploidy consistent with the NIPS result is considered confirmation of a positive NIPS and therefore of an affected fetus. A full study (as defined by the ACMG laboratory guidelines[12]) on CVS or amniocentesis demonstrating a normal karyotype would not typically warrant additional metaphase cell counts or other analyses. However, a mosaic result on CVS should not be considered confirmatory. There are known physiological limitations of CVS that include the possibility of CPM and rare case reports of complete discordancy between the CVS karyotype and the fetal karyotype.[13–16] While NIPS can be performed in the late first trimester of pregnancy, and CVS is a possibility for confirmatory studies (and often desired by the patient due to timing), CVS may simply reflect the same DNA/cells that were detected by NIPS, as both are derived from the placenta.[17] Certain aneuploidies, including trisomy 13 and

monosomy X, are more likely to be found in the mosaic form on CVS, which may influence genetic counseling about the preferred diagnostic test for confirmatory studies.[18] When CVS shows mosaicism for the suspected trisomy, it is impossible to determine if this is CPM or true fetal mosaicism (TFM). Therefore, a mosaic CVS result cannot be treated as confirmation of an affected fetus and a follow-up amniocentesis is warranted, as is recommended in all cases of mosaicism observed on CVS[12,15,16] (**Table 1**).

Similarly, CMA testing on either CVS or amniotic fluid may be used as confirmatory diagnostic testing in cases with positive NIPS results, or as reflex testing in cases with initial normal results from chromosome analysis. Smaller copy-number changes are ideally confirmed by this method. Again, if the NIPS results and CMA results are concordant, no further testing is recommended. However, given that structural information is not available from CMA analysis, a reflex to chromosome analysis may be considered to evaluate the structural arrangement to inform recurrence risks, especially for those cases with trisomies 21 and 13.

On occasion, prenatal diagnostic testing may not be performed due to loss of the pregnancy before testing is possible. In such instances, testing of the products of conception and/or the fetus by either chromosome analysis or CMA should be considered on a case-by-case basis.

Other forms of abnormal result exist, such as "no calls" and the unanticipated findings rendered by special maternal medical circumstances (e.g., obesity, oocyte donations and prior transfusions). These are discussed at length in the revised ACMG position statement[1] but are beyond the scope of this laboratory algorithm.

POSITIVE PREDICTIVE VALUE

While most NIPS laboratories report a greater than 99% specificity and sensitivity for trisomy 21, the positive predictive value (PPV) is essential for patient care. The 2016 ACMG position statement[1] recommends that all laboratories reporting NIPS results also include the PPV, as well as the negative predictive value, detection rate, clinical specificity and fetal fraction. PPV answers an important question: "If NIPS is

Table 1 Prenatal diagnostic testing algorithm following positive NIPS results

NIPS positive for:	Recommended laboratory test	Sample type	Result/recommended further testing	
T13, T18, T21, SCA, other aneuploidy, triploidy	Chromosome analysis	CVS	Normal or abnormal c/w NIPS	No further testing/consider CMA
			Mosaic	Follow-up amniocentesis with mosaicism studies[a]
		AF	Normal or abnormal c/w NIPS or mosaic c/w NIPS	No further testing/consider CMA
Smaller copy-number changes	CMA	CVS or AF	Negative or abnormal c/w NIPS	No further testing
			Abnormal *not* c/w NIPS	Further testing may be warranted dependent on specific finding

AF, amniotic fluid; CMA, chromosomal microarray; CVS, chorionic villus sampling; c/w, consistent with; NIPS, noninvasive prenatal screening; SCA, sex chromosome aneuploidy; T13, trisomy 13; T18, trisomy 18; T21, trisomy 21.
[a]See the text for discussion of further testing options.

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positive, what is the chance that the fetus is affected?" The PPV is affected by the analytic specificity and sensitivity of the test, as well as the prevalence of the disorder in the population.[6,11,19] When the prevalence is age dependent, as it is for trisomies 13, 18 and 21, maternal age is a determining factor in the reliability of the test. Due to the higher incidence of aneuploidy associated with advanced maternal age, a 35-year-old woman with a positive result by NIPS has a higher PPV than a 25-year-old woman with a positive result. In addition, less common disorders, such as trisomy 13 and trisomy 18, would be expected to have lower PPVs than the more common trisomy 21. Very rare disorders (e.g., microdeletion syndromes and smaller copy-number changes), which are typically not associated with increased maternal age, would be expected to have even lower PPVs.[7] The PPV can also be determined by comparing the NIPS results with the diagnostic testing results, with the caveat that these studies involve relatively low numbers of cases with wide confidence intervals.[20–23]

SOURCES OF DISCORDANT RESULTS

The source of cfDNA in the maternal circulation is primarily of maternal origin, with a much lower proportion (typically around 10%) being derived from degraded trophoblastic cells of the placenta.[24] The primary reason for discordant NIPS and diagnostic cytogenetic testing is that the DNA tested is not solely representative of the fetus. This could be due to CPM or to a resorbed or unrecognized twin pregnancy. Furthermore, it has been reported that discordant results can be due to variations in the maternal DNA contribution, including low-level sex chromosome and autosomal chromosome mosaicism, maternal malignancies, and maternal copy-number variants.[9,17,25–27] It is well known that some women may have low-level age-related losses and gains of the X chromosome.[28,29] There are a few reports of concurrent maternal malignancies when multiple or rare aneuploidies (e.g., autosomal monosomies) are detected by NIPS.[30,31] Other reasons for discordance might be technical or statistical.[9] Since analytic algorithms differ between testing platforms and providers, there could be inconsistency in the reporting of aneuploidy results from the same pregnancy reported from different laboratories due to the utilization of different cutoffs, z-scores and/or comparison to different normalization controls. By necessity, reporting algorithms include screen-positive cases that are true negatives, to ensure that nearly all true positives would be identified by the screening test.

CPM AND TFM

When mosaicism is detected by CVS, cytogenetics laboratories attempt to distinguish between CPM and TFM. In general, regardless of the chromosome involved, this requires follow-up amniocentesis and often an extended chromosome analysis of this specimen with adherence to standard guidelines for distinguishing between pseudomosaicism and TFM.[12] This extended analysis could include screening additional cells (or colonies) from independent cultures. Screening additional metaphase cells, however, has its limitations, and a very low level of fetal mosaicism can essentially never be ruled out. Theoretically, analyzing 15 amniotic fluid colonies from at least two independent coverslips will rule out a 19% level of mosaicism at the ninety-fifth confidence interval, while screening an additional 15 colonies will rule out a 10% level of mosaicism.[32] Alternatively, interphase FISH for the mosaic aberration found at CVS might be useful, although it should be noted that laboratories need to validate and establish cutoff values for positivity for each probe utilized. Any value below these cutoff values or thresholds would be considered negative.[33] CMAs may also be ordered as part of the follow-up testing, although detection of low-level mosaicism may be more challenging than by chromosome analysis and/or interphase FISH analysis[34] (**Table 1**).

UNIPARENTAL DISOMY OF KNOWN IMPRINTED CHROMOSOMES

CPM can occur as a result of either postzygotic nondisjunction or aneusomy rescue. Given the latter, it is important to determine if the normal cell line represents uniparental disomy if an imprinted chromosome is involved.[16] In these cases, discordance between the positive NIPS result and the diagnostic test result should be followed up with testing appropriate for detecting uniparental disomy of the particular chromosome of interest.

NIPS RESULTS WITH MULTIPLE ANEUPLOIDIES OR RARE ANEUPLOIDIES

Although reportedly rare, any NIPS result that is positive for more than one aneuploidy or one that shows rare aneuploidies, such as an autosomal monosomy, should include consideration of the possibility of a maternal malignancy. A wide variety of maternal malignancies have been described in the literature in association with unusual NIPS results[30,31] and there are currently no guidelines for clinical evaluation following these rare results. Further evaluation and referral to an oncologist may be warranted.

SMALLER COPY-NUMBER CHANGES

Some NIPS laboratories offer screening for rare microdeletion syndromes and smaller copy-number changes. Again, diagnostic testing is necessary in these cases, particularly as most will be falsely positive due to lower PPVs, and some may represent variants of uncertain significance. In most cases that are positive by NIPS for smaller copy-number changes, the breakpoints and the base pair coordinate positions and sizes are not provided or reported by the testing laboratory.[35,36] As a result, specific microdeletion FISH is not the appropriate diagnostic test, due to the possibility of incorrect or incomplete FISH probe coverage. In the vast majority of cases, a whole-genome CMA analysis should be used to determine the true fetal result. As well, it should be noted that maternal contribution may also

play a role in discordant results, either due to low-level maternal mosaicism or maternal copy-number changes[27] (Table 1).

NIPS cases positive for imprinted genetic disorders (e.g., Angelman or Prader–Willi syndrome) may come with the acknowledgment that the laboratory cannot distinguish between a deletion and uniparental disomy of the region in question. In such cases, methylation analysis, including methylation-specific multiplex ligation-dependent probe amplification or similar methodology is the appropriate diagnostic test to confirm the fetal result. It should be noted that methylation may not be complete for all loci at the time of CVS, and amniocentesis or neonatal testing may be warranted. Laboratories performing methylation analyses should be consulted regarding the appropriate specimen type and requirements.

Table 2 Postnatal diagnostic testing algorithm following positive NIPS results

NIPS positive for	Clinical phenotype	Recommended laboratory test	Result/recommended further testing
T13, T18, T21, other aneuploidy, triploidy	Normal	No testing needed	N/A
	Abnormal c/w NIPS	Blood chromosome analysis	Abnormal or mosaic c/w NIPS: no further testing; Normal: additional cell counts or interphase FISH or CMA
	Abnormal *not* c/w NIPS	CMA	Further testing may be warranted depending on specific findings
SCA or discrepant sex chromosomes	Normal	Blood chromosome analysis	Abnormal or mosaic c/w NIPS: no further testing; Normal: no further testing
	Abnormal c/w NIPS or abnormal *not* c/w NIPS	Blood chromosome analysis	Abnormal or mosaic c/w NIPS: no further testing; Normal: further testing may be warranted depending on the phenotype
Smaller copy-number changes	Normal or abnormal	CMA	Abnormal c/w NIPS: parental studies, if indicated; Negative: no further testing; abnormal not c/w NIPS: Further testing may be warranted depending on specific findings

AF, amniotic fluid; CMA, chromosomal microarray; CVS, chorionic villus sampling; c/w, consistent with; N/A, not applicable; NIPS, noninvasive prenatal screening; SCA, sex chromosome aneuploidy; T13, trisomy 13; T18, trisomy 18; T21, trisomy 21.

Box 1 Points to consider following positive noninvasive screening results

- NIPS is a screening test. It is not a diagnostic test. Diagnostic testing is recommended as a follow-up for any positive NIPS result.
- The fetal contribution of the cfDNA studied by NIPS is of presumed placental origin and, therefore, NIPS results may not be representative of the fetus.
- Sources of discordant NIPS results include CPM, a resorbed or unrecognized twin, maternal chromosome abnormalities (either mosaic or nonmosaic), maternal malignancy, technical issues including low fetal fraction, or statistical errors.
- Mosaic CVS results should not be considered confirmation of a positive NIPS result. Follow-up amniocentesis is recommended.
- Chromosome analysis on follow-up amniotic fluid specimens with screening of additional cells, FISH, and/or CMA analyses may be considered to detect possible TFM in discordant cases. It should be understood that while the chance that TFM is present can be reduced to relatively low levels, it cannot be completely ruled out.
- CMA is recommended as follow-up testing for any smaller copy-number changes that are reported as positive by NIPS.
- Specific uniparental disomy analyses on CVS or amniotic fluid cells are recommended for any imprinted regions or chromosomes reportedly involved in positive NIPS cases with discordant results.
- For patients with screen-positive NIPS results, posttest access to genetic counseling by a genetics professional and accurate, balanced and up-to-date information are essential for guiding management.
- For unusual positive NIPS results (e.g., monosomy, or multiple or rare aneuploidies), an oncology consultation for possible maternal malignancy may be warranted.

NEWBORNS

In some cases, parents with a fetus suspected to have an anomaly by NIPS will decline diagnostic testing and choose the option of a neonatal assessment. If possible, at birth, a genetics consultation should be requested and a detailed physical examination performed. In NIPS cases positive for trisomies 13, 18 or 21, normal findings on a physical examination by a clinical geneticist may be sufficient to preclude further testing. Any suspicion of an abnormal phenotype related to the aneuploidy in question should prompt a cytogenetics evaluation. When warranted, an extended chromosome analysis to rule out low levels of mosaicism or FISH may be performed. If the neonate has an abnormal physical examination that is not suggestive of the trisomy in question, CMA is recommended. For sex chromosome aneuploidies, chromosome analysis or CMA is recommended, with the possibility of additional interphase FISH analysis if mosaicism is suspected. Any NIPS result indicating smaller copy-number changes should be confirmed by CMA. Extensive testing of placental tissue is not recommended, as this is not important in the clinical care of the infant. In most cases, peripheral blood chromosome analysis of the infant's mother to screen for mosaic sex chromosome gains or losses also may not be relevant and is typically not needed for patient care or for reproductive management.[28,29] However, maternal chromosome analysis or CMA may be warranted depending on the maternal phenotype or medical history.

Finally, sex designation by NIPS may be discordant with physical examination. While sex designation by NIPS is relatively accurate, there are cases of XX or XY NIPS results with the opposite sexed infant.[37,38] Blood chromosome analysis is recommended (**Table 2**). Clinical findings suggestive of a disorder of sexual differentiation may warrant follow-up by CMA or an appropriate gene panel.

IMPORTANCE OF GENETIC COUNSELING

Pretest counseling by the provider offering NIPS should include both the advantages and limitations of this screening test, as well as the alternatives.[1] For patients with positive NIPS results, posttest access to genetic counseling by a trained genetics professional is essential for guiding management. To ensure an informed decision regarding testing and diagnostic follow-up, patients undergoing this screening should be provided with up-to-date, balanced and accurate information about the limitations of NIPS, the implications of both negative and positive NIPS results, the potential for false positives and false negatives, and the role of diagnostic testing. Patients should understand that diagnostic testing is both available and voluntary. Furthermore, the education of providers is of paramount importance.

Several points to consider following a positive noninvasive screening result are listed in **Box 1**.

DISCLOSURE

REFERENCES

1. Gregg AR, Skotko BG, Benkendorf JL, et al. Noninvasive prenatal screening for fetal aneuploidy, 2016 update: a position statement of the American College of Medical Genetics and Genomics. *Genet Med* 2016;18:1056–1065.
2. Palomaki GE, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med* 2011;13:913–920.
3. Chiu RWK, Lo YMD. Noninvasive prenatal diagnosis empowered by high-throughput sequencing. *Prenat Diagn* 2012;32:401–406.
4. Palomaki GE, Deciu C, Kloza EM, et al. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study. *Genet Med* 2012;14:296–305.
5. Chen EZ, Chiu RW, Sun H, et al. Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. *PLoS ONE* 2011;6:e21791.
6. Bianchi DW, Parker, RL, Wentworth J, et al. DNA sequencing versus standard prenatal aneuploidy screening. *N Engl J Med* 2014;370:799–808.
7. Wapner RJ, Babiarz JE, Levy B, et al. Expanding the scope of noninvasive prenatal testing: detection of fetal microdeletion syndromes. *Am J Obstet Gynecol* 2015;212:332.e1–332.e9.
8. Helgeson J, Wardrop J, Boomer T, et al. Clinical outcome of subchromosomal events detected by whole-genome noninvasive prenatal testing. *Prenat Diagn* 2015;35:999–1004.
9. Bianchi DW, Wilkins-Haug L. Integration of noninvasive DNA testing for aneuploidy into prenatal care: what has happened since the rubber met the road? *Clin Chem* 2014;60:78–87.
10. Society for Maternal-Fetal Medicine (SMFM) Publications Committee. #36: renatal aneuploidy screening using cell-free DNA. *Am J Obstet Gynecol* 2015;212:711–716.
11. The American College of Obstetricians and Gynecologists and the Society of Maternal Fetal Medicine. Committee Opinion Number 640: cell-free DNA screening for fetal aneuploidy. *Obstet Gynecol* 2015;126:e31–e37.
12. American College of Medical Genetics. Standards and Guidelines for Clinical Genetics Laboratories, Section E4, Prenatal Diagnosis, 2009 edn. http://www.acmg.net/StaticContent/SGs/Section_E_2011.pdf.
13. Crane JP, Cheung SW. An embryogenic model to explain cytogenetic inconsistencies observed in chorionic villus versus fetal tissue. *Prenat Diagn* 1988;8:119–129.
14. Ledbetter DH, Zachary JM, Simpson JL, et al. Cytogenetic results from the US collaborative study on CVS. *Prenat Diagn* 1992;12:317–345.
15. Hahnemann JM, Vejerslev LO. European collaborative research on mosaicism in CVS (EUCROMIC) – fetal and extrafetal cell lineages in 192 gestations with CVS mosaicism involving single autosomal trisomy. *Am J Med Genet* 1997;70:179–187.
16. Gardner RJM, Sutherland GR, Shaffer LG. *Chromosome Abnormalities and Genetic Counseling*, 4th edn. Oxford University Press: New York, 2012:444–452; 351–374.
17. Ashoor G, Poon L, Ayngelaki A, et al. Fetal fraction in maternal plasma cell-free DNA at 11-13 weeks' gestation: effect of maternal and fetal factors. *Fetal Diagn Ther* 2012;31:237–243.
18. Grati FR, Bajaj K, Malvestiti F, et al. The type of feto-placental aneuploidy detected by cfDNA testing may influence the choice of confirmatory diagnostic procedure. *Prenat Diagn* 2015;35:994–998.
19. Mersy E, Smits LJM, van Winden LAAP, et al. Noninvasive detection of fetal trisomy 21: systematic review and report of quality and outcomes of diagnostic accuracy studies performed between 1997 and 2012. *Hum Reprod Update* 2013;19:318–329.
20. Dar P, Curnow KJ, Gross SJ, et al. Clinical experience and follow-up with large scale single-nucleotide polymorphism-based noninvasive prenatal aneuploidy testing. *Am J Obstet Gynecol* 2014;211:527.e1–527.e17.
21. Cheung SW, Patel, A, Leung TY. Accurate description of DNA-based noninvasive prenatal screening. *N Engl J Med* 2015;372:1675–1677.
22. Meck JM, Kramer Dugan E, Matyakhina L, et al. Noninvasive prenatal screening for aneuploidy: positive predictive values based on cytogenetic findings. *Am J Obstet Gynecol* 2015;213:214.e1–214.e5.
23. Wang JC, Sahoo T, Schonberg S, et al. Discordant noninvasive prenatal testing and cytogenetic results: a study of 109 consecutive cases. *Genet Med* 2015;17:234–236.

24. Bianchi DW. Circulating fetal DNA: its origin and diagnostic potential – a review. *Placenta* 2004; 60:S93–S101.
25. Wang Y, Chen Y, Tian F, et al. Maternal mosaicism is a significant contributor to discordant sex chromosomal aneuploidies associated with noninvasive prenatal testing. *Clin Chem* 2014;60:251–259.
26. Curnow KJ, Wilkins-Haug L, Ryan A, et al. Detection of triploid, molar, and vanishing twin pregnancies by a single-nucleotide polymorphism-based noninvasive prenatal test. *Am J Obstet Gynecol* 2015;212:79.e1–79.e9.
27. Snyder MW, Simmons LE, Kitzman JO, et al. Copy-number variation and false positive prenatal aneuploidy screening results. *N Engl J Med* 2015;372:1639–1645.
28. Horsman DE, Dill FJ, McGillivray BC, Kalousek DK. X chromosome aneuploidy in lymphocyte cultures from women with recurrent spontaneous abortions. *Am J Med Genet* 1987;28:981–987.
29. Nowinski GP, Van Dyke DL, Tilley BC, et al. The frequency of aneuploidy in cultured lymphocytes is correlated with age and gender but not with reproductive history. *Am J Hum Genet* 1990;46:1101–1111.
30. Osborne CM, Hardisty E, Devers P, et al. Discordant noninvasive prenatal testing results in a patient subsequently diagnosed with metastatic disease. *Prenat Diagn* 2013;22:609–611.
31. Bianchi DW, Chudova D, Sehnert AJ, et al. Noninvasive prenatal testing and incidental detection of occult maternal malignancies. *JAMA* 2015;314:162–169.
32. Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95%, and 99% confidence limits and comments on use. *Am J Hum Genet* 1977;29:94–97.
33. Mascarello JT, Hirsch B, Kearney HM, et al. Section E9 of the American College of Medical Genetics technical standards and guidelines: fluorescence in situ hybridization. *Genet Med* 2011;13:667–675.
34. South ST, Lee C, Lamb AN, Higgins AW, Kearney HM. Working group for the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee. ACMG Standards and Guidelines for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013. *Genet Med* 2013;15:901–909.
35. Yaron Y, Jani J, Schmid M, Oepkes D. Current status of testing for microdeletion syndromes and rare autosomal trisomies using cell-free DNA technology. *Obstet Gynecol* 2015;126:1095–1099.
36. Yatsenko SA, Peters DG, Saller DN, et al. Maternal cell-free DNA-based screening for fetal microdeletion and the importance of careful diagnostic follow-up. *Genet Med* 2015;17:836–838.
37. Bianchi DW, Parsa S, Bhatt S, et al. Fetal sex chromosome testing by maternal plasma DNA sequencing: clinical laboratory experience and biology. *Obstet Gynecol* 2015;125:375–382.
38. Devaney SA, Palomaki GE, Scott JA, Bianchi DW Noninvasive fetal sex determination using cell-free fetal DNA. A systematic review and meta-analysis. *JAMA* 2011;306:627–636.

APPENDIX 2
(See following page)

Section E6.1–6.4 of the ACMG technical standards and guidelines: chromosome studies of neoplastic blood and bone marrow–acquired chromosomal abnormalities

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Cytogenetic analyses of hematological neoplasms are performed to detect and characterize clonal chromosomal abnormalities that have important diagnostic, prognostic, and therapeutic implications. At the time of diagnosis, cytogenetic abnormalities assist in the diagnosis of such disorders and can provide important prognostic information. At the time of relapse, cytogenetic analysis can be used to confirm recurrence of the original neoplasm, detect clonal disease evolution, or uncover a new unrelated neoplastic process. This section deals specifically with the standards and guidelines applicable to chromosome studies of neoplastic blood and bone marrow–acquired chromosomal abnormalities.

6.1 GENERAL CONSIDERATIONS

6.1.1 Cytogenetic analyses of neoplastic blood and/or bone marrow–acquired clonal chromosomal abnormalities have been increasingly important in the clinical management of patients with hematological neoplasms. At time of diagnosis, cytogenetic abnormalities assist in the diagnosis of such disorders and can provide important prognostic information.[1] Furthermore, cytogenetic analysis can provide crucial information regarding specific genetically defined subtypes of these neoplasms that have targeted therapies. At time of relapse, cytogenetic analysis can be used to confirm recurrence of the original neoplasm, detect clonal disease evolution, or uncover a new unrelated neoplastic process.

6.1.2 These cytogenetic analyses include conventional G-banded chromosome analysis, fluorescence in situ hybridization (FISH),

and/or chromosomal microarray (CMA). Laboratories should work closely with oncologists and pathologists to determine the order of testing required to obtain relevant cytogenetic information in a cost-effective manner.

6.1.3 Laboratories offering cytogenetic analyses for hematological neoplasms should be familiar with the various chromosomal abnormalities associated with the different neoplasms and their clinical significance. The laboratory should be able to provide a robust analytical and interpretative service for the various hematological neoplasms. All results should be, to the extent possible, interpreted in the context of the clinical, pathologic, and molecular findings.[1,2]

6.1.4 Tissue processing, analytical variables, and turnaround time (TAT) should be determined by the laboratory based on

the indication for cytogenetic referral (e.g., initial diagnosis versus follow-up studies, pre- versus posttransplant studies, and lymphoid versus myeloid malignancies) and the clinical application of the cytogenetic results (e.g., selection of therapy).

6.1.5 Molecular genetics analyses are essential for diagnosis of some hematological neoplasms, and several molecular mutations, not detectable by cytogenetic analyses, provide important diagnostic and prognostic information. These are outside the scope of the current guidelines.

6.1.6 For quality assurance, the laboratory should monitor the numbers and types of hematological neoplasms received, percentage of cases with abnormal results, cell culture success rate, success rate of FISH and CMA studies, TAT, and correlation of FISH and CMA data with G-banded chromosome analysis results.

6.2 SPECIMEN COLLECTION AND PROCESSING
6.2.1 Specimen collection
6.2.1.1 Only those cells involved in the neoplastic process will harbor the abnormalities being sought. Therefore, the specimen type and culture techniques utilized should optimize the probability of detecting an abnormal clone.

6.2.1.2 In most cases, bone marrow is the tissue of choice for cytogenetic analyses of suspected hematological neoplasms. In some circumstances, alternative specimens may be used, including the following:

a. Peripheral blood specimens may yield informative results when the circulating blast cell percentage is higher than 10%. In general, the abnormal clone can be identified in such specimens, albeit not as often as in bone marrow. Peripheral blood or bone marrow can be used in chronic lymphocytic leukemia (CLL).
b. Bone marrow core biopsy specimens.
c. Bone marrow smears and core biopsy touch imprints can be used for interphase FISH.
d. Lymph node biopsy material or biopsy material from a suspected lymphoid mass are the preferred tissue in all lymphomas.
e. Cerebrospinal fluid.
f. Extramedullary leukemia (myeloid sarcoma, chloroma) tissue biopsy.

6.2.1.3 Specimens should be collected under sterile conditions in sodium heparin tubes for chromosome and/or FISH analyses. Bone marrow aspirate and biopsy specimens should preferably be collected in an appropriate transport medium tube with sodium heparin. The concentration of sodium heparin should be ~20 U/ml of specimen (per either bone marrow volume alone or per total volume of bone marrow and transport medium combined). EDTA tubes can be used for procedures that require genomic DNA extraction.

6.2.1.4 The volume of bone marrow available will differ for adults and children. An approximate specimen of 1 to 3 ml should be requested. During specimen procurement, several

draws are likely to be withdrawn. Because the first draw is more concentrated with neoplastic immature bone marrow cells, it is recommended that cytogenetics receive the first or second draw whenever possible.

6.2.1.5 Specimens should be received by the laboratory as soon as possible, ideally within 24 hours. Also, it is recommended that specimens be maintained at ambient temperature during transit. Extreme temperatures should be avoided.

6.2.1.6 If the specimen size precludes cell culture and conventional G-banded chromosome analysis, bone marrow smears or core biopsy touch imprints can be used for interphase FISH analysis.

6.2.2 Specimen processing
6.2.2.1 The laboratory should process the specimen as soon as possible after it is received. The methods that will be used to analyze the specimen should be determined prior to processing whenever possible. If chromosome analysis is requested, cell culture will be required. If FISH and/or CMA analyses are requested, a portion of the specimen can be used for direct harvest of interphase cells and/or genomic DNA extraction.

6.2.2.2 If a bone marrow core biopsy is obtained, it should be disaggregated to generate a cell suspension. This can be achieved by mechanical mincing and/or enzymatic digestion using collagenase. Culture conditions are the same as those for a bone marrow aspirate.

6.2.2.3 Cell culture conditions should be optimized for the specific hematological neoplasm suspected:

a. Acute leukemias, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute biphenotypic leukemia: Unstimulated short-term cultures are recommended. If sufficient specimen is received, at least two cultures should be initiated, including direct, overnight, and/or 24-hour cultures. In pediatric ALL, an additional unstimulated 48-hour culture can be useful in characterizing the abnormal karyotype. The seeding density is usually 1 to 3 million cells per ml of medium.
b. Myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN): Same as acute leukemias.
c. Plasma cell dyscrasias, including multiple myeloma (MM) and plasma cell leukemia: Unstimulated 24- and 72-hour cultures as well as 120-hour IL-4-stimulated culture are recommended.[3] For FISH and/or CMA analyses, if the bone marrow plasma cells percentage (as determined by flow cytometry) is below a certain cutoff value, plasma cell separation is recommended to enrich for the CD138^{+} plasma cell fraction.[4,5] The laboratory needs to establish its cutoff value for plasma cell enrichment.
d. Chronic lymphoproliferative disorders: Depending on the immunophenotype, additional cultures with B- or T-cell mitogens may be helpful. In CLL and other mature B-cell neoplasms, CpG-oligonucleotide cell stimulation

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is recommended and has been shown to enhance the detection of clonal chromosomal abnormalities.[6,7]

e. Well-differentiated T-cell disorders (e.g., T-cell leukemias, T-cell lymphoma, Sézary syndrome, and mycosis fungoides): T-cell mitogens may be helpful.

6.3 ANALYSIS

6.3.1 Conventional G-banded chromosome analysis

6.3.1.1 Cell selection: metaphase cells should not be selected for analysis solely on the basis of good chromosome morphology. In general, the technologist should select an area of the slide to begin the analysis and then examine metaphase cells as they appear consecutively in the microscope field, only skipping cells for which extremely poor morphology precludes chromosome identification. This technique can also be performed using automated metaphase finders by examining metaphase cells consecutively captured by the system. Sufficient cells should be analyzed or examined to maximize the detection of an abnormal clone and establish the clonality of the abnormality found. For each abnormal clone identified, clonal cells with the best chromosome morphology should be analyzed, captured, and karyotyped to provide the most accurate breakpoint assignment.

When cells are skipped because of poor morphology, it is important to attempt to count the number of chromosomes. This is particularly true for possible hyperdiploid or hypodiploid pediatric ALL and hyperdiploid plasma cell dyscrasias. In addition, attempts should be made to identify possible structural chromosomal abnormalities, particularly if the disease under consideration is associated with a specific recurring abnormality (e.g., the t(9;22) in chronic myeloid leukemia (CML)).

6.3.1.2 Number of cells evaluated: The number of metaphase cells analyzed versus the number of cells counted or scored should be appropriate for the type of the study (e.g., initial diagnosis or follow-up studies) and the purpose of the study (e.g., detection of residual disease or response to therapy, monitoring for clonal evolution, or monitoring of allogeneic transplant engraftment).

6.3.1.3 Initial diagnostic studies:

a. Analysis: Analyze a minimum of 20 cells from unstimulated cultures. For the mature B- and T-cell disorders, a combination of unstimulated and mitogen-stimulated cultures may be appropriate as described. Unstimulated CLL cultures infrequently yield CLL-related clonal chromosomal abnormalities; however, they can reveal MDS-related clonal abnormities since some of these patients might have co-morbid MDS because of either prior therapy or age-related. Similarly, unstimulated 24-h MM cultures can reveal co-morbid MDS-related clonal abnormalities.

b. Documentation:
- For the abnormal cells:
  - If *only one* abnormal clone is present: two karyotypes.

- If *more than one* related abnormal clone is present: two karyotypes of the stemline and one of each sideline.
- If *unrelated clones* are present: two karyotypes for each stemline and one for each associated pertinent sideline.
- In instances when the sideline contains complex abnormalities, two karyotypes of each sideline may be required for better documentation.
- For the normal cells:
  - If *only normal* cells are present: two karyotypes.
  - If *normal and abnormal cells* are present: one karyotype of a normal cell.

6.3.1.4 Follow-up studies of patients who have had a previous cytogenetic study: For the following analytic guidelines, it is assumed that the laboratory has documentation of the patient's previous cytogenetic results. If the study has been performed elsewhere and there is minimal information available, it is recommended that, except for patients seen for the first time posttransplant, the analysis be considered the same as an initial diagnostic workup (see above).

I. Patients who *have not* undergone allogeneic hematopoietic cell transplantation:

a. Analysis: analyze 20 cells. If all cells are normal, additional cells may be scored for a specific abnormality by G-banding or FISH if pathology is positive for the diagnosis in question. For some patients, follow-up cytogenetic study is ordered to rule out a therapy-associated malignancy (e.g., MDS) rather than disease recurrence.

b. Documentation:
- For cases with both normal and abnormal cells or only abnormal cells:
  - One karyotype of a normal cell, if such a karyotype was not documented in a previous study by the laboratory; otherwise, one normal metaphase spread.
  - One or two karyotypes from each abnormal clone for a minimum total of two karyotypes.
- For cases with all normal cells:
  - Two karyotypes.

II. Patients who *have* undergone an allogeneic hematopoietic cell transplantation for whom donor versus recipient origin of the cells can be determined (by sex chromosome complement or cytogenetic heteromorphisms):

For studies aimed solely at determining engraftment status, molecular methods and/or interphase FISH (in the case of opposite sex transplant) are more sensitive than G-banded chromosome analysis and are the preferred methodologies. Therefore, in consultation with the referring physician, cancellation of test

requests for G-banded chromosome analysis for engraftment status should be considered.

During the course of the cytogenetic analysis, it will become evident whether there is chimerism for donor and recipient cells. It is expected that there will be different approaches used by different laboratories to address these studies.

- If *only donor* cells are present:
  a. Analysis: analyze 20 cells.
  b. Documentation: document two karyotypes for each cell line. In such cases, one is documenting either the constitutional karyotype (normal or abnormal of the donor) or the rare event of a malignant process arising in a donor cell.

- If *donor and recipient* cells are present:
  a. Analysis: Analyze recipient cells completely for previously identified clonal chromosome abnormalities and any newly acquired abnormalities. In some cases there may be structural chromosomal abnormalities secondary to chromosome breakage or rearrangement induced by the pretransplant conditioning regimen. The laboratory should distinguish clonal from nonclonal changes and determine the significance of new abnormalities as much as possible.
  Analyze all recipient cells present out of 20 cells analyzed. Evaluate each recipient cell for the presence of the abnormality present prior to transplantation (i.e., the diagnostic abnormality). Depending on the number of recipient cells present among the initial 20 metaphase cells scored, additional recipient cells may be analyzed completely and/or scored for the presence of the diagnostic abnormality.
  Donor cells: analyze two donor cells if donor cells have not been analyzed in previous studies. Otherwise, simply score these cells as being of donor origin and count.
  b. Documentation: for the recipient cells: Two karyotypes of the stemline and one of each sideline. For the donor cells: If donor cells have been documented previously, then provide a single metaphase spread. If donor cells have not been documented previously, then provide two karyotypes.

- If *only recipient* cells are present:
  a. Analysis: analyze 20 cells following the guidelines set forth above with respect to the characterization of secondary abnormalities.
  b. Documentation: same as noted above for abnormal recipient cells.

III. Patients who *have* undergone an allogeneic hematopoietic cell transplantation for whom donor and recipient cells *cannot* be determined:
   Analysis: analyze 20 cells. As in case scenarios outlined here, follow guidelines for recipient cells as set forth above.

6.3.2 FISH analysis

6.3.2.1 Interphase FISH analysis may be used as a primary testing methodology in conjunction with G-banded chromosome analysis for the evaluation of hematological neoplasms. FISH studies may be indicated to (i) provide a rapid result to aid in the differential diagnosis or planning of therapy; (ii) detect a cryptic chromosomal abnormality or gene rearrangement, especially when G-banded chromosome analysis yields normal results; (iii) detect clinically significant gene amplification, which may also require metaphase FISH analysis to document the tandem nature of this rearrangement on the same chromosome or the presence of double minutes; (iv) provide an alternative diagnostic method when no metaphase cells are obtained by blood or bone marrow cultures; and (v) detect abnormalities in samples that are not adequate or not suitable for G-banded chromosome analysis.

6.3.2.2 Characterization of the initial diagnostic interphase FISH abnormal signal pattern is important and will allow future monitoring of the patient's disease.

6.3.2.3 Metaphase FISH analysis and/or sequential G-banded chromosome analysis to metaphase FISH analysis may be useful and provides a useful methodology to characterize variant chromosomal abnormalities or gene rearrangements as demonstrated by a variant abnormal interphase FISH signal pattern.

6.3.2.4 Analysis and documentation of FISH studies should be in accordance with Section E9 of these standards and guidelines for clinical genetics laboratories.

6.3.3 CMA analysis

6.3.3.1 CMA analysis can add valuable information that will support and supplement both G-banded chromosome analysis and FISH. It can detect small cryptic clinically significant copy number changes (CNCs) in various hematological neoplasms. Additionally, CMA SNP platforms can also detect copy-neutral loss of heterozygosity (cnLOH). However, this technology cannot detect balanced chromosomal rearrangements.

6.3.3.2 The clinical utility of genome-wide CMAs in cancer diagnostics is growing rapidly. This technology is being used to better identify high-risk patients and predict clinical outcomes. In view of the rapid introduction of CMAs into clinical practice, it is important that laboratories stay up-to-date with this technology.

6.3.3.3 Analysis and documentation of CMA studies should be in accordance with Section E11 of these standards and guidelines for clinical genetics laboratories.

6.3.4 Recommended cytogenetic analysis scheme in hematological neoplasms

6.3.4.1 *Acute leukemias*

Bone marrow is the preferred specimen for acute leukemias, but peripheral blood can be used when >10% circulating blast cells are present.[8] Interphase FISH analysis performed on bone marrow smears or core biopsy touch imprints is an alternative in cases with a dry tap and/or hemodiluted bone marrow aspirate and absent/low circulating blast cells. A strong collaboration with the oncologist and pathologist is important for

establishing the order of testing and additional tests that should be undertaken.[9]

1. AML
   - G-banded chromosome analysis should prefer-ably be performed first. However, interphase FISH analysis for *KMT2A (MALL)* gene rearrangement is highly recommended on all diagnostic AML samples because these abnormalities are often cryptic and have a pronounced prognostic impact.
   - In case of a successful normal chromosome analysis with a clear diagnosis of AML by morphology and flow cytometry, additional interphase and metaphase FISH analyses are recommended to exclude cryptic rearrangements. Depending on the morphology and flow cytometry results, the following FISH probes can be added:
     a. *RUNX1-RUNX1T1 (AML1-ETO)* fusion probes
     b. *CBFB* rearrangement or *CBFB-MYH11* fusion probes: inv(16) and t(16;16) resulting in *CBFB-MYH11* fusion can be subtle in cases with sub-optimal G-banded chromosomes quality
     c. *KMT2A (MLL)* rearrangement probes
     d. *PML-RARA* fusion probes: *PML-RARA* fusion is diagnostic of acute promyelocytic leukemia (APL), which is usually strongly suspected at diagnosis based on the patient's presentation and blast cell morphology. A *RARA* break-apart probe can be used to detect variant transloca-tions in which *RARA* fuses with a different partner
   - In case of an incomplete/unsuccessful chromosome analysis or if the laboratory is unable to maintain a short TAT for chromosome analysis, then the fol-lowing probes can be bundled in an AML FISH panel, which should be performed on the diagnostic specimen:
     a. *RUNX1-RUNX1T1 (AML1-ETO)* fusion probes
     b. *CBFB* rearrangement or *CBFB-MYH11* fusion probes
     c. *KMT2A (MLL)* rearrangement probes
     d. –5/5q– probes
     e. –7/7q– probes
     f. *PML-RARA* fusion probes: if there is suspicion of APL based on the patient's presentation and blast cell morphology
   - *MECOM (EVI1)* rearrangement probes should be considered when chromosome analysis is suggestive of an inv(3) or t(3;3).
   - Recent CMA studies revealed acquired CNCs and region of cnLOH that add independent prognostic impact in AML. CMA analysis can detect CNCs that are more specific to primary AML, whereas others are more specific to therapy-related AML.[10] In addi-tion, regions of cnLOH are more often detected in

patients with normal karyotypes than with abnor-mal karyotypes.[11,12]

2. ALL
   - B-lineage ALL is more frequent, accounting for 85% of pediatric ALL and 75% of adult ALL.[1]
   - In pediatric/young adult B-lineage ALL, G-banded chromosome analysis should be performed simulta-neously with interphase FISH analysis using a panel that includes the following probes:
     a. *BCR-ABL1* fusion probes
     b. *KMT2A (MLL)* rearrangement probes
     c. *ETV6-RUNX1* fusion probes: for *ETV6-RUNX1* fusion, *ETV6* deletion, and iAMP21 (intrachro-mosomal amplification of chromosome 21)
     d. Centromeric probes for chromosomes 4 and 10: for trisomies of chromosomes 4 and 10
   - In adult B-lineage ALL, G-banded chromosome analysis should be performed simultaneously with interphase FISH analysis using the following probes:
     a. *BCR-ABL1* fusion probes
     b. *KMT2A (MLL)* rearrangement probes
   - In both pediatric and adult B-lineage ALL, and depending on the blast cell morphology, flow cytom-etry, chromosome analysis, and FISH results, addi-tional interphase FISH testing should be considered, including:
     a. *CRLF2* rearrangement probes: for *P2RY8-CRLF2* fusion and *IGH-CRFL2* fusion (Ph-like ALL)[13]
     b. *PDGFRB* rearrangement probes (Ph-like ALL)[13]
     c. *CDKN2A/B* (9p21.3) probe: 9p21.3 deletion is common in both B- and T-lineage ALLs, but its prognostic significance has been debated; how-ever, it provides a clonal target for future moni-toring of the patient's disease in the absence of other FISH targets
     d. *PAX5* (9p13.2) probe
   - *MYC* rearrangement and/or *IGH-MYC* fusion probes should be considered in both pediatric and adult ALL, where the morphology and flow cytom-etry results are suggestive of B-cell ALL (Burkitt leu-kemia variant)
   - In T-lineage ALL, G-banded chromosome analysis should be performed first. Interphase FISH analysis is optional and could include the following probes:
     a. *BCR-ABL1* fusion probes: for *BCR-ABL1* fusion and *ABL1* amplification
     b. *KMT2A (MLL)* rearrangement probes
   - In ALL, CMA analysis can be very helpful for detecting cryptic CNCs, with proven relevance to diagnosis, prognosis, and therapeutic response.[14-16] Examples include deletions involving *PAX5* and *IKZF1* genes. It can also help clarify the structure of complex chromosomal rearrangements. Finally,

CMA SNP platforms can detect whole-chromosome cnLOH due to "doubling" of a near-haploid or low hypodiploid clone, which manifests in the form of a hyperdiploid or near-triploid karyotype. The prognosis of these two entities is very different.

6.3.4.2 Myelodysplastic syndromes

- Bone marrow is the preferred specimen for MDS.[17] Interphase FISH analysis performed on bone marrow smears or core biopsy touch imprints is an alternative in cases with a dry tap and/or hemodiluted bone marrow aspirate. A strong collaboration with the oncologist and pathologist is important in MDS cases, where other non-neoplastic hematological disorders can have a similar presentation.
- G-banded chromosome analysis should preferably be performed first. In case of an incomplete/unsuccessful chromosome analysis or if the laboratory is unable to maintain a short TAT for chromosome analysis, the following probes can be bundled in an MDS FISH panel,[18] which should be performed on the diagnostic specimen:
  a. -5/5q- probes
  b. -7/7q- probes
  c. Centromeric probe for chromosome 8: for trisomy 8
  d. 20q- probe
- Recent data suggest that MDS exhibits abundant clonal CNCs and cnLOH, often in the setting of a normal metaphase karyotype and with no previously identified clonal markers. CMA analysis is proving to be very useful in uncovering these genomic aberrations in MDS.[19,20] Examples include cryptic 5q deletions distal to the EGR1 gene (5q31). These can be missed by G-banded chromosome and FISH analyses.[21]

6.3.4.3 Myeloproliferative neoplasms and myelodysplastic syndromes/myeloproliferative neoplasms.

This is a heterogeneous group of clonal stem disorders that is broadly divided into three groups.[9,22] The first is the classical MPN group, which includes CML (BCR-ABL1 fusion positive), polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia not otherwise specified, mastocytosis, and MPN unclassified. The second group includes myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1. The third is the MDS/MPN group, which includes chronic myelomonocytic leukemia, atypical CML (BCR-ABL1 fusion negative), juvenile myelomonocytic leukemia, and MDS/MPN unclassified.

1. CML
   - Bone marrow is the preferred specimen for CML; however, peripheral blood may be used if the level of blasts is >10%.
   - The t(9;22)(q34;q11.2) is detectable in 90–95% of CML cases at diagnosis. The remaining 5–10% of

cases have either a variant t(9;22) or a cryptic BCR-ABL1 fusion undetectable by chromosome analysis.
   - Therefore, both G-banded chromosome analysis as well as interphase FISH analysis using BCR-ABL1 fusion probes should be performed simultaneously at diagnosis.
   - It is important to establish whether additional chromosome abnormalities are present at diagnosis, including an additional der(22), i(17q), and trisomy 8. These are warning signs that might be associated with inferior overall survival and increased risk of progression to accelerated phase.[23,24]
   - The CML National Comprehensive Cancer Network (NCCN) guidelines recommend that cytogenetic studies (both G-banded chromosome and BCR-ABL1 fusion FISH analyses) and quantitative RT-PCR BCR-ABL1 fusion testing be performed at diagnosis. If no BCR-ABL1 fusion can be detected, molecular testing for mutations associated with other myeloproliferative conditions is indicated.

2. Other MPNs
   - Bone marrow is the preferred specimen for other MPNs; however, peripheral blood may be used if there is peripheral involvement. With few exceptions, cytogenetic abnormalities are usually not specific in other MPNs. Typical abnormalities of myeloid neoplasms are usually observed and can be useful in demonstrating evidence of clonality.
   - Interphase FISH analysis performed on bone marrow smears or core biopsy touch imprints is an alternative in cases with a dry tap and/or hemodiluted bone marrow aspirate. A strong collaboration with the oncologist and pathologist is important.
   - The exclusion of BCR-ABL1 fusion is necessary for the differential diagnosis of other MPNs from CML.
   - Other specific FISH probes recommended in other MPNs based on the pathology input include FIP1L1-PDFGRA fusion, PDGFRB rearrangement, and FGFR1 rearrangement probes in myeloid/lymphoid neoplasms with eosinophilia. MPNs with these gene rearrangements can be treated with targeted therapies (i.e., tyrosine kinase inhibitors).

6.3.4.4 Plasma cell dyscrasias

- A bone marrow specimen is required for MM. For FISH and/or CMA analyses, plasma cell separation is recommended to enrich for the CD138+ plasma cell fraction in bone marrow samples with low plasma cell percentages (see Section 6.2.2.3).[4,5]
- G-banded chromosome analysis should be performed (as described above) simultaneously with interphase FISH analysis using a panel that includes the following probes:[25–27]
  a. 1q21.3 probe (including CKS1B): for 1q21 copy gain, which has been linked to adverse prognosis

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b. 13q14.2q14.3 probes (including *RBI*): 13q14.2q14.3 deletion is common in MM but, when detected only by FISH, it is not predictive of survival in the absence of other adverse cytogenetic abnormalities. However, it provides a clonal target for future monitoring of the patient's disease in the absence of other FISH targets. 13q deletion detected by G-banded chromosome analysis still retains its prognostic value

c. *IGH* rearrangement probes: if *IGH* is rearranged, including the classical gene disruption as well as deletion of either the 5′ or 3′ region of *IGH*, then reflex to *IGH-FGFR3, IGH-CCND1*, and *IGH-MAF* fusion probes.

d. *TP53* (17p13.1) probe

e. Probes for three of the odd-numbered chromosomes often trisomic in hyperdiploid MM (e.g., chromosomes 5, 9, 11, 15, and 19)

- The use of CMA analysis on the enriched plasma cell fraction has been shown to be very valuable in detecting clinically relevant CNCs.[28-30]

6.3.4.5 Chronic lymphocytic leukemia

- CLL is a mature B-cell neoplasm diagnosed by B-cell count, morphology, and flow cytometry. Cytogenetically, either peripheral blood or bone marrow can be used in CLL. G-banded chromosome analysis should be performed simultaneously with interphase FISH analysis.[31] CLL cell stimulation in culture using CpG-oligonucleotides greatly improves the detection rate of clonal cytogenetic abnormalities by G-banded chromosome analysis.[6,7]

- To assign the patient into clinically relevant prognostic subgroups, the following panel of FISH probes is recommended:
  a. *ATM* (11q22.3) probe
  b. Centromeric probe for chromosome 12: for trisomy 12
  c. 13q14.3 probe (including D13S319)
  d. *TP53* (17p13.1) probe

- FISH can also be useful for the differential diagnosis with mantle cell lymphoma (MCL), for which FISH using the *IGH-CCND1* fusion probes is recommended.

- In CLL, CMA analysis has proven to be very effective in detecting CNCs and cnLOH at genomic regions with established prognostic significance, and it provides a much higher resolution compared to G-banded chromosome and FISH analyses.[32,33] Examples include 13q14 deletions, which are quite heterogeneous.[34] Moreover, clinically relevant genomic alterations in CLL involve mostly deletions and duplications, whereas most balanced translocations are relatively rare and are of unclear significance.

6.3.4.6 B- and T-cell lymphomas

- For all lymphomas, the preferred tissue is lymph node or biopsy material from a suspected lymphoid mass. If fresh material is available, G-banded chromosome analysis is recommended.
- Interphase FISH analysis using relevant probes performed on lymph node tissue sections, fine needle aspirate smears, and/or touch imprints should be included.
- For lymph node cytogenetic analysis in lymphomas, see Section E6.5-6.8.
- Bone marrow or peripheral blood analysis will not detect clonal chromosomal abnormalities if there is no evidence of infiltration. For FISH analysis, bone marrow smears or core biopsy touch imprints can be used.

6.4 TAT AND REPORTING

6.4.1 TAT
6.4.1.1 Specific chromosomal abnormalities are crucial for establishing a diagnosis and have direct relevance to specific treatment. Therefore, an effort should be made to expedite communicating the cytogenetic analyses results to the oncologist. It is recommended that the cytogenetics laboratory should have a written policy describing how cases are prioritized in the laboratory.

6.4.1.2 TAT guidance:

a. Initial diagnostic workup: It is strongly recommended that the preliminary result should be reported within 7 calendar days, and the final results should be reported within 21 calendar days.
b. Follow-up studies: It is strongly recommended that the final results should be reported within 21 calendar days.
c. FISH studies: Reporting the FISH results within 3–5 working days from the time of receiving the specimen is recommended whenever possible.

6.4.2 Reporting
6.4.2.1 The most recent edition of the International System for Human Cytogenetic Nomenclature (ISCN) should be used to report the cytogenetics results.[35]

6.4.2.2 The number of cells analyzed (both normal and abnormal) should be documented in the final report.

6.4.2.3 For CMA analysis, clones and subclones cannot be ascertained with certainty; however, the percentage of cells (levels of mosaicism) can be provided to give an estimate of possible clones/subclones and clonal diversity.

6.4.2.4 If a potential nonmosaic constitutional abnormality is observed, analysis of a PHA-stimulated peripheral blood sample during remission is strongly recommended to confirm that the abnormality is constitutional and not clonal.

6.4.2.5 At the time of initial diagnosis, finding a single abnormal metaphase cell, even one that is potentially significant, cannot be used as evidence of clonality unless there is strong supporting evidence of clonality for the same abnormality by either FISH or other molecular technique.

6.4.2.6 The final cytogenetic report of hematological acquired chromosomal abnormalities should contain the following information:

1. Patient identification using two different identifiers
2. Patient medical record number and/or laboratory identification number
3. Referring physician
4. Sample information (type, date of withdrawal and receipt, and date of report)
5. Reason for referral or suspected diagnosis
6. ISCN nomenclature of cytogenetic studies performed
7. Narrative description of the abnormalities observed, including modal chromosome number in each clone (to the extent possible), and numerical and structural abnormalities. The report should comment on the clinical significance of the abnormalities observed, including clinically relevant genes involved, possible disease association, and prognostic significance.
8. Literature references to support the clinical interpretation and to provide helpful information for the oncologist.

DISCLOSURE
All of the authors direct clinical cytogenetics laboratories that run the tests discussed in the current standards and guidelines on a fee-for-service basis.

REFERENCES
1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (eds). *WHO Classification of Tumours: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues 2008 (2008). 4th edn.* IARC Press: Lyon, France.
2. Mitelman F, Johansson B and Mertens F (eds). Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2015). http://cgap.nci.nih.gov/Chromosomes/Mitelman.
3. Hernández JM, Gutiérrez NC, Almeida J, et al. IL-4 improves the detection of cytogenetic abnormalities in multiple myeloma and increases the proportion of clonally abnormal metaphases. *Br J Haematol* 1998;103:163–167.
4. Stevens-Kroef M, Weghuis DO, Croockewit S, et al. High detection rate of clinically relevant genomic abnormalities in plasma cells enriched from patients with multiple myeloma. *Genes Chromosomes Cancer* 2012;51:997–1006.
5. Shin SY, Jang S, Park CJ, et al. Application of an immune-magnetic cell sorting method for CD138-positive plasma cells in FISH analysis of multiple myeloma. *Int J Lab Hematol* 2012;34:541–546.
6. Heerema NA, Byrd JC, Dal Cin PS, et al.; Chronic Lymphocytic Leukemia Research Consortium. Stimulation of chronic lymphocytic leukemia cells with CpG oligodeoxynucleotide gives consistent karyotypic results among laboratories: a CLL Research Consortium (CRC) Study. *Cancer Genet Cytogenet* 2010;203:134–140.
7. Shi M, Cipollini MJ, Crowley-Bish PA, Higgins AW, Yu H, Miron PM. Improved detection rate of cytogenetic abnormalities in chronic lymphocytic leukemia and other mature B-cell neoplasms with use of CpG-oligonucleotide DSP30 and interleukin 2 stimulation. *Am J Clin Pathol* 2013;139:662–669.
8. Weinkauff R, Estey EH, Starostik P, et al. Use of peripheral blood blasts vs bone marrow blasts for diagnosis of acute leukemia. *Am J Clin Pathol* 1999;111:733–740.
9. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009;114:937–951.
10. Itzhar N, Dessen P, Toujani S, et al. Chromosomal minimal critical regions in therapy-related leukemia appear different from those of de novo leukemia by high-resolution aCGH. *PLoS One* 2011;6:e16623.
11. Walter MJ, Payton JE, Ries RE, et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. *Proc Natl Acad Sci USA* 2009;106:12950–12955.
12. Parkin B, Erba H, Ouillette P, et al. Acquired genomic copy number aberrations and survival in adult acute myelogenous leukemia. *Blood* 2010;116:4958–4967.
13. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med* 2014;371:1005–1015.
14. Collins-Underwood JR, Mullighan CG. Genomic profiling of high-risk acute lymphoblastic leukemia. *Leukemia* 2010;24:1676–1685.
15. Simons A, Stevens-Kroef M, El Idrissi-Zaynoun N, et al. Microarray-based genomic profiling as a diagnostic tool in acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 2011;50:969–981.
16. Baughn LB, Biegel JA, South ST, et al. Integration of cytogenomic data for furthering the characterization of pediatric B-cell acute lymphoblastic leukemia: a multi-institution, multi-platform microarray study. *Cancer Genet* 2015;208:1–18.
17. Cherry AM, Slovak ML, Campbell LJ, et al. Will a peripheral blood (PB) sample yield the same diagnostic and prognostic cytogenetic data as the concomitant bone marrow (BM) in myelodysplasia? *Leuk Res* 2012;36:832–840.
18. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 2012;120:2454–2465.
19. Heinrichs S, Kulkarni RV, Bueso-Ramos CE, et al. Accurate detection of uniparental disomy and microdeletions by SNP array analysis in myelodysplastic syndromes with normal cytogenetics. *Leukemia* 2009;23:1605–1613.
20. Thiel A, Beier M, Ingenhag D, et al. Comprehensive array CGH of normal karyotype myelodysplastic syndromes reveals hidden recurrent and individual genomic copy number alterations with prognostic relevance. *Leukemia* 2011;25:387–399.
21. MacKinnon RN, Kannourakis G, Wall M, Campbell LJ. A cryptic deletion in 5q31.2 provides further evidence for a minimally deleted region in myelodysplastic syndromes. *Cancer Genet* 2011;204:187–194.
22. Vardiman J, Hyjek E. World health organization classification, evaluation, and genetics of the myeloproliferative neoplasm variants. *Hematology Am Soc Hematol Educ Program* 2011;2011:250–256.
23. Palandri F, Testoni N, Luatti S, et al. Influence of additional cytogenetic abnormalities on the response and survival in late chronic phase chronic myeloid leukemia patients treated with imatinib: long-term results. *Leuk Lymphoma* 2009;50:114–118.
24. Fabarius A, Leitner A, Hochhaus A, et al.; Schweizerische Arbeitsgemeinschaft für Klinische Krebsforschung (SAKK) and the German CML Study Group. Impact of additional cytogenetic aberrations at diagnosis on prognosis of CML: long-term observation of 1151 patients from the randomized CML Study IV. *Blood* 2011;118:6760–6768.
25. Dimopoulos M, Kyle R, Fermand JP, et al.; International Myeloma Workshop Consensus Panel 3. Consensus recommendations for standard investigative workup: report of the International Myeloma Workshop Consensus Panel 3. *Blood* 2011;117:4701–4705.
26. Munshi NC, Anderson KC, Bergsagel PL, et al.; International Myeloma Workshop Consensus Panel 2. Consensus recommendations for risk stratification in multiple myeloma: report of the International Myeloma Workshop Consensus Panel 2. *Blood* 2011;117:4696–4700.
27. van de Donk NW, Sonneveld P. Diagnosis and risk stratification in multiple myeloma. *Hematol Oncol Clin North Am* 2014;28:791–813.
28. Agnelli L, Mosca L, Fabris S, et al. A SNP microarray and FISH-based procedure to detect allelic imbalances in multiple myeloma: an integrated genomics approach reveals a wide gene dosage effect. *Genes Chromosomes Cancer* 2009;48:603–614.
29. Avet-Loiseau H, Li C, Magrangeas F, et al. Prognostic significance of copy-number alterations in multiple myeloma. *J Clin Oncol* 2009;27:4585–4590.
30. Walker BA, Leone PE, Chiecchio L, et al. A compendium of myeloma-associated chromosomal copy number abnormalities and their prognostic value. *Blood* 2010;116:e56–e65.
31. Zenz T, Mertens D, Döhner H, Stilgenbauer S. Importance of genetics in chronic lymphocytic leukemia. *Blood Rev* 2011;25:131–137.
32. Hagenkord JM, Monzon FA, Kash SF, Lilleberg S, Xie Q, Kant JA. Array-based karyotyping for prognostic assessment in chronic lymphocytic leukemia: performance comparison of Affymetrix 10K2.0, 250K Nsp, and SNP6.0 arrays. *J Mol Diagn* 2010;12:184–196.
33. O'Malley DP, Giudice C, Chang AS, et al. Comparison of array comparative genomic hybridization (aCGH) to FISH and cytogenetics in prognostic evaluation of chronic lymphocytic leukemia. *Int J Lab Hematol* 2011;33:238–244.
34. Ouillette P, Collins R, Shakhan S, et al. The prognostic significance of various 13q14 deletions in chronic lymphocytic leukemia. *Clin Cancer Res* 2011;17:6778–6790.
35. Shaffer LG, McGowan-Jordan J, Schmid M (eds). *An International System for Human Cytogenetic Nomenclature (ISCN) 2013.* S. Karger: Basel, Switzerland.

ERRATUM: Section E6.1–6.4 of the ACMG technical standards and guidelines: chromosome studies of neoplastic blood and bone marrow–acquired chromosomal abnormalities

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On page 5, in the left column, first paragraph under "AML," a gene name is misspelled. The correct gene name is "*KMT2A* (*MLL*)." The publisher regrets the error.

APPENDIX 3
(See following page)

Section E6.5–6.8 of the ACMG technical standards and guidelines: chromosome studies of lymph node and solid tumor–acquired chromosomal abnormalities

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Cytogenetic analysis of tumor tissue is performed to detect and characterize chromosomal aberrations to aid histopathological and clinical diagnosis and patient management. At the time of diagnosis, known recurrent clonal aberrations may facilitate histopathological diagnosis and subtyping of the tumor. This information may contribute to clinical therapeutic decisions. However, even when tumors have a known recurrent clonal aberration, each tumor is genetically unique and probably heterogeneous. It is important to discover as much about the genetics of a tumor at diagnosis as is possible with the methods available for study of the tumor material. The information gathered at initial study will inform follow-up studies, whether for residual disease detection, determination of relapse and clonal evolution, or identifying a new disease clone.

This updated Section E6.5–6.8 has been incorporated into and supersedes the previous Sections E6.4 and E6.5 in Section E: Clinical Cytogenetics of the 2009 Edition (Revised 01/2010), American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories. This section deals specifically with the standards and guidelines applicable to lymph node and solid tumor chromosome analysis.

6.5 GENERAL CONSIDERATIONS

6.5.1 Genetic analysis of solid tumors and lymphomas at diagnosis provides information critical for diagnosis and patient management.[1,2] Analysis of tumor tissues may be accomplished by conventional chromosome analysis, fluorescence in situ hybridization (FISH) analysis, chromosomal microarray (CMA) analysis, molecular analysis, or a combination of methodologies. Because the genetic information aids in the differential diagnosis and provides direction for the most appropriate therapeutic management, including targeted therapies, tumor materials should be studied with available methods to gain as much

information as possible at the time of initial study. At a time of suspected disease recurrence or metastasis, the initial genetic data will be used to confirm recurrence or metastasis, assess clonal disease evolution, or reveal a new malignant process.

The method(s) chosen for evaluation of a tumor at the time of biopsy or resection will depend on the differential diagnosis, clinical indications, available tissue, available methodologies, and initial histopathology of the tumor tissue.

For disease staging, tumor samples may be accompanied or followed by other tissue samples for analysis, such as bone marrow and cerebrospinal fluid.

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6.5.2 The laboratory director and staff should be familiar with the chromosomal and molecular aberrations associated with tumor types/subtypes and their clinical significance. **Supplementary Tables S1–S5** online include common solid tumor and lymphoma chromosomal aberrations with known genes, potential FISH targets, clinical significance, and references.

6.5.3 Pediatric tumors should be cytogenetically analyzed whenever sufficient fresh tissue is available. Karyotyping, although low-resolution, provides a view of the entire genome. This genome view allows detection of cytogenetic aberrations that are commonly disease- or disease subtype–specific and have prognostic and therapeutic significance. Genetic analysis of adult tumors is indicated whenever such analysis may provide diagnostic, prognostic, or treatment-related information, especially if targeted therapies are available for the disorder undergoing study.

6.5.4 Methods for the processing of tumor material should be determined by the cytogenetic laboratory based on available clinical and pathologic findings. Laboratories should work with the oncologist and pathologist to determine the method(s) to gain the most genetic information cost-effectively. The laboratory should seek information about the suspected diagnosis and tissue type at the time of sample receipt to choose the most appropriate testing and tissue culture method(s) and to determine if DNA should be isolated from the fresh tumor. **Supplementary Table S6** online provides tumor nomenclature for tumor culture method selection.

6.5.5 Conventional cytogenetic, FISH, CMA, gene mutation panel, or sequencing analysis may be used as a primary or secondary method of evaluation of the tumor tissue. Multiple technologies may be needed for specific tumor types. The availability of fresh tissue, the differential diagnosis, a need for rapid diagnostic information, and the type of information needed should be used to prioritize testing such as conventional cytogenetic analysis, FISH, CMA, and/or mutation analysis.

6.5.6 Cytogenetic and molecular analysis results must be interpreted within the context of the pathologic and clinical findings.

6.5.7 For quality assurance, the laboratory may monitor the number and types of tumors received, the percentage of tumors with abnormal results, the cell culture success rate, and the success rate for FISH and CMA studies.

6.5.8 The presence or absence of specific aberrations should be available to the physician as soon as is feasible to contribute to the patient's plan of care.

6.6 SAMPLE COLLECTION AND PROCESSING

6.6.1 Sample collection

6.6.1.1 Tumor samples should be collected in a sterile manner. For conventional cytogenetic analysis, the tissue sample must be fresh. The sample selected for cytogenetic analysis should be "pure" tumor if possible, without necrosis. The sample must not be placed in fixative or frozen. Samples to be evaluated solely by FISH or CMA analysis may be fixed, frozen, or paraffin-embedded. If CMA analysis or sequencing is requested at the time of biopsy, DNA should be isolated from fresh tumor or formalin-fixed paraffin-embedded tumor rather than cultured tumor cells because clonal aberrations may be lost during cell culture. Cultured tumor cells may be used for isolation of DNA if the karyotype is clonally abnormal. The use of formalin-fixed paraffin-embedded samples for FISH and DNA isolation allows a pathologist to identify and mark optimal areas of tumor to examine, specify the percentage of tumor in an area, and/or identify areas of necrosis or stromal tissue to avoid.

6.6.1.2 The laboratory should request a sample size of 0.5 to 1 cm³. If less tissue is available, the laboratory should accept as much as can be provided. If the sample size is very limited (e.g., fine needle aspirate or needle core biopsy), coverslip cultures are often successful. If the sample size precludes cell culture and conventional cytogenetic evaluation, touch preparations, cytospins, or paraffin-embedded tissue sections may be used for FISH analysis, or DNA may be isolated for CMA or sequencing analysis. See Section E6.5.2.

6.6.1.3 Fresh tumor should be transported in culture medium to the cytogenetics laboratory as soon as possible for immediate processing.

6.6.2 Sample processing

6.6.2.1 The cytogenetic laboratory should process the tumor sample as soon as possible after it is received. Prior to processing, it should be clear what methods will be used to analyze the sample (e.g., chromosome analysis, FISH, CMA, sequencing). If the sample is to be processed for CMA or sequencing, select a portion of the sample for DNA isolation. If the sample is for FISH analysis, touch preparations may be made or direct harvest performed. If the sample is for chromosome analysis, tissue culture will be required.

6.6.2.2 The fresh tumor sample should be inspected and details of the sample size, color, and attributes recorded. The time of sample collection and the time of sample receipt in the laboratory should be documented.

6.6.2.3 The cytogenetics laboratory should expect the sample submitted by a pathologist to be most representative of the tumor as determined by gross examination. However, if the fresh sample received by the laboratory is large and appears heterogeneous, portions of the sample may be cultured separately. If obvious normal, necrotic, or vascular tissues are present, the tumor should be separated from nontumor tissue for processing. Obvious necrotic tissue should be removed to reduce enzymatic damage induced by dying cells. If the tumor cannot be distinguished from normal or necrotic tissue, caution should be exercised and the entire sample processed.

6.6.2.4 For tissues from a body region with high concentrations of bacteria (e.g., tonsils, gut), treatment of the sample prior to disaggregation with antibiotic and/or antifungal solutions and addition of antibiotic and/or antifungals to the medium may be prudent.

6.6.2.5 Disaggregation methods should be optimized for different tissue types:

a. Disaggregation of solid tumor samples for tissue culture is needed. Mechanical and/or enzymatic methods may

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be used. If sufficient tumor material is submitted, both methods of disaggregation are recommended. For some tumor types, different growth characteristics can be seen with exposure to collagenase versus no exposure to collagenase. If sufficient material is available, cultures should be initiated with and without enzyme exposure.

b. Disaggregation of lymphoid tissues into single cell suspension is necessary before culture initiation. The lymphoid cells in most tissues are readily disaggregated by mechanical means such as mincing with scalpels or curved scissors. The use of these methods is often advantageous if the tissue is easily dissociated because it will keep the loss of cells to a minimum and may help minimize stromal contamination because stromal cells are often locked in fibrous connective tissues. If cells are not readily liberated by mechanical means, enzymatic digestion may be necessary. When using enzymatic digestion, the tissue must first be minced and then incubated with the enzyme solution (e.g., collagenase) for 20 minutes to 16 hours depending on how quickly cell release occurs.

6.6.2.6 Culture methods, culture medium, and culture conditions should be chosen to best support the type of tumor received.

a. The diagnosis and histopathology of a tumor can be helpful in determining culture and harvest methods. Different cell types can be expected to respond differently with growth medium, harvest method, and other factors (Table 6). If the diagnosis is unknown at culture initiation, it can be helpful to know whether the pathologist would classify the tumor as a "small round cell tumor" (SRCT), which includes lymphoproliferative disorders. SRCTs can be successfully grown in suspension, whereas non-SRCTs are best grown with monolayer (flask or coverslip) culture methods. Most, but not all, SRCTs (e.g., lymphoproliferative disorders) will also grow in monolayer culture. If adequate tissue is obtained, both culture types should be initiated for SRCTs. For very small tumor samples, coverslip cultures are recommended. Duplicate cultures should be established whenever possible.

b. For lymphoid tissues, disaggregated cells are cultured in suspension using appropriate supportive growth medium. Tumor cells are spontaneously dividing; however, mitogens may be used for lymphoid disorders to encourage proliferation of the desired cell type.

6.6.2.7 Experience with solid tumor culture will provide the laboratory with information regarding optimal growth conditions and harvest methods for different tumor types.

a. It can be helpful for the laboratory to maintain a database that documents how the different tumor types have grown and which culture and harvest conditions yield abnormal clones. This database can then be searched for

optimal processing and harvesting methods for any new tumor received in the laboratory.

b. Short culture durations are preferred to optimize the mitotic index of early dividing tumor cells and to avoid growth of normal tissues. Depending on the amount of available tissue, a combination of direct, 24-hour, and/or 48-hour cultures are most often utilized for lymphoid disorders. Short-term cultures (e.g., direct or overnight cultures) may also be used in conjunction with longer-term cultures to capture actively dividing cells from solid tumors.

c. Frequent (daily) observation of cells in culture is needed to determine cell growth rate and optimal time to harvest. Tumor cells should be harvested as soon as possible upon adequate growth to capture early dividing tumor cells and to prevent overgrowth by chromosomally normal cells.

d. Conditions used for cell harvest will vary among tissue types (e.g., mitotic inhibitors) used (e.g., colcemid, velban, ethidium bromide), their concentration, and exposure duration, and they should be established by each laboratory.

6.7 ANALYTICAL METHODS
6.7.1 Conventional G-banded chromosome analysis
6.7.1.1 Cell selection. Analysis of metaphase chromosomes should include cells with both good and poor chromosome morphology when attempting to identify an abnormal clone. Once identified, clonal cells with the best chromosome morphology should be analyzed, karyotyped, and imaged to provide the most accurate breakpoint assignments.

Cells that cannot be completely analyzed because of poor morphology should be scanned for obvious structurally abnormal chromosomes and abnormal chromosome counts.

Clonal abnormalities should be documented in two independent cultures, if possible, to ensure that an in vitro culture artifact is not mistakenly identified as a clinically significant abnormality.

6.7.1.2 Analytic standards
6.7.1.2.1 Initial diagnostic studies
a. Analysis
   i. Analyze 20 metaphase cells and/or a sufficient number of cells to characterize all abnormal clones and subclones.
   ii. If all cells show a complex karyotype where each cell is different, then analyze at least 10 cells with karyotyping.
b. Documentation
   i. For abnormal cells:
      1. If only one abnormal clone is present: two karyotypes.
      2. If more than one related abnormal clone is present: at least one karyotype of the stemline and at least one of each sideline.

3. If unrelated clones are present: at least one karyotype for each stemline and one for each associated pertinent sideline.
   ii. For normal cells:
      1. If only normal cells are present: two karyotypes.
      2. If normal and abnormal cells are present: one karyotype of a normal cell plus karyotypes for abnormal clone(s) as described.

6.7.1.2.2 Follow-up studies may be performed to assess stage of disease at the time of diagnosis or at the time of tumor recurrence.
   a. Analysis
      i. Analysis should include a minimum of 20 metaphase cells.
      ii. Additional cells may be scored for a specific abnormality identified in the diagnostic sample.
      iii. In addition to looking for the known clonal aberration(s) from the diagnostic study, analysis of a sample after therapy should be performed with awareness of the possibility of new aberrations signifying clonal evolution and/or a new clonal process (i.e., therapy-related malignancy).
      iv. FISH analysis may be considered in lieu of conventional chromosomal analysis for diagnoses characterized by an abnormality for which FISH testing is available.
   b. Documentation
      i. If both normal and abnormal cells or if only abnormal cells are present:
         1. One or two karyotypes from each abnormal clone with a minimum of two karyotypes.
         2. One karyotype of a normal cell, if a normal karyotype was not documented in a previous study.
         3. If only normal cells are present: two karyotypes.

6.7.2 FISH analysis
6.7.2.1 FISH analysis may be used for primary, supplementary, or follow-up evaluation
   a. As a primary method for tumor evaluation, FISH is useful when (i) fresh tumor tissue is not available; (ii) rapid diagnostic information is needed to narrow the differential diagnosis; (iii) gene amplification or rearrangement for diagnostic or prognostic and/or therapeutic purposes is to be determined; (iv) no metaphase cells are obtained by culture of tumor material; or (v) conventional cytogenetic analysis yields a normal result.
   b. Supplemental FISH may be used as an adjunct to the initial conventional chromosomal analysis or CMA analysis to: (i) document a specific molecular event (e.g., gene rearrangement or fusion); (ii) provide a rapid result to aid in the differential diagnosis or planning of therapy; (iii) to assess gene copy number,; (iv) clarify level of clonality; or (v) confirm a microarray variant.

c. Follow-up FISH studies may be indicated to assess recurrent disease or disease progression and/or to differentiate recurrence of a tumor from a new disease process.
   i. If initial studies failed to identify the clonal process unique to the tumor, then follow-up studies may provide another opportunity.

6.7.2.2 Characterization of interphase FISH aberrations and FISH signal patterns. Characterization of interphase FISH aberrations and the FISH signal patterns in diagnostic samples is useful for future monitoring of disease. Gene fusions may confirm a specific tumor diagnosis. If a particular patient's tumor has a unique FISH signal pattern, documentation of the pattern at diagnosis can prevent misinterpretation of FISH analysis at follow-up.

6.7.2.3 Sample types. Sample types that may be used for FISH include (i) paraffin-embedded tissue sections; (ii) touch preparations (TP); (iii) cytospin preparations; (iv) cultured or direct harvest tumor cells; (v) fixed cytogenetically prepared cells; or (vi) fresh-frozen tumor tissues.
   a. Paraffin-embedded tissue[3]
      i. Before scoring a paraffin-embedded FISH slide, it is crucial for a pathologist to review a hematoxylin and eosin–stained slide and delineate the region of tumor cells that should be scored because it can be difficult to differentiate normal cells from malignant cells using only DAPI counterstain. The technologist should be clear, before scoring the slide, where the malignant cells of interest are located on the slide.
      ii. Formalin-fixed, paraffin-embedded tissue is acceptable for FISH analysis. Tissues preserved in B5 fixative or decalcified are not suitable for FISH.
      iii. Tumor sections cut 3 to 4 µm thick and mounted on positively charged organosilane-coated (silanized) slides work well. The cytogenetics laboratory should request several unstained sections and one hematoxylin and eosin–stained sequentially cut section from the submitting laboratory.
   b. Touch preparations
      i. A pathologist should make the TP or should be involved in selecting the tissue for TPs.
      ii. TPs are helpful when tissue architecture is not crucial.
      iii. TPs should be made by lightly touching the piece of tumor to a glass slide without smearing, followed by air drying.
   c. Cytospin preparations
      i. Cytospin preparations are useful for a concentration of samples with very low cellularity (e.g., cerebrospinal fluid).
   d. Fixed cytogenetically prepared cells
      i. Such preparations have multiple uses for both interphase and metaphase FISH evaluation including confirmation and clarification of suspected

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chromosome aberrations or characterization of an apparently abnormal clone. Metaphase cell evaluation may help clarify specific chromosome rearrangements.

e. Fresh-frozen tumor tissues
   i. Such tissues may be useful in sequential analysis of recurring tumors or in evaluation of archived samples.

6.7.2.4 Documentation. Analysis and documentation of FISH results should be in accordance with Section E9 of these Standards and Guidelines for Clinical Genetics Laboratories.[4]

6.7.3 CMA analysis

6.7.3.1 CMA can provide valuable information to supplement that of chromosomal and FISH analyses. Isolated tumor DNA hybridized to whole-genome copy number and/or single-nucleotide polymorphism microarrays allows detection of loss, gain, and amplification of regions of DNA, which may not otherwise be detected. Single-nucleotide polymorphism probes allow detection of large regions of loss of heterozygosity, which may harbor tumor-suppressor genes.[5]

6.7.3.2 Sample types that may be used for CMA analysis include (i) fresh tumor tissue; (ii) paraffin-embedded tumor tissue; (iii) frozen tumor; and (iv) cultured cells, chromosomally characterized when possible.

a. Fresh tumor tissue
   i. If the tumor is homogeneous, fresh tumor is the optimal sample for CMA and can be procured at the time of sample processing for chromosomal analysis. A small piece of identified tumor should be transferred to the microarray laboratory as soon as possible for DNA isolation. For heterogeneous tumors with areas of necrosis, normal tissue, or prominent stoma, DNA isolation from histologically characterized formalin-fixed paraffin-embedded material may be needed to ensure that isolated DNA is from the tumor.
b. Paraffin-embedded tumor
   i. A pathologist should review the hematoxylin and eosin-stained section of the tumor to identify an area of concentrated tumor for DNA isolation.
c. Fresh-frozen tumor
   i. Frozen stored tumor should provide high-quality DNA for CMA. A pathologist's review of the original H&E-stained slides can assure the frozen sample contains adequate tumor.
d. Cultured tumor cells
   i. Tumor cells that have been placed into culture may be used for DNA isolation and CMA as long as they remain viable. An early decision to use cells for CMA is best to minimize growth of normal tissue components.
   ii. DNA from cultured and harvested tumor cells that have been chromosomally characterized as abnormal may be used for CMA.

6.7.3.3 Documentation: analysis and documentation of CMA studies should be in accordance with Section E11 of these Standards and Guidelines for Clinical Genetics Laboratories.[5]

6.8 TURNAROUND TIME AND REPORTING

6.8.1 Turnaround time

6.8.1.1 TAT should be appropriate for clinical utility. The cytogenetics laboratory may want to have a written policy describing how tumor cases are prioritized (with respect to each other and with respect to other sample types) such that the genetic information provided can be used for patient management.

6.8.1.2 TAT guidance:
   a. Because of the multiplicity of tumor types and the different tumor growth characteristics in culture, TATs will vary. However, the final report for each tumor should be available as soon as possible given such factors. Final results should be available within 28 calendar days.
   b. Tumor FISH analysis results should be available within 1 to 4 days for most tumors and within 7 days for paraffin-embedded tumors.
   c. Preliminary verbal reports may be appropriate for some case studies. If preliminary results are communicated, then the date of preliminary report should be documented in the final report. The content of the preliminary report should be documented if it differs significantly from that of the final report.

6.8.2 Reporting

6.8.2.1 The most recent edition of the International System for Human Cytogenetic Nomenclature should be used to report the chromosomal, FISH, CMA, and sequencing results.[6]

6.8.2.2 Cells analyzed (both normal and abnormal) should be documented in the final report.

6.8.2.3 If an aberration is suspected to be constitutional, analysis of a phytohemagglutinin (PHA)-stimulated blood sample during remission is recommended to clarify the constitutional versus clonal nature of the aberration so genetic counseling may be recommended as appropriate.

6.8.2.4 The final report(s) for tumor samples should contain the following information:

1. Patient identification using two different identifiers
2. Patient medical record number and/or laboratory identification number
3. Name of referring physician
4. Sample information (type, dates of collection and receipt, date of report)
5. Reason for referral or suspected diagnosis
6. International System for Human Cytogenetic Nomenclature of all studies performed
7. Narrative description of the aberrations observed. The report should associate results if more than one study was performed on the same tissue. The interpretation should correlate the genetic testing results with the histopathology report and patient-specific clinical information.

Discussion can include the clinical significance of the results for the diagnosis, prognosis, and/or therapeutic management of the patient with reference to current literature.

8. Literature references should be included to support the interpretation and to provide helpful information for the health-care provider.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

DISCLOSURE
All of the authors direct clinical cytogenetics laboratories that run the tests discussed in the current standards and guidelines on a fee-for-service basis.

REFERENCES
1. Cooley LD and Wilson KS. The cytogenetics of solid tumors. In: Gersen SL and Keagle MB (eds). *The Principles of Clinical Cytogenetics 2013. 3rd edn.* Springer: New York.
2. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (eds). *WHO Classification of Tumours: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues 2008. 4th edn.* IARC Press: Lyon, France.
3. Zordan A. Fluorescence in situ hybridization on formalin-fixed, paraffin-embedded tissue sections. *Methods Mol Biol* 2011;730:189–202.
4. Mascarello JT, Hirsch B, Kearney HM, et al.; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. Section E9 of the American College of Medical Genetics technical standards and guidelines: fluorescence in situ hybridization. *Genet Med* 2011;13: 667–675.
5. Cooley LD, Lebo M, Li MM, Slovak ML, Wolff DJ; Working Group of the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee. American College of Medical Genetics and Genomics technical standards and guidelines: microarray analysis for chromosome abnormalities in neoplastic disorders. *Genet Med* 2013;15: 484–494.
6. Shaffer LG, McGowan-Jordan J, Schmid M (eds). *An International System for Human Cytogenetic Nomenclature (ISCN) 2013.* S. Karger: Basel, Switzerland.

Tumor	Chromosome Aberrations	Genes Involved	Significance	References (PMID)
PEDIATRIC TUMORS				
• **GLIAL**				
- **Pilocytic astrocytoma grade I**	Most are normal; Loss 19p most common; Gain:5, 6, 7, 8, 9, 11,12,15, 17, 19, 20, 22; Loss: 9q, 22q	*KIAA1549/BRAF* dup *BRAF* 7q34, amp*HIPK2* 7q34	*BRAF* dup prognosis variable; More common in cerebellum than supratentorial; Often with *NF1*, benign, non-aggressive	17086101 18716556 25664944 26378811 19016743
- **Diffuse astrocytoma grade II**	Rearrangement: 2p, 7q Gain: 7q, 8q Loss: 19p	*MYBL1*	Germline mutations of *TP53*	15269292 25461780 25664944 26061751 23633565
- **Anaplastic astrocytoma grade III**	Gain: 1q, 5q, 7q; Loss: 6q, 9p, 10q, 12q, 13q, 17p, 22q; Mutation:*TP53*	*FGFR1* *CDKN2A/B* *SMARCB1*	Gain of 1q may correlate with worse prognosis	11290570 25461780 25231549 25727226
- **Glioblastoma**	Gain: 1p, 1q, 2q, 3q, 7p, 16p, 17q, 21q Loss: 6q, 8q, 10q, 11q, 13q, 16q, 17q,22q	*EGFR* *CDKN2A/B* *PTEN, IDH1, IDH2*	Amplification of genes worse outcome; *IDH1, IDH2* mutations longer survival; H3 mutations poor prognosis; Gain 3q, changes in 7, 10, 9p, 13q, 19 associated with glioblastoma progression	22064882 11290570 25461780 25727226 25752754 25231549 25754088 26328271
- **Diffuse intrinsic pontine glioma**	Gain: 1q, 7p, 7q Loss: 10q	amp*MYCN, MDM4, PDGFRA, EGFR, IRS2 CDKN2A, PTEN*	Poor prognosis	22064882 23293772 24705252
• **MENINGIOMA**	Loss: 22q most common Loss: 1p, 14 as progresses, then loss of 4p, 6q, 7p, 10q, 11p, 18q, other whole arm loss	*NF2* mutations Loss *CDKN2A/B*	Loss 1p and other whole arm loss seen with progression / higher grade, additional losses as progresses further	20015288 23528542 21988727
• **EPENDYMOMA**	Rearrangement: 11q13.1 Gain: 1q, 7, 9	amp *ERBB2* Loss or	Gain 1q, homozygous loss	15269292 20516456

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	Loss: 6q, 9p, 10, 11q, 13q, 17p, 19q	mutation*NF2*, *TP53, MEN1*	*CDKN2A* poor survival; Gain 9, 15q, 18, loss 6q favorable survival	24553141 24939246 20425037 21840481
- **Spinal**	Loss: 22q	homozygous loss *NF2* Loss *MEN1*	More common in adults	23528542
- **Intracranial**	Rearrangement: 2p Gain: 1q Loss: 6q23, 22q		Loss 6q have longer survival; Gain 1q poor survival, more common in pediatrics	24939246 20516456
• **MEDULLOBLASTOMA**	amp *MYCN, MYC* i(17)(q10) Loss: 10q	*amp**MYCN, MYC; BRCA2, ERBB2, SUFU, ERRB4, PTCH, APC*mutations	amp *MYCN, MYC* worse outcome	15269292 20425037 22358457 24264598 20823417
- **WNT pathway**	Loss: 6, 6q	*CTNNB1, AXIN1, APC*	Very good prognosis, more common in children	22134537 19255330 24493713 22358457
- **SHH pathway**	amp *GLI2, MYCN* Gain: 3q Loss: 9q, 10q	*GLI2, MYCN PTCH1, SMO, SUFU,*	Good in infants, intermediate in children and adults	22134537 26195713 24651015 12068298 24077351
- **Group 3**	amp *MYC* Gain: 1q, 7, i(17)(q10), 18q Loss: 5q, 8, 10q, 11p, 16q	*amp MYC*	Poor prognosis, not seen in adults	22134537 19255330 22832581 25043047
- **Group 4**	amp *CDK6, MYCN* Gain: 7, 17q, i(17q), 18q, Loss: X, 8, 11p	*amp CDK4, MYCN*	Intermediate prognosis, mostly in children	22134537 19255330 22832581 25043047
• **CHOROID PLEXUS**				
- **Carcinoma (CPC)**	Gain: 1, 4, 7, 12p, 12q, 20p, 20q Loss: 3q22, 5p, 5q, 6p21, 18p, 18q, 22q	*PDGFR* *SMARCB1, TP53*	>36 mo of age more gains, <36 mo of age more losses, Loss 12q shorter survival; Mutation *TP53*more aggressive; Gain 9 and loss 10 associated with	24478045 23172371 11891207 9242217

			prolonged Survival	
- **Papilloma (CPP)**	Gain: 5p, 5q, 6q, 7p, 7q, 8q, 9p, 12, 14, 20 Loss: 10q	*PDGFR, TWIST1, TP53, NOTCH2, NOTCH3*		23172371 11891207 12237235 24478045 9242217 25575132
- **Supratentorial primitive neuroectodermal (sPNET)**	ampPDGFRA, MYB, KIT Gain: 1q, 9q, 15, 18 Loss: 9, 13q, 19q Homozygous loss: CDKN2A/B	*ampPDGFRA, KIT, MYB;* Mutation or loss *TP53, PTEN CDKN2A/B*	del 9p21.3 correlates with metastasis	20425037
- **Atypical teratoid/rhabdoid (AT/RT)**	Loss: 22q	*SMARCB1*	Distinguish from other tumors	23074045
ADULT TUMORS				
• **GLIAL**				
- **Grade I**	Gain: 7, 19, 20 Loss: 10, 22, X or Y			15269292 26061751
- **Diffuse astrocytoma, Grade II**	Loss: 17p (*TP53*), 17q (*NF1*), 6q, 13q, 22q	*TP53, ATRX IDH1, IDH2, RB1*	Survival of ~7 years	15269292 25664944 26061751
- **Anaplastic, Grade III**	Gain: 1q, 7p, 7q, 8p Loss: 1p, 6q, 9p, 10p, 10q, 13q, 14, 17p, 19q, 22	*ampEGFR, MDM2; CDKN2A/B, PTEN, TP53, RB1, IDH1, IDH2,*	Survival of ~4 years	15269292 11290570 26061751
- **Glioblastoma, Grade IV**	amp *PIK3C2B, MDM4, EGFR,* *MET, MYC, CDK4, GKI1, MDM2* Rearrangement: 1, 6, 7, 9, 11, 13, 16, 19 Gain: 4q, 7p, 7q, 19, 20q Loss: 1p, 6q, 9p 10p, 10q, 13q, 17p, 22q Homozygous loss *TP73, LRRC47, DFFB, CDKN2A/B, CACNA1B*	amp *MDM2,CDK4* amp*EGFR,* *PTEN, RB1, TP53* *CDKN2A/B, TP73, LRRC47, DFFB*	Short term survival, aggressive tumor, associated with *PTEN* loss, amp *EGFR*	11290570, 19609742 25461780
• **OLIGODENDROGLIAL**	der(1;19)(q10;p10)	*FUBP1*	Favorable outcome with	15269292

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	Rearrangement: 4, 6, 7, 11, 13, 15, 18, 22 Loss: 1p, 4p, 4q, 9p, 13q, 18, 19q Mutation *IDH1*	*CDKN2A/B, CIC* *IDH1, IDH2*	der(1;19); Malignant progression with amp*CDK4*, or homozygous loss of*CDKN2A/B* or *RB1*	17047046 24918277 25664944 26061751
• OLIGOASTROCYTIC	der(1;19)(q10;p10)	*IDH1, IDH2*	Favorable outcome with der(1;19) and/or *IDH1, IDH2*	22039037 25143301
• MENINGIOMA	Gain: 1q25-32 Loss: 22; Higher grade loss 1p, 6q24-qter, 9p, 10, 14q, 18q	*NF2, CDKN2A/B*	*NF2* loss or mutation homozygous loss *CDKN2A/B* with progression; Loss 6q and 14q common in recurrent tumors	20015288 23528542 25347344
• EPENDYMONA				
- Spinal	Gain 5p, 7, 12. Loss 6p, 13, 14q, 10 Mostly deletions, del22q	*NF2*	More common in adults, better survival than intracranial	24939246 23528542
- Intracranial	3 groups: 1) gain 9, 15q, 18 or loss 6 2) balanced or one abnormality 3) gain 1q, loss *CDKN2A/B*	*CDKN2A/B*	1) good prognosis 2) intermediate risk 3) poor prognosis	24939246 21840481 20516456
• CHOROID PLEXUS				
- Papilloma (CPP)	Gain 5q, 6q, 15q, 18q Loss 22q	*ARL4A*	Indolent	12237235 11891207 23172371 24478045 25575132
- Atypical (aCPP - grade II)	Gain: 7, 20, 9, 12, 8, 18, 11, 15, 19 Loss: very few	*ARL4A*	Indolent	25575132
- Carcinoma (CPC - grade III)	Loss: 3, 6, 11, 16, 17p, 22q	*TP53, GTPBP2 RSPH9, VEGFA, RBFOX1*	aggressive, poor outcome, with del/mutation *TP53* worse prognosis	25575132

Supplemental Table 2. Genitourinary tumors with diagnostic or clinically significant chromosome aberrations

Tumor	Chromosomal Aberrations	Genes Involved	Significance	References (PMID)
RENAL				23161685
• **Renal cell carcinoma (RCC)**				
- **Clear cell RCC**	der(3)t(3;5)(p11p21;q11q35) Gain: 5q, 7, 12, 20 Loss: 3(3p12p14, 3p21 and 3p25), 4p, 5q, 8p, 9p(p13p22), 13q, 14q, Y	*VHL, PBRMI, PTH1R, IGH*	Loss 3/3p with gain 5q favorable prognosis; Loss 3/3p and loss 5q correlated with metastasis	19124809 15122209 12407697
- **Papillary RCC**	Gain: 7, 12, 16, 17, 20 Loss: 9p(p21.3), Y	*CDKN2A, MET*	Adult papillary RCC	15122209 12407697
- **t(X;V)(p11.23;V) RCC**	t(X;1)(p11.23;q23.1) t(X;1)(p11.23;p34.3) t(X;17)(p11.23;q25.3) t(X;17)(p11.23;q23.1) inv(X)(p11.23q13.1)	*PRCC/TFE3* *SFPQ/TFE3* *ASPSCR1/TFE3* *CLTC/TFE3* *NONO/TFE3*	More common in pediatric RCC; Balanced t(X;17) in RCC vs unbalanced t(X;17) in ASPS	15122209 12407697
- **t(6;11) RCC**	t(6;11)(p21.1;q13.1)	*TFEB/ALPHA*	Pediatric/young adult RCC	15644781
• **Chromophobe**	Loss: 1, 2, 6, 10, 13, 17, 21 (monosomies)		Distinguish from oncocytoma	15122209
• **Oncocytoma**	t(5;11)(q35;q13.3) t(9;11)(p23;q13.3) Gain: 7 Loss: 1(1p), 14, Y	*CCND1*	Benign; Distinguish from chromophobe; Chromosome 1 abnormality more common in bilateral tumors	15122209 12407697
• **Wilms tumor (Nephroblastoma)**	der(16)t(1;16)(q10;p10) Gain: 1q,6, 7, 8, 12, 13, 18 Loss: 1p, 7p, 11p13, 16q, 17p, 22	*TP53, WT1, WTX, CTNNB1* mutations	Unfavorable histology; Augmented chemotherapy if loss 1p, 16q	21248786 12407697 11835232 21882282
• **Clear cell sarcoma (CCSK)**	t(10;17)(q22.3;p13.3)	*YWHAE/FAM22E*	t(10;17) in 12% of CCSK; t(10;17) also in endometrial stromal cell sarcoma	22294382
• **Congenital mesoblasticnephrom**	t(12;15)(p13.2;q25.3)	*ETV6/NTRK3*	Diagnostic	12407697 11801301

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a (CMN)	Gain: 11, 17, 20			
• **Rhabdoid tumor (RTK)**	Loss: 11p15.5, 22 (22q11.23)	*SMARCB1*	Diagnostic; 11p loss may be secondary to 22q loss	8824720 12407697
• **Mucinous tubular and spindle cell carcinoma (MTSCC)**	Loss: 1, 4, 6, 8(8p), 9(9p), 13, 14, 15, 22		Favorable prognosis	12429795
		FH		
• **Bellini duct carcinoma (Collecting duct carcinoma)**	Gain: 3 Loss: 1 (1q32), 6 (6p), 8p, 14, 15, 21q, 22		Aggressive	12407697 15122209
• **Papillary adenoma**	Gain: 7, 17 Loss: Y		Distinguish from papillary RCC	12407697

PROSTATE

- **Adenocarcinoma**	del(21)(q22.2q22.3) t(7;21)(p21.2;q22.3) t(17;21)(q21.31;q22.3) t(3;21)(q27.2;q22.3) t(8;21)(q24.22;q22.2) t(7;V)(p21.2;V) t(17;V)(q21.31;V) t(3;V)(q27.2;V) t(4;6)(q22;q15) Gain: 7 (7q31), 8q24 Loss: 8p21.3, 10q23.31, 13q, 17p13.1	*TMPRSS2/ERG* *TMPRSS2/ETV1* *TMPRSS2/ETV4* *TMPRSS2/ETV5* *NDRG1/ERG* *ETV1* *ETV4* *ETV5* *MYC, PTEN, TP53,LPL*	*ERG* amp *TMPRSS2/ERG* fusion+ poor OS *PTEN-, TP53-* poorest OS; Hormone independence and poor prognosis with gain 8q	23161685 18563191 18563191 17437846 12837920 19402094

BLADDER   23161685

- **Urothelial cell carcinoma (Transitional cell carcinoma)**	Gain: (Pseudodiploidy/pseudotetraploidy) Loss: 8p21.3, 9 (9p21.3), 11 (11p), 13q, 14q, 15q, 17p	*LZTS1, CDKN2A*	Loss of 9/9p early event; Homozygous deletion *CDKN2A* higher grade & stage; recurrence, progression; Loss 8p, 11/11p, 13q, 14q, 17p and tetraploidy late stage/invasive	16110317 11888856

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- **Bladder squamous cell carcinoma**	Gain: 7 Loss: 3p, 8p, 9 (9p), 17p		Loss 9/9p also early event	11888856

REPRODUCTIVE

- **Endometrial stromal cell sarcomas**	t(7;17)(p15.2-15.1;q11.2) t(6;7)(p21.32;p15.2-15.1) t(6;10)(p21.32;p11.22) t(6;10;10)(p21.32;q22;p11.22) t(10;17)(q22.3;p13.3)	*JAZF1/SUZ12JAZF1/PHF1* *EPC1/PHF1* *YWHAE/FAM22E*	Distinguish from non-EST uterine tumors; t(10;17) also common in renal clear cell sarcoma	21420714 24342291
- **Endometrial carcinoma**	i(1q) Gain: 1q, 2, 7, 10		More complex abnormalities seen in serous versus endometrioid carcinomas	7736425 9115961 8174089
- **Uterine leiomyomata**	t(6;V)(p21.31;V) or other rea(6p21.31) t(12;14)(q14.3;q23-24) del(7)(q22q23)	*HMGA1* *HMGA2*	Only 40% of uterine leiomyomata exhibit abnormal karyotypes; *MED12* mutations in ~80% of 46,XX myomas	16504804

GERM CELL (GCT)

- **Postpubertal GCTs**	i(12p) Gain: 1q, 7, 8, 12p, 21, 22, X Loss: 1p, 4, 5, 11q, 13q, 18	*RET/NCOA4*	i(12p), amp(12p) distinguishes GCTs; Mediastinal GCT associated with Klinefelter syndrome	9461002 15738984
- **Prepubertal GCTs**	Gain: 1q, 2p, 3p, 13, 16p, 20q Loss: 1p, 4q, 6q		12p gain rare in prepubertal GCT distinguishes from adult GCT; Prepubertal GCT karyotypes generally less complex compared to adult GCTs	24577549

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Supplemental Table 3. Gastrointestinal, dermal and neural crest tumors with diagnostic or clinically significant chromosome aberrations

Tumor	Chromosomal Aberrations	Genes Involved	Significance	References (PMID)
GASTROINTESTINAL				
• GIST	Loss: 1p, 14q, 15q, 13q, 22q Gain: 1q, 12q	*KIT, PDGFRA*	*KIT, PDGFRA* mutation diagnostic, response to TKIs	18623623, 18671247 16452129, 15095270 12072198, 20470368 15580284, 23942094
• LIVER				
- Hepatoblastoma	Gain: 1q, 2, 2q, 8, 20, der(4)t(1;4) Loss: 4q	Unknown genes	Distinguish from HCC, HMH	15981236, 20461752 25525853
- Hepatic mesenchymal hamartoma (HMH)	t(11;19)(q13;q13.4) t(19;V)(q13.4;V)	Unknown genes	Distinguish from hemangioma or malignant tumor	15325096
• SALIVARY GLAND				
- Pleomorphic adenoma	t(3;8)(p22.1;q12.1), t(12;V)(q14.3;V) Gain: 8	*CTNNB1/PLAG1, HMGA2*	Diagnostic; benign tumor	17693184, 15920557 18828159, 20055685 22987447
- Ca-ex-PA	*HMGA2, MDM2* amplification	*HMGA2, MDM2*	Amplification contributes to malignant transformation of PA	15920557, 22287457 22297681
- Mucoepidermoid cancer	t(11;19)(q21;p13.11) in 40-80%; Gain: 7, 8, X Loss: 6q	*CRTC1/MAML2*	Malignant; t(11;19) assoc with low / intermediate grade tumors	18486532, 16444749 23583282, 22847156
- Warthin's tumor	t(11;19)(q21;p13.11) in low percentage	*CRTC1/MAML2*	Benign; t(11;19) w/ metaplasia	18647217
DERMAL				
- DFSP and variants (GCF, Bednar, other)	t(17;22)(q22;q13.1), der(22)t(17;22) or r(22)t(17;22)	*COL1A1/PDGFB*	Diagnostic for DFSP; response to TKIs	20637435, 17124411 19890351, 12550751 21111450, 12661001 23327733
- Hidradenoma	t(11;19)(q21;p13.11), Gain: 7, 8, X Loss: 6q	*MAML2/CRTC1*	Clear cell variant	17334997, 15729701

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- **Cutaneous melanoma**	Gain: 1q, 6p, 7, 8q, 11q, 17q, 20q Loss: 6q, 9, 9p, 10q	*CDKN2A, BRAF, PTEN*	*CDKN2A*	21732770, 21876842 25207365
- **Uveal melanoma**	Loss: 3 Gain: 8q	*GNA11, GNAQ*	Monosomy 3 correlates with metastatic disease	21083380, 1309305 22415057

BREAST

- **Invasive intraductal**	dmin, hsr	*ERBB2* amp	Improved outcome with targeted therapy	19548375, 22417857 23539740
- **Secretory Breast**	t(12;15)(p13.2;q25.3)	*ETV6/NTRK3*	Favorable; distinguish from other breast lesions	22129193, 23944930

LUNG

- **NSCLC**	*EGFR* high copy number or amplification, Loss: 3p	*EGFR*	Response to TKIs	21670455, 21400669 20472851
	Gain: 7 *MET* amplification inv(2)(p21p23.2) *ALK, ROS1, RET* rearrangements	*MET* *EML4/ALK*	Response to TKIs	25492085, 25055117 25806222 22311682, 22282074 25288236, 25077070 25806222

NEURAL CREST

- **Neuroblastoma**	del(1p) with or without *MYCN* amplification	1p	Unfavorable	22146831, 16306521, 19401703
	2p24.3 11q deletion	*MYCN* amp 11q23 band region	Unfavorable Unfavorable, inversely associated with *MYCN*	15571958, 19401703 16306521, 19401703
	Low *ALK* expression Gain 17q with or without *MYCN* amp	*ALK* 17q	Unfavorable Unfavorable	21492432 22146831, 19171713
	del(3p)	In assoc with del(11q), lack *MYCN* amp	Older age at diagnosis, unfavorable	12538451, 15800319
	Triploidy without above aberrations		Favorable	19401703

amp-amplification; Ca-ex-PA-Carcinoma ex Pleomorphic Adenoma; DFSP-dermatofibrosarcoma protuberans; dmin-double minutes; GCF-giant cell fibroblastoma; GIST-gastrointestinal tumor; HCC-hepatocellular carcinoma; hsr-homogeneously stained regions; NSCLC-non-small cell lung carcinoma; TKI-tyrosine kinase inhibitor

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Supplemental Table 4. Bone and soft tissue tumors with diagnostic or clinically significant chromosome aberrations

Tumor	Chromosome Aberrations	Genes Involved	Significance	References (PMID)
BONE TUMORS				
- **Aneurysmal bone cysts**	t(1;17)(p34.3;p13.2) t(3;17)(q21.3;p13.2) t(9;17)(q22.31;p13.2) t(16;17)(q21;p13.2) t(17;17)(p13.2;q21.33)	THRAP3/USP6 CNBP/USP6 OMD/USP6 CDH11/USP6 COL1A1/USP6	Benign lesions but locally aggressive. Recurrences are common	11408073 11441369 15044915 15735689
- **Chondrosarcoma**	Structural abnormalities: 1, 6 , 9, 12, 13, and 15 Gains and losses: 5, 7, 8, and 19		Presence of chromosome abnormalities correlates with increasing histological grade with complex aberrations mainly seen in high-grade disease 13q loss is an independent factor for metastasis, regardless of the tumor grade and size	8402563 10629543 11793445 11793371
- **Enchondroma**	Broad range of chromosomal abnormalities but chromosome 6 and 12 are more frequently affected		A common benign hyaline cartilaginous lesion	1458512 8402563 9452264 12606137 12742153
- **Osteochondroma**	Germ line losses; 8q24.11or 11p11.2	EXT1or EXT2	Most common benign bone tumor	7507706 9576285
- **Osteosarcoma**	Gains: 1q21, 3p26, 6p, 8q, 12p12p13, 14q24qter, 17p11p12, Xp12, and Xp11.2p21 Losses: 6q, 13q, and 17p	RB1 and TP53	1q21 and 8q gains are associated with shorter survival 13q and 17p losses are poor prognostic signs	8344751 8636759 9140456 9685858 11950895
- **Parosteal Osteosarcoma**	12q13q15 amplification, ring chromosomes	CDK4, MDM2	Low grade malignant potential	22749040 20196171
SOFT TISSUE TUMORS				
• **Adipocytic tumors**				
- **Lipoma**	Translocations involving 12q14.3 t(3;12)(q28;q14.3) most common	HMGA2 rearrangements HMGA2/LPP most common	65% of cases. Distinguish from liposarcoma	1988102 8453640 8812423 9403060

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				9530339
	Losses: 13q11q22, and 6p21p23 rearrangements		15-20% of lipomas without 12q14.3 rearrangements	
- **Chondroid lipoma**	t(11;16)(q13.1;p13.12)	C11orf95/MKL2	Diagnostic	20607705
- **Lipoblastoma**	Rearrangements involving 8q12.1	PLAG1 rearrangements	Rare benign soft tissue tumor of embryonal fat	10987300 11549588
	Gains: 8 with or without 8q12.1 rearrangements			
- **Liposarcoma** a) **Well-differentiated liposarcoma**	Ring and giant marker chromosomes usually involving 12q14q15	MDM2, CDK4, and HMGA2 amplification	Low-grade malignancy that may recur locally but doesn't metastasize	1568170 8353809 8387391
b) **Myxoidliposarcoma**	t(12;16)(q13.3;p11.2)	FUS/DDIT3	Diagnostic	7503811 7805034 8510758
c) **Pleomorphic liposarcoma**	Complex karyotypic changes with no characteristic abnormalities		Highly malignant tumor	9591630
- **Spindle cell lipoma/Pleomorphic lipoma**	Losses: 2q21, 6q14q21, 10p, 13q, 16q22qter, and 17p		Distinguish from liposarcoma	7798294
• **Fibroblastic/Myofibroblastic tumors**				
- **Angiofibroma**	t(5;8)(p15.33;q13.3)	AHRR/NCOA2	Diagnostic. Benign tumor with local recurrence	22337624
- **Adult fibrosarcoma**	Complex karyotypes with no consistent abnormality detected			
- **Infantile fibrosarcoma**	t(12;15)(p13.2;q25.3)	ETV6/NTRK3	Diagnostic	1582636
	Gains: 8, 11, 17, and 20			
- **Inflammatory myofibroblastic tumor**	t(1;2)(q21.3;p23.2) t(2;2)(p23.2;q12.3) t(2;17)(p23.2;q23.1) t(2;19)(p23.2;p13.12)	TPM3/ALK RANBP2/ALK CLTC/ALK TPM4/ALK	Rare soft tissue tumor at the edge between benign and malignant	10383129 10934142 12112524 12661011
- **Low-grade fibromyxoid sarcoma**	t(7;16)(q33;p11.2) in 70% of cases	FUS/CREB3L2 associated with	Diagnostic	11106828 12960807

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	Supernumerary ring chromosome in 25% of cases	both abnormalities		
- **Solitary fibrous tumor**	12q13 rearrangements	*NAB2/STAT6*	Diagnostic	23761323

• Smooth muscle tumor

- **Leiomyosarcoma**	Complex karyotypes with no consistent abnormality detected	Involvement of *TP53, FANCA, RB1, PTEN,* and *ROR2*		8622088 12827608

• Skeletal muscle tumors

- **Alveolar rhabdomyosarcoma**	t(2;13)(q36.1;q14.11) t(1;13)(p36.13;q14.11) Amplifications: 1p36, 2p24, 2q34qter, 12q13q15, 13q14, and 13q31	*PAX3/FOXO1* *PAX7/FOXO1*	Diagnostic translocations	8098985 8275086 11607823 12039929
- **Embryonal rhabdomyosarcoma**	Complex karyotypes with gains: 2, 8, and 13 Loss of heterozygosity at 11p15.5			8764111 12506174 18985676
- **Spindle cell or sclerosing rhabdomyosarcoma**	t(6;8)(q22.1;q13.3) t(8;11)(q13.3;p15.3)	*VGLL2/NCOA2* *TEAD1/NCOA2*	Congenital/infantile rhabdomyosarcoma	26501226 23463663

• Tumors of uncertain differentiation

- **Angiomatoid fibrous histiocytoma**	t(12;16)(q13.12;p11.2) t(12;22)(q13.12;q12.2) t(2;22)(q33.3;q12.2)	*FUS/ATF1* *EWSR1/ATF1* *EWSR1/CREB1*	Diagnostic translocations	15884099 17188428 17724745 18094413
- **Alveolar soft part sarcoma**	der(17)t(X;17)(p11.23;q25.3)	*ASPSCR1/TFE3*	Diagnostic	1423174 11169942 11244503
- **Clear cell sarcoma of the soft tissue**	t(12;22)(q13.12;q12.2)	*EWSR1/ATF1*	Diagnostic	8401579 8552387
- **Desmoplastic small round cell tumor**	t(11;22)(p13;q12.2)	*EWSR1/WT1*	Diagnostic	1314522 8374894 8187063
- **Ewing sarcoma**	t(11;22)(q24.3;q12.2)	*EWSR1/FLI1*	90% of cases	3163261

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				1522903
	t(21;22)(q22.2;q12.2)	*EWSR1/ERG*	Variant translocations in 5%	8022439
	t(7;22)(p21.2;q12.2)	*EWSR1/ETV1*	of cases	7700648
- **Undifferentiated small round cell sarcoma**	t(4;19)(q35.2;q13.2) t(10;19)(q26.3;q13.2) t(X;19)(q13.1;q13.2) inv(X)(p11.4p11.22)	*CIC/DUX4* *CIC/DUX4L3* *CIC/FOXO4* *BCOR/CCNB3*	*EWSR1* negative, aggressive	25683183 25007147 24215322
- **Extraskeletal myxoid chondrosarcoma**	t(9;22)(q22.33;q12.2) t(9;17)(q22.33;q12) t(9;15)(q22.33;q21.3)	*EWSR1/NR4A3* *TAF15/NR4A3* *TCF12/NR4A3*	Diagnostic translocations	3967207 10602520
- **Extrarenal rhabdoid tumor**	Germ line deletions or translocations involving 22q11.23	*SMARCB1*	Poor prognosis	8092393 8545590 9892189 26216536
- **Synovial sarcoma**	t(X;18)(p11.23;q11.2) t(X;18)(p11.22;q11.2)	*SS18/SSX1* *SS18/SSX2*	Diagnostic translocations	3461881 3030536 3030537 7951320 9428816

Tumor	Chromosomal Aberrations	Genes Involved	Significance	References (PMID)
• B-CELL				
- Burkitt lymphoma	t(8;14)(q24;q32) t(2;8)(p12;q24) t(8;22)(q24;q11.2)	*IGH/MYC* *IGK/MYC* *IGL/MYC*	Characteristic *MYC* disruption Variant translocations	4113130 946170
	Gain: 1q21-q25, 7, 8, 12, 13q, 18 Loss: 6q, 13q, 17p		Secondary changes /Unfavorable: 13q	18923440 1869243 21207210 19895612
- Diffuse large B-cell lymphoma (DLBCL)	t(3;14)(q27;q32) t(2;3)(p12;q27) t(3;22)(q27;q11.2)	*IGH/BCL6* *IGK/BCL6* *IGL/BCL6*	Characteristic*BCL6* disruption, variant translocations	8235596 8506375
	Gain:X, 3, 5, 7, 9, 12, 18, 1q23-q31, 1q31-q44, 3q, 6p, 7p, 7q31-q32, 8q2-2q24, 11q12-q13, 12q14-q24, 18q11q21, 22q12-qter		Unfavorable: 3, 1q Favorable: 5, 7q	11850073 15350300 10337996 12181265 21418177
	Loss: Y, 4, 6, 13, 15, 17, 1p36pter, 2p23pter, 4q32qter, 6q21q25, 8p12pter, 9p21pter, 11q23qter, 12p12p13, 14q23qter, 17p12p13, 18q21qter	*CDKN2A,TP53*	Unfavorable: 9p, 17p	18765795 20813005 17881637
	Rearrangement: 4p13, 6p22, 7p13, 8q24, 11q23, 13q14, 15q22, 17q11, 18q21	*MYC* *BCL2*	Unfavorable: *MYC* disruption *BCL2* disruption	21490439 24740248 22189900 15215171
- Follicular lymphoma	t(14;18)(q32;q21) t(2;18)(p12;q21) t(18;22)(q21;q11.2)	*IGH/BCL2* *IGK/BCL2* *IGL/BCL2*	Characteristic *BCL2* disruption, Variant translocations	7579360 2129304
	Gain: X, 3, 5, 7, 8, 12, 18 (18q) Loss: 1p36, 6q21, 6q23q26, 9p21, 10q22q24, 17p13	*CDKN2A, PTEN,TP53*	Unfavorable: X, 7, 12, 18, der(18)t(14;18) Unfavorable: 6q, 9p21, 10q22-q24, 17p13	10389925 8049424 9087572 9616165 17699855
	Rearrangement: 1p, 3q27, 6q23q26, 8q24, 11q, 12q13q15	*BCL6* *MYC*	*BCL6* disruption Unfavorable: *MYC* disruption	8167331 16075463 14736281
- Mantle cell lymphoma	t(11;14)(q13;q32)	*IGH/CCND1*	Characteristic *CCND1*	8499640

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			disruption	
	Gain:3(3q), 12(12q)		Unfavorable: 3q, 12	
	Loss: Y, 1p, 6q, 9(9p), 10q, 11q, 13q, 17p, 18	CDKN2, TP53	Unfavorable: 9p, 13q14, 17p	9559341 21945515
	Rearrangement:			
	t(2;12)(p12;p13)	IGK/CCND2		
	t(12;14)(p13;q32)	IGH/CCND2		16861358
	t(6;14)(p21;q32)	IGH/CCND3		18391076
	8q24, 3q27	MYC, BCL6	Unfavorable: MYC disruption, complex karyotype	15138714
- **B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)**	Gain: 3, 12, 18, 2p24p25, 3q26q27, 8q24	MYC	Characteristic 12 gain Unfavorable: MYC	11486330
	Loss: 6q, 9p, 11q22, 13q, 14q24q32, 17p	CDKN2A, ATM IGH, TP53	Favorable: isolated 13q14.3 deletion Unfavorable: 6q, 17p, 9p	18477041 21749360 23001040
	Rearrangement:			
	t(9;14)(p13;q32)	IGH		10784387
	t(11;14)(q13;q32)			23581835
	t(14;19)(q32;q13.3)			21502423
	8q24, 18q21	MYC, BCL2	Unfavorable: MYC disruption	19246886
- **Splenic marginal zone lymphoma**	Gain: 3q			11146574
	Loss: 6q, 7q31q32, 8p, 17p	TP53	Unfavorable: 7q, 8p/17p together	10329610 21115979 20479288 22816737
- **Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)**	t(11;18)(q21;q21)	BIRC3/MALT1		10979968 10907943
	t(14;18)(q32;q21)	IGH/MALT1		12406890
	Gain:3 (3q), 9q, 18 (18q)		Unfavorable: partial or complete trisomy 18	17606442 16512826
	Loss: 6q, 9p, 17p	CDKN2A,TP53	Unfavorable: 9p, 17p	17525089
	Rearrangement: 1p22, 2q, 3p14.1	BCL10, FOXP1		20352431 9178679 10845924 15703784
- **Nodal marginal zone lymphoma**	Gain: 3, 7, 12, 18			16405665
	Loss: 6q, 11q	ATM		8547655 16156859
	Rearrangement:			
	t(11;14)(q23;q32)	IGH/DDX6		22965301
	1q, 1p, complex karyotype			

- **Lymphoplasmacytic lymphoma**	Gain: 3, 12, 18 Loss: 6q, 7q, 13q, 17p		Unfavorable: 6q	23477936 12351413
- **Primary mediastinal (thymic) large B-cell lymphoma**	Gain: 2p15, 9p24, Xp11.4p21, Xq24q26	*REL, JAK2*		11241792 8608249 15572583 17728785
- **ALK-positive DLBCL**	Translocation: t(2;17)(p23;q23), variants	*ALK*		19521280 17509395
- **B-cell, unclassifiable** - **with features intermediate between DLBCL and Burkitt lymphoma**	Rearrangement:3q27, 8q24, 14q32, 18q21 Complex karyotypes	*BCL6, MYC, IGH,BCL2*	Unfavorable: *MYC* disruption combined with *BCL2* or *BCL6* (double hit)	23600716 25696840
- **B lymphoblastic lymphoma**	t(9;22)(q34;q11.2) t(4;11)(q21;q23), *KMT2A* variants t(12;21)(p13;q22) Gain:X, 4, 10, 14, 17, 21	*BCR/ABL1* *KMT2A/AFF1* *ETV6/RUNX1*	Unfavorable Unfavorable Favorable Favorable: 4, 10, 17	16304368 11071360 22580999 11392884
- **B or T lineage lymphoblastic lymphoma**	Rearrangement:8p11	*FGFR1*	Unfavorable	23594707
• **T-CELL**				
- **Anaplastic large cell, *ALK*–positive (ALCL) lymphoma**	t(2;5)(p23;q35), variants Gain: X, 7, 9, 17p, 17q24-qter Loss: Y, 4q13-q21, 6q, 17, 11q14, 13q	*ALK*	Favorable	25533804 18385450 20660290 18275429
- **Anaplastic large cell, *ALK*–negative (ALCL)** - **lymphoma**	Gain: 1q, 3p, 6p21, 7 Loss: 6q13p21, 13q, 15, 16pter, 16qter, 17p13			18275429 15111330
- **Angioimmunoblastic** - **T-cell lymphoma**	Gain: X, 3 (3q), 5 (5q), 11q14, 19, 21, 22q Loss: 6q, 13q		Unfavorable: X	22586046 17044049 18341637 7919378 12780782 8636776
	Rearrangement: 1p		Unfavorable: 1p31p32, Complex karyotypes	
- **Peripheral T-cell lymphoma, NOS**	Gain: 1q, 3p, 5p, 7q22q31, 8q24qter,			18341637 7987800

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	11q13, 17q, 12p13, 22q			8286748
				10700871
	Loss: 4q, 5q, 6q, 9p, 10q,11p11, 12q, 13q, t(14;19)(q11;q13), TCRA/D variants		Favorable: 5q, 10q, 12q	
		TCRA/TCRD		15111330
	t(5;9)(q33;q22)		Follicular variant	17696193
				17582237
				16341044
- T lymphoblastic lymphoma	t(10;11)(p13;q14)	PICALM/MLLT10		16826225
	t(7;14)(p14;q32)	TRG,TCL1A		24966976
	Loss: 9p	CDKN2A		
	Rearrangement: 7p14, 7q35, 14q11.2, 14q32, 11q23	TCRG, TCRB, TCRA/D, IGH, KMT2A		17369128
- Hepatosplenic T-cell lymphoma	Gain: 7, i(7q), 8			9264394
				11807981
	Loss: X, Y			16941150

- **HODGKIN**

- Classical Hodgkin lymphoma	Gain: 2p, 4p16, 4q23q24, 9p23-p24, 12q			10676635
	Polyploid			10190942
	Loss: 1p, 3p, 6q, 7q			
	Rearrangement: 1p36, 6q15, 6q21, 7q22, 7q32, 8q24, 11q23, 12q24, 13p11, 14p11, 14q32, 15p11, 19p13			21325169
				17510532
	complex karyotypes	IGH	Arising from follicular lymphoma	
- Nodular lymphocyte, predominant	Rearrangement: 3q27	BCL6		15339680,
	Similar rearrangements as in DLBCL- Complex karyotype			16049307
	Gain: Polyploid			16353293

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Supplemental Table 6. Tumor nomenclature for solid tumor culture method selection

Tumors types may histologically be divided into small round cell tumors (SRCTs) and non-small round cell tumors (NSRCTs) based on cellular features. SRCTs may grow in suspension or attach to the culture dish and grow as a monolayer. NSRCTs will not grow in suspension. When the sample is received in the lab, if the histopathologic diagnosis is not yet known, it can be helpful if the pathologist can tell you if the tumor is a 'SRCT' for the purposes of initiating cultures. Some tumors may grow with either method. If sufficient sample is provided for a SRCT, initiate cultures using both methods. If a very small amount of tumor is received, a coverslip culture is best. Observation of growth will allow one to determine if cells attach or float. If cells float and form balls, a suspension microharvest can be done. Suspension direct or overnight harvest may provide material for FISH if culture growth fails.

Suspension only tumors
  Lymphoma or other lymphoproliferative disorders
  Histiocytosis
  Plasmacytoma
Suspension and monolayer - Small round cell tumors
  Ewing sarcoma or peripheral primitive neuroectodermal (pPNET)
  Medulloblastoma or central primitive neuroectodermal tumor (PNET)
  Neuroblastoma
  Osteosarcoma
  Retinoblastoma
  Rhabdomyosarcoma
Monolayer Culture - Non-small round cell tumors
  Brain tumors
    Astrocytoma
    Choroid plexus tumors
    Ependymoma
    Glial tumors, glioblastoma, ganglioglioma
    Meningioma
    Oligodendroglioma
  Mesenchymal tumors or sarcomas or "spindle cell" tumors
    Clear cell sarcoma
    Desmoplastic small round cell tumor
    Fibrosarcoma
    Hemangiosarcoma
    Hepatoblastoma, hepatocellular carcinoma
    Leiomyosarcoma, leiomyoma
    Liposarcoma, lipoma
    Malignant fibrous histiocytoma (MFH)
    Mesothelioma
    Synovial sarcoma
    Wilms tumor
  Germ cell tumors
    Embryonal carcinoma, yolk sac tumors
    Seminoma
    Teratoma
  Epithelial tumors (carcinomas)
    Breast
    Gastrointestinal
    Lung
    Prostate
    Renal cell

APPENDIX 4
(See following page)

Section E9 of the American College of Medical Genetics technical standards and guidelines: Fluorescence in situ hybridization

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Disclaimer: These standards and guidelines are designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality laboratory genetic services. Adherence to these standards and guidelines does not necessarily ensure a successful medical outcome. These standards and guidelines should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific clinical circumstance presented by the individual patient or specimen. It may be prudent, however, to document in the laboratory record the rationale for any significant deviation from these standards and guidelines.

Abstract: This updated Section E9 has been incorporated into and supersedes the previous Section E9 in Section E: Clinical Cytogenetics of the 2008 Edition (Revised 02/2007) American College of Medical Genetics Standards and Guidelines for Clinical Genetics Laboratories. This section deals specifically with the standards and guidelines applicable to fluorescence in situ hybridization analysis. *Genet Med* 2011: 13(7):667–675.

Key Words: *fluorescence, hybridization, FISH, standards, guidelines*

E9 FLUORESCENCE IN SITU HYBRIDIZATION

E9.1 General considerations

Fluorescence in situ hybridization (FISH) analyses can be performed on metaphase cells or on interphase nuclei. Metaphase studies are usually performed to gain information about chromosome structure that is not readily ascertainable by conventional banding techniques. Thus, metaphase studies are frequently considered an adjunct to conventional chromosome analysis. Common examples of metaphase analyses include detection of microdeletions, detection of cryptic rearrangements involving the ends (subtelomere regions) of chromosome arms, and characterization of structural abnormalities. Although meta-phase FISH could be used to assess mosaicism, clinical situations for which this would be needed are rare.

Interphase FISH studies are performed to detect and, often, to quantify the presence of specific genomic targets in nondividing cells. Because mitotic cells are not required, interphase analysis makes it practical to examine large numbers of cells and cells from samples that have low (or no) mitotic index. Changes in the relative position of FISH signals in interphase nuclei can be used to detect rearrangements even though the chromosomes involved cannot be directly visualized. With careful design of the FISH probe sets and with the large number of nuclei that can be examined, FISH testing is often so sensitive as to make repeated chromosome analysis unnecessary for disease monitoring. Note, however, that FISH detects only its intended targets and may give no information about additional abnormalities that may signal disease progression or secondary disease. Examples of interphase FISH analyses include detection of aneuploidy in uncultured amniocytes and detection/quantification of abnormalities associated with neoplastic processes in hematological and solid tumor specimens.

It is recognized that technology and probe development may proceed at such a rapid pace that the standards and guidelines may not specifically address all situations. It is the laboratory director's responsibility to ensure quality assurance and proper pre- and postanalytical practices that are consistent with the general guidelines presented later.

These guidelines are not intended to address interphase FISH used in preimplantation genetics.

E9.2 Regulatory requirements

E9.2.1 Test ordering

As with other high-complexity tests, FISH tests may be ordered only by physicians and by other persons authorized by applicable state law.

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Disclosure: The authors declare no conflict of interest.

E9.2.2 Regulatory classification of FISH probes

With respect to the US Food and Drug Administration (FDA) regulation, FISH probes generally fall into one of four categories:

- Probes/kits whose analytical performance and clinical utility have been approved by the FDA (for in vitro diagnostics).
- Stand-alone probes manufactured according to good manufacturing practices and regulated for clinical use by the FDA as "analyte-specific reagents" (ASRs). FDA regulations prohibit manufacturers from making claims regarding the analytical performance or clinical utility of ASRs.
- Probes labeled for "research use only" (RUO) or for "investigational use only" (IUO) are subject to FDA approval but have not been approved by the FDA for clinical use. Laboratories may consider whether such probes could be used under the practice of medicine exemption or an investigative device exemption. When reporting results of tests that use RUOs or IUOs, the laboratory must disclose the FDA status of these reagents.
- Probes developed and used exclusively in-house, and not sold to other laboratories, are not actively regulated by the FDA at the present time. However, because they may be regulated in the future, the laboratory director should be aware of all applicable federal oversight requirements. A laboratory making its own probes should meet the standards set forth under Section G (Clinical Molecular Genetics).

Clinical laboratories should establish the performance characteristics for each test that uses such probes (42 CFR §493.1213). FDA regulations require the inclusion of a disclaimer on all reports for tests using probes that have not received FDA approval, 21 CFR §809.30(e).

Probes that have been approved by the FDA must be used exactly according to the manufacturer's instructions. Because the performance characteristics of the probe/kit have been approved by the FDA, the laboratory need only ensure that the probe/kit is operating within the performance specifications stated in the product insert. Any changes to the procedure or substitution of reagents included in the FDA approved kit invalidate the approved status and make the laboratory responsible for establishing the performance characteristics of the test.

E9.2.3 Regulation of genetic testing laboratories

E9.2.3.1. Center for Medicare and Medicaid Services (formerly called Health Care Financing Administration), through CLIA '88, regulates all clinical laboratories and their practices. Thus, all laboratories providing FISH testing for clinical purposes are subject to Center for Medicare and Medicaid Services regulations and subject to inspection by Center for Medicare and Medicaid Services or other organization with "deemed" status.

E9.2.3.2. Many laboratories are also subject to regulation by state/local agencies and/or agencies representing the states from which their clinical samples may originate.

E9.2.3.3. Although the FDA has recently claimed responsibility for regulating laboratory developed tests, how this will impact FISH testing is, as yet, not clear.

E9.3 Development/validation of FISH tests

In the present context, a "test" is defined by the specific use of a probe or concurrent use of a set of probes, rather than by the generic "FISH" technology. Documentation of test validation is required under CLIA '88 for any test placed into clinical service after September 1994. In general, validation requirements for a FISH test will depend on its intended use.

Questions that should be considered in test development/validation include the following:

- Is the test intended to detect a condition that should be present in every cell (qualitative testing) or is it intended to detect a condition that may be present in only some cells (quantitative testing)?
- Is the test intended to detect the presence/absence of the DNA sequence complementary to the probe's sequence or is it intended to detect a change in the relative position of targeted sequences (break-apart and fusion probe sets)?

Tests that fall into the latter category will also have the potential to yield information relating to the presence/absence of targeted sequences.

Because the effectiveness of a FISH test can vary with the type of tissue examined, the laboratory director should consider whether separate validations for each tissue type are warranted. Separate validations are always required if the test will be used for conventional cytogenetic preparations and preparations from paraffin-embedded tissues.

E9.3.1 Familiarization procedures

Factors such as reagent (including probe) concentrations and the temperature and timing of denaturation, hybridization, and slide washing contribute to the intensity of the probe signal and to the intensity of nonspecific fluorescence. Establishing the optimum conditions is an empirical process and is the first step in test development and validation.

For some FISH tests, there may be a limited number of alternative signal patterns, all of which can be anticipated before test development. For others and, in particular, for tests intended to detect abnormalities associated with neoplasia, there may be a large number of alternative signal patterns. In the latter situation, it may be helpful to identify alternative, unanticipated, signal patterns with a pilot study involving a small cohort of samples before beginning the validation process. If behavior of a new probe set is somewhat different from others of the same design (e.g., dual fusion and break apart), the pilot study might also help identify adjustments that need to be made to scoring criteria.

Other than for probes sold as FDA-approved reagents, there is no requirement for a manufacturer to demonstrate that the probe/probe set actually detects the abnormality of interest. For this reason, the laboratory should evaluate a known abnormal sample as part of its test development process. If this is not possible, the laboratory may wish (in some states, may be required) to include a disclaimer in the test report that acknowledges the fact that the test's ability to detect the abnormality has not been confirmed.

E9.3.2 Probe localization

There are three methods that may be used to confirm that probes detect their intended targets. For any FISH probe, hybridization with concurrent 4',6-diamidino-2-phenylindole banding or sequential G-/R-/ or Q-banding can be used to confirm that the probe's signal is located over the intended chromosomal region. For break-apart and fusion probe sets, a sample known to contain the abnormality of interest could also be used. The latter approach has the advantage of also confirming the probe set's ability to detect the abnormality and the advantage of confirming localization at the molecular level rather than the chromosomal region level.

Score a minimum of five metaphase cells to verify that each probe used in the test hybridizes to the appropriate chromosome target(s) and to no other chromosomes. Any source of meta-

phase cells may be used, but it is advisable to use cells prepared in a manner that, as closely as possible, mirrors the way cells will be prepared for clinical testing. To exclude cross-hybridization with loci on the Y chromosome, cells used for probe localization should be from male subjects whenever possible.

Use of a cell line containing the region of interest as a uniquely identifiable metaphase target (e.g., structural rearrangements and trisomy) is also an acceptable means for confirming correct localization of the probe as long as the cell line contains at least one copy of each chromosome (including the Y).

In addition to confirming that the probe targets the expected chromosome region, the localization process should also confirm that the probe mix is not contaminated with another probe and that the probe does not hybridize to other targets. Probes with significant cross-hybridization to other targets should not be used.

E9.3.3 Probe sensitivity and specificity

Probe sensitivity and specificity should be established by analysis of the hybridization of the probe to at least 40 chromosomes targeted by the probe. For autosomal targets, this will usually require scoring 20 metaphase cells. For targets located on sex chromosomes, this will usually require scoring 40 metaphase cells. If, as is often the case for many commercially available probes, the probe has perfect sensitivity and specificity (see later), no more than 40 targets need to be evaluated. If the sensitivity or the specificity is <100%, either the hybridization and evaluation should be repeated or the total number of targets evaluated should be increased to 100.

Cells from at least five chromosomally characterized males should be examined. To conserve probe, the patients may be pooled, but the laboratory should be aware that pooling may lead to overrepresentation of one individual's cells in this assessment.

E9.3.3.1. Probe sensitivity is the percentage of scorable metaphase chromosomes with the expected probe signal. A probe with perfect (100%) sensitivity will produce a detectable signal over the expected region of every target chromosome examined. A sensitivity of at least 95% is recommended for all probes used in clinical testing.

Assessment of the sensitivity for probes targeting repeated sequences is complicated by normal population variation in the size of the target. In rare individuals, the target may be difficult, or impossible, to detect. If such targets are used for clinical testing, recognition of this variation and the limitation it poses for interpretation of results should be documented during probe validation. The laboratory director should be aware of any probe limitations when interpreting results.

E9.3.3.2. Probe specificity is the percentage of all scored signals that occur at the expected location. A probe with perfect (100%) specificity will never produce signal over any chromosomal region other than the expected region on the target chromosome. Specificity is calculated by dividing the number of times the signal is seen at the correct chromosome location by the total number of signals seen over all chromosome locations. For clinical testing of metaphase cells, at least 98% of the signals should be located exclusively over the targeted region.

Targets that are comprised of repeated sequences may be especially prone to cross-hybridization. Adjustments to probe concentration and/or stringency of the hybridization may be required to achieve the desired specificity.

For testing of metaphase cells, the probe is sufficiently validated for use in the same sample type if its sensitivity and specificity are as high as recommended. The probe's sensitivity

and specificity are effectively equivalent to the test's analytical sensitivity and specificity (see later), and these values can be used to estimate the likelihood that a mixture of signal patterns is due to mosaicism.

For testing of interphase nuclei (e.g., detection of aneuploidy in uncultured amniocytes or detecting acquired changes in neoplasia), development of reporting criteria requires further evaluation, as follows.

E9.3.4 Analytical sensitivity and specificity

Although probe sensitivity and specificity are measures of how well a FISH probe detects a specific chromosomal target, analytical sensitivity and specificity are measures of how effectively a test based on one or more probes detects a particular condition. If the condition is the presence of a FISH signal at the targeted location in a metaphase chromosome, probe sensitivity/specificity is equivalent to analytical sensitivity/specificity. If the condition is aneusomy, deletion/duplication or change in relative position of loci in interphase nuclei, factors other than the probe's sensitivity/specificity will also affect the test's ability to detect the condition of interest. For example, if a test based on a single probe is used to detect deletion of a locus, the test's effectiveness will be a function of the probe's sensitivity/specificity, but it will also be a function of signal size and nucleus size. Larger signals and smaller nuclei will increase the chance that two separate signals will appear to be a single signal. Analytical sensitivity/specificity may also be a function of the probe design and FISH strategy. Single-fusion translocation probe sets have relatively low specificity because coincidental juxtaposition of signals can mimic the abnormal gene fusion condition. An extra signal or a dual fusion strategy has greater specificity because there are few biological or technical conditions that can mimic the abnormal condition.

Analytical sensitivity is a measure of a test's ability to detect the analyte (condition) of interest. Analytical specificity is a measure of a test's ability to detect only the analyte of interest. Neither analytical sensitivity nor analytical specificity can be directly measured for most FISH applications because there is usually not a more accurate method for quantifying the presence/absence of the analyte. However, in FISH, the measurement of concern is usually the limit of detection, a term that is used interchangeably with analytical sensitivity by some authors.[1] The most practical method for establishing a FISH test's limit of detection is to calculate the upper limit of the abnormal signal pattern in normal cells. This upper limit constitutes the "normal cut-off value."

E9.3.5 Calculation of normal cut-off values

Three statistical methods have been used to calculate the upper limit of the confidence interval for abnormal FISH signal patterns. Unfortunately, none of the three is without drawbacks. Most widely used are the confidence interval around the mean and the inverse beta function. Less frequently, maximum likelihood has been used to calculate cut-off values. Although the latter may be most appropriate due to the fact that it makes no assumptions about the distribution of the data, the calculation itself is so complex as to make this approach unsuitable for most assays. Mean ± confidence interval and inverse beta functions are readily available in spreadsheet programs and, thus, are widely used despite the fact that the distribution of values in most FISH databases fits neither the normal distribution nor the binomial distribution. As currently used,[2] the inverse beta function may lead to conservative (high) cut-off values that yield some false-negative results and very few false-positive results. The confidence interval around the mean may lead to stringent

(low) cut-off values that yield few false-negative results at the expense of producing more false-positive results.

Because of these limitations, none of the three methods in current use is ideal for all applications. The laboratory should choose a method for calculating normal cut-off values that is compatible with its statistical analysis capabilities and with its FISH testing repertoire. When interpreting abnormal signal patterns, the laboratory should be aware of their method's inherent limitations. Regardless of the calculation used, borderline-positive and borderline-negative results should always be interpreted with great caution and in the context of other clinical and laboratory findings.

E9.3.6 Construction of the normal database

A confidence limit of at least 95% is desirable for FISH analyses. See the study by Dewald et al.[3] for a discussion of the relationship between analytical sensitivity, frequency of the abnormal cell type, and the number of cells required to detect the abnormal cell type with a specified degree of confidence. In general, the evaluation of larger numbers of cells will lead to greater confidence in the ability to detect rare cell types.

For acquired abnormalities, an acceptable normal database should include at least 200 nuclei examined from at least 20 individuals who have no indication of having the condition/disease of interest. Databases that will be used for interphase analysis of presumed nonmosaic constitutional microduplications/microdeletions should be based on at least 50 nuclei from at least five individuals known not to have the abnormality of interest. Note that these databases only give information about the expectation for the abnormal signal pattern in normal cells and that an abnormal result for many nonmosaic microduplications should involve a much higher proportion of cells.

Databases that include more individuals may yield fewer false-positive results if the normal cut-off is calculated with the inverse beta method and fewer false-negative results if the confidence interval around the mean is used. The number of cells examined for database samples should reflect the number of cells that will be examined during the analysis itself. For FISH assays that have a low likelihood of yielding an abnormal signal pattern in normal cells, the assay's ability to detect low-frequency abnormal cells will improve if the number of nuclei examined during validation and analysis is larger.

Database samples should be analyzed using methods established during the familiarization step by staff members who would normally be involved in this testing. If an automated scanner is used for this testing, concurrent analysis by staff and the scanner should be performed. If the two data sets differ significantly, the automated scanner should be adjusted and the slides rescanned until the difference is insignificant.

A database and its resulting normal cut-off values are specific to the methodology and, to a lesser extent, to the personnel and equipment used in the laboratory that developed the database. Thus, a laboratory should not use a database developed by any other laboratory.

E9.3.7 Construction of an abnormal database

If the goal of testing is simply to detect the presence of abnormal cells, an abnormal database may have limited value. However, if the test will also be used to discriminate samples comprised entirely (or largely) of abnormal cells from samples with a mixture of cells, an abnormal database is also warranted. For instance, in prenatal detection of Down syndrome, one might want to discriminate nonmosaic trisomy 21 from mosaic trisomy 21 due to the fact that the phenotypic consequences of the latter are less predictable. An abnormal database based on patients shown by conventional cytogenetics to have nonmosaic trisomy 21 would be one method for distinguishing between the two.

If an abnormal database is developed, the process used for development of the normal database should be followed except for the fact that the control samples would all be drawn from known affected individuals.

E9.3.8 Paraffin-embedded FISH analyses

For paraffin-embedded tissues, FISH may be performed either on 3–6 μm sections or on nuclei extracted from thick sections or cores from paraffin blocks. FISH performed on sections has the advantage of preserving specimen architecture, thus allowing the analysis to be focused on neoplastic tissue. However, sectioning causes nuclear truncation, resulting in possible loss of signals in some nuclei. The nuclear extraction technique yields whole nuclei, but nuclei from neoplastic cells cannot be distinguished from normal nuclei; therefore, nuclear extraction should not be used for specimens in which tissue architecture is integral to interpretation, such as *HER2 (ERBB2)* FISH in breast cancer.

Regardless of the preparation technique used, analyses performed on paraffin-embedded tissue should use their own databases. A database developed for detecting *MYC/IGH* gene rearrangements in conventionally prepared marrow should not be used for paraffin-embedded lymph nodes. Databases should be established based on tissue sections of consistent thickness, and this same thickness should be maintained for testing of all specimens. For example, a database determined using 6 μm sections should not be used for testing specimens that are cut at a thickness of 3 μm.

FISH testing of paraffin-embedded tissue using enumeration probes is generally not suitable for the detection of low-level mosaicism or minimal residual disease due to the fact that nuclear truncation and decreased hybridization efficiency will lead to relatively high normal cut-off values. However, this limitation may not apply to paraffin-embedded assays that rely on break-apart or fusion probe strategies. For paraffin-embedded FISH assays that are not used for detection of low-level mosaicism or minimal residual disease, databases may be based on fewer normal samples and on the analysis of a smaller number of cells. For example, the analysis of 50 nuclei from five normal samples each may be suitable for neoplasms or constitutional cases that are not expected to show genetic heterogeneity and in which a large percentage of the sample is expected to be composed of the cells of interest (e.g., a diagnostic sarcoma specimen) or when neoplastic cells can be distinguished from nonneoplastic cells. One hundred nuclei may be desirable for neoplasms known to exhibit genetic heterogeneity or in which neoplastic cells may be focally present against a background of nonneoplastic cells (e.g., certain lymphomas). However, scoring is best approached by scanning the entire area of hybridization for abnormal signal patterns and by correlating any abnormal FISH findings with histology.

A tissue source that mimics, as closely as possible, the tissues for which the assay is intended should be used for the database (e.g., tonsil for tests likely to involve lymph nodes).

Because metaphase cells are absent and specific chromosomes cannot be recognized in paraffin-embedded preparations, probe sensitivity and probe specificity cannot be directly assessed. Nevertheless, assessment with conventional cytogenetic preparations is recommended due to the fact that if a probe demonstrates suboptimal sensitivity and specificity on metaphase chromosome preparations, it is not likely to be acceptable for evaluation of paraffin-embedded tissue.

If the test will be used for detecting deletions, duplications, or genomic amplification, an internal control (second probe labeled in a different color) should be included in the probe mixture.

E9.3.9 Test precision

In FISH, test precision is a measure of the quantitative agreement between repeated assessments of the same sample. A test with perfect precision will find exactly the same percentage of abnormal cells in a given sample every time the test is performed.

Precision is usually not assessed for FISH tests due to the fact that inherent biological variation in samples confounds such assessment. The laboratory should be aware that FISH tests do not have perfect precision. Hence, when a test value falls just under or just over the cut-off value established for normal controls, the lack of perfect precision may contribute to a false-negative or a false-positive result. Care should be taken in reporting results near the cut-off values.

Appreciation of a test's precision can be achieved by comparing the analytical scores obtained from two different test readers. The laboratory director should have a method to measure agreement between readers and indirectly assess test precision and reproducibility. Discrepancies between two independent reads are often attributable to scoring technique, which should be controlled through training and on-going technologist competency assessment.

Note that varying culture conditions and, in particular, varying the length of the cell culture period may impact a test's precision and that these conditions should be controlled by following the laboratory's standard operating procedure.

E9.3.10 Probes included in FDA-approved kits

E9.3.10.1. Reagents sold in the form of FDA kits must be used exactly as described by the manufacturer or the approval status is invalidated. Demonstrating that a change in the recommended procedure yields no difference in probe signal intensity does not constitute revalidation of a kit. In effect, any change in the procedure results in a new test that must be validated, as appropriate, according to sections 9.3.1–9.3.9.

E9.3.10.2. If an FDA-approved kit is used for testing tissues other than those validated by the manufacturer, either the kit must be revalidated according to sections 9.3.1–9.3.9 or the test report must include a disclaimer that identifies the tissue for which the kit is approved and must note the fact that the kit has not been approved for other tissues.

E9.3.10.3. Although further validation is not needed when an FDA-approved kit is used according to the manufacturer's instructions, laboratories should confirm that the kit performs as expected by analyzing at least 10 samples whose status with respect to the test's targeted abnormality is known. At least one of these samples should have the abnormality of interest.

E9.3.11 Validation of probes used for characterization of copy number imbalances detected by microarray (array comparative genomic hybridization and single nucleotide polymorphism microarrays)

Whenever possible, characterization of array results and assessment of parent carrier status should be conducted with industry-standard FISH assays using probes already validated in the laboratory. It is recognized, however, that many such studies will require the use of novel FISH reagents prepared from the molecular constructs used in the array or from available constructs/clones overlapping the genomic region in question. Such reagents should be prepared as described for "home brew" probes (section E9.2.2.4) and should, at a minimum, be validated for localization and for probe sensitivity and specificity (sections E9.3.2–E9.3.3.2).

Before a FISH probe is used for copy number microarray follow-up, specific genomic coordinates of the construct should be documented and understood relative to the copy number change in question. Gross mapping of a FISH clone to a cytogenetic band is insufficient for precise molecular identification. When used following bacterial artificial chromosome-based copy number microarray, it is strongly recommended that the molecular identity of a "home brew" FISH clone be verified either by the commercial source of the clone or preferably by the laboratory reporting the results. For example, one could end sequence the clone or confirm an expected internal fragment by polymerase chain reaction.

Because oligonucleotide-based array findings are generally represented by numerous independently synthesized oligonucleotides, FISH characterization of an oligonucleotide array result generally provides independent confirmation of a probe's molecular identity.

It is generally not feasible to establish an extensive normal control database for probes used for characterization of copy number microarray findings. For nonmosaic abnormalities that can be confidently appreciated in metaphase preparations, the results of probe sensitivity/specificity assessment in normal controls are sufficient to document the normal condition (see section E9.3.3). If the abnormality in question is a duplication that can only be appreciated by interphase analysis, probe behavior in a minimum of 50 interphase cells from a representative normal control (or control pool) should be scored. This can be accomplished by adding interphase analysis to the sensitivity/specificity assessment as outlined in section E9.3.3. Very small tandem duplications (<500 kb) may not be resolvable by FISH and may require alternate methodologies (e.g., dual color FISH, fiber FISH, quantitative polymerase chain reaction, and multiplex ligation-dependent probe amplification) for assessment.

E9.4 Analytical standards

E9.4.1 General considerations

In many FISH tests, two or more targets/loci are routinely examined in a single assay. For tests that target only one locus, inclusion of a second probe is still recommended. The second probe provides an internal control for hybridization efficiency and can be used to tag the chromosome of interest or used to distinguish polysomy from polyploidy. If a probe is used for a target that might not be present in every sample (e.g., targets on the Y chromosome), another sample that is known to have the probe target should be run in parallel with the patient sample. When an internal control is not used, reverse banding on metaphase preparations should be used to confirm chromosomal location in all tests using the probe.

The laboratory should have a system for evaluating the technical quality of the slides used for FISH analyses. Factors such as disease state, tissue source, and age of the slides/fixed materials may result in nonspecific fluorescence or adversely impact the quality of the probe hybridization. Slides with poor technical quality should either not be examined or should be examined and interpreted with great caution. The laboratory should also have a written procedure for scoring that includes which cells should/should not be scored and methods for discriminating one signal from two.

The following analytical standards for testing presume that sensitivity and specificity are at least as recommended in section E9.3.3. If lower, a corresponding increase in the number of cells scored to attain comparable confidence levels is required.

Analytical criteria for FDA-approved probes supersede the general recommendations provided later.

E9.4.2 Metaphase FISH analyses

E9.4.2.1. Metaphase selection for analysis should be based on the observed hybridization of the control probe(s) and the target-specific probe to metaphase chromosome(s). Metaphases showing chromosome-bound background (signals located over nontarget sites) should not be scored.

E9.4.2.2. For nonmosaic microdeletion analyses, a minimum of 10 metaphase cells should be analyzed. If any metaphases are discordant, 10 additional metaphases should be examined. If suboptimal hybridization quality is a potential source of the discordance, the hybridization should be repeated. Assuming the probe's sensitivity and specificity meet the standards noted earlier, observation of three cells with loss of the same signal is, most likely, evidence of mosaicism.

E9.4.2.3. Because these abnormalities are often difficult to visualize in metaphase cell preparations, testing for microduplications should be based, at least in part, on the analysis of interphase nuclei (interpretation requires a reference database; see section E9.3.6). A minimum of 50 interphase nuclei should be examined.

E9.4.2.4. Concurrent testing of all chromosome subtelomere regions is usually performed in a format in which each probe mix is applied to a small region on the slide(s). Because few mitoses may be available in these regions, it is acceptable to examine five metaphase cells for each probe mix so long as abnormal findings are confirmed by the examination of at least 10 metaphase cells (may require a second, independent hybridization).

E9.4.2.5. For characterization of nonmosaic marker chromosomes or unidentified chromosome regions in derivative chromosomes, a minimum of five metaphase cells should be examined for each probe used in the characterization.

E9.4.2.6. Results of metaphase FISH analysis should be confirmed by at least two experienced individuals, one of whom may be the laboratory director.

E9.4.3 Interphase FISH analyses

E9.4.3.1. Selection of nuclei for analysis should be based on the observed hybridization of the probe(s). Nuclei that are broken, overlapped, or that have significant background "noise" should not be scored. If the assay uses more than one probe, different fluorochrome colors should be used to allow differentiation of the individual targets.

As noted in section E9.3.3.1, care should be exercised in the interpretation of results from studies based on repeated sequence probes. Although rare, individuals exist who have a low copy number of a repeat on one homolog. This could result in misleading results due to reduced hybridization and/or signal intensity. Whenever possible, concurrent examination of available metaphase cells should be performed in interphase analyses that use repeated sequence probes.

The presence of contamination by maternal cells (in prenatal cases), bacteria, or fungus can lead to false-positive or false-negative results. Routine processes to identify these contaminants are recommended, such as evaluating spun pellet for visible blood, which can indicate maternal cell contamination,

or evaluating slides for nonspecific background signals that could indicate fungal or bacterial contamination.

E9.4.3.2. For analysis of nonmosaic constitutional abnormalities (e.g., aneuploidies and microdeletions/microduplications), a minimum of 25 nuclei should be scored by each of two readers. If the scores from the two readers are discordant, the case should be read by a third qualified individual, or the test should be repeated.

If a result does not meet laboratory established reporting criteria, the study should be repeated. If no additional material is available, a third analysis (at least 50 nuclei) by a qualified individual can be performed in an attempt to account for questionable results (e.g., poor hybridization or background on a portion of the slide).

E9.4.3.3. Interphase FISH may be used as an adjunctive test to assess levels of mosaicism/chimerism in cell lines with abnormalities previously established by standard banded chromosome and/or metaphase FISH analysis. In this circumstance, at least 50 interphase nuclei should be examined.

E9.4.3.4. For analysis of acquired abnormalities, the total number of nuclei examined should reflect the number of nuclei examined in establishing the normal cut-off values (see E9.3.6). Half of the nuclei should be scored by each of two readers.

Exceptions to this requirement could be made if the abnormal cell type was extremely common in the test specimen. The laboratory director may establish conditions whereby the analysis of such specimens could be terminated before the standard number of nuclei is reached. See section E9.5.3.3.

E9.4.4 Paraffin-embedded FISH analyses

E9.4.4.1. For analysis of paraffin-embedded tissues, selection of nuclei should be based on location of cells of interest (e.g., if there are neoplastic cells and normal stroma on the same section, caution must be taken to score the appropriate cell type). Analysis of paraffin-embedded neoplastic specimens usually involves morphologic interpretation that requires participation by a pathologist. In some instances, depending on the type of specimen and amount of neoplastic tissue present, prehybridization identification (marking relevant neoplastic regions) by a pathologist may be sufficient to ensure analysis of appropriate cells. For some specimens, such as those containing a small amount of tumor admixed with abundant stroma or those in which in situ neoplasia needs to be distinguished from invasive cancer (e.g., breast cancer), this approach may not be sufficient and a pathologist may need to review the posthybridization slide at the microscope or captured images of the regions scored at a magnification that allows morphologic assessment. In specimens in which genetic heterogeneity could be present, such as in the setting of *HER2* amplification assessment in breast cancer, the entire area of hybridization should be evaluated.[4] If areas containing an abnormal signal pattern are identified outside of regions previously marked by a pathologist, those areas should be reviewed by a pathologist to determine the clinical relevancy of the observation. With any paraffin-embedded FISH assay, interaction between the individuals scoring the FISH slide and a pathologist is strongly encouraged if there are any findings in question.

E9.4.4.2. Preparations from paraffin-embedded tissues tend to show more variability in hybridization quality and background fluorescence than conventional cytogenetic preparations. For this reason, care must be taken to score only areas with optimal probe hybridization. Areas with high tissue autofluorescence that could obscure signals should also be avoided. Signal scoring should involve focusing through the entire section to detect signals in different planes. Scoring of overlapping nuclei should be avoided.

Some types of probes are more problematic than others when used on paraffin-embedded tissues. For example, assessment of deletions in paraffin-embedded tissue is more difficult than assessment of gene rearrangements using break-apart or dual-fusion probe strategies. Evaluation for deletions should be performed with an appropriate control probe (e.g., use of a centromere or opposite arm probe). For tests not using an FDA-approved kit, distinguishing polyploidy from true amplification should also be evaluated in the context of an internal control probe on the same chromosome as the test probe.

E9.4.4.3. Appropriate internal control probes may not be readily available (e.g., amplification controls). In such cases, a negative (e.g., no amplification) and a positive (e.g., known amplification) control sample should be included in the analytical process.

E9.4.5 Analytical considerations for FISH following copy number microarray results

E9.4.5.1. In general, FISH used to confirm or visualize abnormal findings identified by copy number microarrays should follow the analysis guidelines established in sections E9.2.4.2 and E9.2.4.3. The following special considerations apply.

- E9.4.5.1.1: Whenever possible, parental FISH analyses should be performed by the same laboratory that performed the initial microarray and FISH evaluation of the proband. When this is not possible, the second laboratory should carefully review the array data to determine whether a suitable, previously validated probe is available. If a previously validated probe is not available, the laboratory should evaluate a specimen from the proband for validation and for positive control purposes. Without confirmation of the probe signal pattern in the proband, one cannot be certain that the probe used is capable of detecting the abnormality in question nor can subtle abnormalities such as small duplications be adequately interpreted in the parental samples.
- E9.4.5.1.2: For probes with which the laboratory has limited or no clinical experience, it is recommended that a normal control be run concurrently with patient material.
- E9.4.5.1.3: When a mosaic condition is suspected (e.g., copy number imbalances near the centromere or hybridization parameters suggestive of mosaicism), it is recommended that 30 metaphase cells be examined. Additionally, because the abnormality may represent a mosaic condition underrepresented in stimulated T cells, it is recommended that at least 50 interphase nuclei be examined in cases where metaphase FISH is nonconfirmatory. FISH examination of unstimulated preparations may be helpful.

E9.5 Interpretation and reporting

E9.5.1 General considerations

E9.5.1.1. For each FISH test performed, the report should, whenever possible, clearly and prominently state that the result is normal/negative or abnormal/positive. Other language such as "inconclusive," "equivocal," "borderline," or "suspicious for" may be used for those situations where the result is not clearly normal or abnormal.

E9.5.1.2. In addition to information required on all clinical test reports, FISH test reports should identify the probe(s) used (either gene symbol or locus symbol), the manufacturer of each probe, and the number of cells evaluated. For FISH studies performed as a follow-up to copy number microarray testing,

the linear position of the probe construct, with corresponding genome build, should be referenced.

The report should also include a detailed description of the test results. Test results should also be described using the current International System for Human Cytogenetic Nomenclature. If multiple FISH assays are reported simultaneously, a separate nomenclature string should be used to describe the results of each.

E9.5.1.3. If a test yields normal results, images (photographic or digital) of two representative normal cells should be obtained. If the test yields abnormal results, images of at least two cells representing each of the abnormal signal patterns should be obtained. Images of normal cells are not required if there is a mixture of normal and abnormal cells.

For concurrent evaluation of all chromosome subtelomere regions, a normal result may be documented by a single image for each probe mix. If an abnormal result is obtained, a minimum of two images should be obtained to document each abnormal cell type.

E9.5.1.4. Pursuant to 21 CFR §809.30(e), the following specific disclaimer must be included in reports of all FISH testing using ASRs:

> "This test was developed and its performance characteristics determined by [laboratory name] as required by CLIA '88 regulations. It has not been cleared or approved for specific uses by the U.S. Food and Drug Administration."

The wording of the above statement is mandatory and should not be changed. However, because the statement may cause some confusion regarding whether such tests are clinically necessary and reimbursable, laboratories may wish to add clarifying language, such as the following, after the disclaimer:

> "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical diagnostic purposes. It should not be regarded as investigational or for research."

Laboratories also may wish to add language such as the following, if accurate:

> "Pursuant to the requirements of CLIA '88, this laboratory has established and verified the test's accuracy and precision."

E9.5.1.5. Limitations of the FISH assay should be stated in the report. For FDA-approved probes/kits, these limitations will be described in the manufacturer's package insert. For tests based on ASRs, RUOs, IUOs, and modification of FDA-approved kits, the following limitations may merit reporting.

E9.5.1.6. If a database for interpreting mosaicism has not been developed for a particular probe (or probe set), caution should be exercised in any conclusion about the presence of mosaicism. Moreover, the test report should clearly state that the test's sensitivity for detecting mosaicism is unknown.

E9.5.1.7. Care should be taken in the interpretation of negative results from studies based on repeated sequence probes because of rare individuals with small numbers of the repeated sequence target.

E9.5.2 Considerations for interpreting metaphase FISH tests

E9.5.2.1. Metaphase FISH analysis provides information only about the probe locus in question. It does not substitute for complete karyotypic analysis.

E9.5.2.2. Care should be taken in the interpretation of results when whole chromosome paints are used to characterize derivative chromosome regions of small size due to the fact that the painting library may not hybridize uniformly across the full length of a target chromosome.

E9.5.2.3. For most known microdeletions, there are also corresponding microduplications. Metaphase FISH analysis is suitable for detection of microdeletions, but microduplication testing should be based, at least in part, on the analysis of interphase nuclei (see E9.3.6 and E9.4.2.3 specifically). Contiguous duplications may result in FISH signals that are very close together, even in interphase.

If microdeletion testing is performed only on metaphase cells and does not include analysis of interphase nuclei, the test report should include a statement indicating that the test cannot exclude the presence of microduplications.

E9.5.2.4. When using metaphase FISH to document a microdeletion in which the missing signal is from a control probe, care should be taken in interpreting results unless the control's sensitivity and specificity were also assessed during the validation process.

E9.5.3 Considerations for interpreting interphase FISH tests

E9.5.3.1. As noted in E9.3.6, cut-off values for interphase FISH analyses are, at best, an estimate of the true upper limit for abnormal signal patterns in the normal population. **For this reason, borderline-positive and borderline-negative results should always be interpreted with great caution and in the context of other clinical and laboratory findings.** For example, bone marrow from a newly diagnosed chronic myeloid leukemia patient would not be expected to yield a borderline-positive result with *BCR/ABL1* FISH analysis. Similarly, one would not expect to have a low-level positive result for the common microduplication syndromes because the duplications are fairly large and because mosaicism is not expected.

E9.5.3.2. If interphase FISH testing is performed on rare sample types or on nonstandard cytogenetic preparations (such as destained, G-banded slides), the laboratory director should consider whether to include a disclaimer about the limitations of these materials in the report. For example, an overwhelmingly positive result with a rearrangement probe set probably needs no qualification in the report but a moderately positive result obtained with a probe used to detect deletions of the chromosome 5 long arm might.

E9.5.3.3. At the laboratory director's discretion, an abnormal interphase FISH result may be reported even though the number of nuclei is less than the standard number for the test. Testing of adequate samples may be terminated prematurely if each of the two readers finds as many, or more, abnormal nuclei as is required to exceed the normal cut-off value (if a full analysis had been performed). Similarly, samples with inadequate numbers of nuclei may be reported as abnormal if the number of abnormal nuclei among the available nuclei exceeds the number of abnormal nuclei that would have been required in a full study.

E9.5.3.4. Interphase FISH for acquired abnormalities may detect potentially abnormal signal patterns that were not anticipated during test development and validation. Such signal patterns should be interpreted with caution and considered in the context of the clinical indications for testing. Metaphase FISH may be helpful for clarifying these signal patterns.

E9.5.3.5. When using interphase FISH to detect a microdeletion or microduplication in which the probe does not target the critical gene responsible for the microdeletion/microduplication syndrome, normal results should be accompanied by a disclaimer stating the limitation of the test. Such a disclaimer may include information as given in the following example:

> "The probe used, however, may give a normal result in cases that are due to very small deletions, point mutations or other genetic etiologies."

E9.5.3.6. For tests not using an FDA-approved kit, the presence/absence of gene amplification should be reported in the context of a control locus or in the context of positive and negative controls. A universal standard for what constitutes FISH evidence of gene amplification does not exist, at present, so the goal of this standard is to prevent polyploidy from being reported as gene amplification.

For some neoplasms, there are published conventions for when amplification should be reported. These are often based on clinical criteria, such as prognosis or response to therapy and, thus, may be disease specific (e.g., the cut-off ratio of >2.2 for *HER2* amplification in breast cancer is different from the cut-off ratio of >4 for amplification of *MYCN* in neuroblastoma). Whenever they are available, guidelines from consensus groups should be used for reporting gene amplification.

If dividing cells are available in the sample, a recommendation for conventional chromosome analysis (to detect homogeneously staining region, double minutes, etc) should be included in the report whenever amplification is detected.

E9.5.4 Considerations for interpreting FISH tests performed on paraffin-embedded tissues

E9.5.4.1. In situations where the fixation procedure is not known (e.g., an archived specimen or one received from an outside institution), and the hybridization fails, a note should be included in the report stating that variables such as type of fixative or age of paraffin block may negatively impact hybridization efficiency.

E9.5.4.2. If interphase FISH testing is performed on paraffin-embedded tissues prepared by another laboratory (i.e., not the same source as the samples used for the database), the possibility that the database may have limited applicability to this material should be acknowledged in the test report. This acknowledgment is not required for FDA-approved kits.

E9.5.5 Interpretive considerations for FISH used following copy number microarray

E9.5.5.1. Because it is impractical to establish normal cut-off values for all FISH tests used in copy number microarray follow-up studies, the laboratory should establish its own standard for interpreting microduplication test results. Two approaches have been used. In the first, the laboratory establishes an arbitrary cut-off (e.g., 50%) above which the results are considered abnormal and below which the result is considered uninformative. In the second, the laboratory establishes a flexible cut-off that is based on some multiple of the frequency of the abnormal pattern in a known normal sample (for instance three times the frequency). Again, the

test is interpreted as either abnormal or uninformative. Reporting the test result as uninformative acknowledges the fact that a normal finding will not always exclude very small duplications. Such duplications may be difficult to distinguish from normal and may require more extensive validation or alternative methodology for confirmation. This limitation should be acknowledged in all test reports in which the FISH analysis fails to confirm the microarray result.

E9.5.5.2. Occasionally, FISH and microarray results may be discordant. When this occurs, the following should be considered in the interpretation and resolution of the discordant findings.

The microarray or FISH data may be artifactual. The quality of the array and FISH data should be reviewed, and testing repeated, if warranted. Additionally, the molecular identity of the FISH probe should be verified, as well as the identity of the clone on the array (for bacterial artificial chromosome-based arrays). The commercial provider of the FISH construct and microarray should be notified of any suspicious manufacturing or labeling errors immediately.

The probe selected may not fully overlap the abnormality. Linear positions of the probe construct and the abnormality defined by the array should be carefully evaluated, using the same genomic build as a reference.

The abnormality in question may be a very small tandem duplication (<500 kb), yielding closely spaced signals that cannot be resolved by interphase FISH. In these cases, alternate confirmation methodologies may be required.

The abnormality identified by microarray may represent a mosaic condition underrepresented in stimulated T cells. See section E9.4.5.1.3.

E9.5.5.3. When parental samples are evaluated to assess the clinical significance of a finding in a proband, it is important to consider that finding the same abnormality by FISH in a parent and proband strongly suggests but does not prove an identical copy number state in both individuals. Laboratories may wish to add a disclaimer to their reports such as the following:

> "Observation of the same abnormality by FISH in a parent and proband strongly suggests, but does not prove an identical copy number state in both individuals. The abnormality may have undergone further modification in the proband, or the parent may have undetected mosaicism for a normal cell line in a tissue not tested."

Other factors that should be considered in assessing clinical significance are discussed more fully in the ACMG laboratory standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants.[5]

E9.6 Quality assurance

E9.6.1

Probe localization, sensitivity, and specificity should be confirmed for each new lot of probe (as described in E9.3.2 and E9.3.3). Evaluation of new lots should include a written statement as to whether the lot passes or fails the quality assessment. Inclusion of a subjective assessment of signal quality is also desirable and may be useful for detecting trends.

E9.6.2

Biannual (twice per year) or continuous quality monitoring verification is required (42 CFR §493.1217) for all FISH assays.

This requirement can be met by continuous monitoring of test results. For example, important test characteristics to monitor might include (1) correct number of signals (i.e., no contamination of probe and no degradation of probe) and (2) no excess background or other technical problems that would preclude interpretation. If continuous monitoring is used, the quality monitors should be assessed and documented at least twice per year.

Alternatively, quality monitoring may be accomplished by incorporating known normal or abnormal samples into the routine workflow of the laboratory and comparing the actual results for those samples to the expected results.

E9.6.3

Changes in equipment and changes in staff (or staff experience) may cause test results to "drift" away from values obtained during the establishment of normal/abnormal databases. The laboratory should have a method for ensuring that previously established normal range cut-offs are still appropriate or should have a plan for assessing the appropriateness of the database on at least an annual basis. One method for accomplishing the latter would be to periodically analyze known normal samples with the intent of adding to (or replacing) sample data in the test's normal database.

E9.6.4 Proficiency testing

Laboratories must participate in proficiency testing (PT) for each FISH method they use at least twice per year. Metaphase FISH, interphase FISH performed on whole nuclei prepared with standard cytogenetic methods, interphase FISH performed on urine specimens, and interphase FISH performed on paraffin-embedded tissue each constitute a method and require their own PT process. If the laboratory does not participate in a commercially available PT program, the laboratory must have a documented alternate means for assessing proficiency.

Commercially available resources for FISH PT are somewhat limited. It is the laboratory director's responsibility to ensure that such resources are sufficient for demonstrating proficiency with the methods used in his/her laboratory and, if they are not, developing alternate means for assessing this proficiency.

E9.6.5 Competency assessment

It is the laboratory director's responsibility to ensure and document that technologists who perform FISH tests are appropriately trained and have demonstrated consistent ability to score cases likely to be assigned to them. At a minimum, each technologist's competency should be assessed annually for each FISH method he/she participates in.

Although color blindness cannot be a condition for staff hiring, color blindness testing is recommended for all laboratory staff participating in the analysis, image capture, and image review for FISH testing.

REFERENCES

1. Armbruster DA, Tillman MD, Hubbs LM. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. *Clin Chem* 1994;40:1233–1238.
2. Wiktor AE, Van Dyke DL, Stupca PJ, et al. Preclinical validation of fluorescence in situ hybridization assays for clinical practice. *Genet Med* 2006;8:16–23.
3. Dewald G, Stallard R, Alsaadi A, et al. A multicenter investigation with interphase fluorescence in situ hybridization using X- and Y- chromosome probes. *Am J Med Genet* 1998;76:318–326.
4. Vance GH, Barry TS, Bloom KJ, et al. Genetic heterogeneity in *HER2* testing in breast cancer: panel summary and guidelines. *Arch Pathol Lab Med* 2009;133:611–612.
5. Kearney HM, Thorland EC, Brown KK, et al. ACMG standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants (CNVs). *Genet Med* 2011;13:680–685.

APPENDIX 5
(See following page)

ACMG Standards and Guidelines for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013

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Microarray methodologies, including array comparative genomic hybridization and single-nucleotide polymorphism–detecting arrays, are accepted as an appropriate first-tier test for the evaluation of imbalances associated with intellectual disability, autism, and multiple congenital anomalies. This technology also has applicability in prenatal specimens. To assist clinical laboratories in validation of microarray methodologies for constitutional applications, the American College of Medical Genetics and Genomics has produced the following revised professional standards and guidelines.

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GENERAL CONSIDERATIONS

Purpose of cytogenomic microarrays

Constitutional cytogenetic abnormalities include aneuploidy (extra or missing chromosomes) and structural aberrations (chromosomal gains and losses, translocations, inversions, insertions, and marker chromosomes). The cytogenomic microarray (CMA) platforms discussed in this guideline are those designed for the detection of DNA copy number gains and losses associated with unbalanced chromosomal aberrations. Regions with an absence of heterozygosity (AOH), also referred to as loss of heterozygosity, regions/runs of homozygosity, or long continuous stretches of homozygosity, may also be detected by platforms with single-nucleotide polymorphism (SNP)-detecting probes. Some regions with AOH may be indicative of uniparental isodisomy or regions of the genome identical by descent.

The utility of this technology for detection of gains and losses in patients with intellectual disabilities, autism, and/or congenital anomalies has been well documented, and CMA is now recommended as a first-tier test for these indications.[1,2]

Advantages of CMAs

The benefits from the use of CMAs for detection of gains and losses of genomic DNA include:

1. Ability to analyze DNA from nearly any tissue, including archived tissue or tissue that cannot be cultured.
2. Detection of abnormalities that are cytogenetically cryptic by standard G-banded chromosome analysis.
3. Ability to customize the platform to concentrate probes in areas of interest.
4. Better definition and characterization of abnormalities detected by a standard chromosome study.
5. Interpretation of objective data, rather than a subjective visual assessment of band intensities.
6. Ability to detect copy neutral AOH with platforms incorporating SNP probes.
7. A ready interface of the data with genome browsers and databases.

Limitations of CMAs

Limitations of the use of CMAs include:

1. For most platforms, the inability to detect genetic events that do not affect the relative copy number of DNA sequences, e.g., molecularly balanced chromosomal rearrangements. However, CMAs may reveal copy number changes in apparently "balanced" chromosomal rearrangements, i.e., gains or losses, at or near the chromosomal breakpoint sites.
2. Low-level mosaicism for unbalanced rearrangements and aneuploidy may not be detected by CMAs. The sensitivity of the microarray for detection of mosaicism will be influenced by the platform, sample type, copy number state, DNA quality, data quality, and size of imbalance.
3. The chromosomal mechanism of a genetic imbalance may not be elucidated.
4. Tetraploidy or other ploidy levels may not be detected or may be difficult to detect.
5. Copy number variations (CNVs) of genomic regions not represented on the platform will not be detected.
6. Current CMA technologies are not designed to detect duplications and deletions below the level of detection according to probe coverage and performance, point mutations, gene expression, and methylation anomalies that may contribute to the patient's phenotype.
7. No microarray platform will detect all mutations associated with a given syndrome. Therefore, it must be understood that failure to detect a copy number alteration at any locus does not exclude the diagnosis of a disorder associated with that locus.

Microarray platform design and manufacture

Different types of CMA platforms are currently available for clinical testing. The probes for these platforms may use either bacterial artificial chromosome–based DNA or oligonucleotide-based DNA. The oligonucleotide-based DNA may be designed to detect only a copy number alteration of a sequence as compared with a control, or may also be able to determine a specific genotype (or allele) associated with the probe (a SNP-detecting probe). The copy number of a probe may be determined either through a directly competitive hybridization of differentially labeled patient and control DNA or a comparative hybridization of the labeled patient DNA to an *in silico* reference set. The copy number data are graphed as a $\log_2$ ratio of the probe intensities, with the expected normalized value equaling "0" (generally associated with two copies of genomic sequence), relative DNA gains having signals of greater intensity ($\log_2 > 0$), and relative DNA losses having less intensity ($\log_2 < 0$). For platforms with SNP-based probes, the copy number alteration should also correlate with the allelic information assuming sufficient coverage of the copy number alteration with SNP-detecting probes. For example, a region present in one copy should only have single SNP alleles identified in the region.

Microarray platform designs may have probes (i) targeted to specific regions of the genome for detection of imbalances known to be associated with congenital anomalies or neurocognitive impairments, (ii) distributed in a genome-wide manner with a specified distribution and spacing, or (iii) placed in both a targeted and genome-wide manner with varying distribution and spacing of probes for specific genomic regions as well as across the genome. The functional resolution of an array will be determined by both the intermarker probe spacing and the number of consecutive probes necessary to confidently identify a true CNV. The functional resolution may be different across different regions of the genome for a given platform due to probe density and may be different for a single copy number gain (two to three copies) versus a single copy number loss (two copies to one copy) of a DNA segment.

The American College of Medical Genetics and Genomics has published specific recommendations for the design and manufacture of CMA platforms.[3] At a minimum, for whole-genome platforms, the design should allow for detection of both gains and losses of 400 kb or larger, genome-wide, with exceptions to this minimal size resolution as necessary due to features of genomic architecture such as segmental duplication-rich regions. It is also desirable to have enrichment of probes targeting dosage-sensitive regions or genes well associated with congenital anomalies or neurocognitive impairments.

All probe descriptions/content and annotations should be openly accessible to the performing laboratory (see also "Annotation/databases" section). Details regarding the microarray design, the synthesis verification, and all quality control (QC) steps taken to validate and assess the performance and reproducibility of the array should be documented and provided by the manufacturer.

FAMILIARIZATION WITH A NEW TECHNOLOGY FOR THE LABORATORY BEFORE VALIDATION

The laboratory with little or no experience with microarray technology should become familiar with all aspects of the new technology before beginning the validation process, regardless of the regulatory status of the array. Familiarization begins with understanding the processes, features, and capabilities of the technology selected. The laboratory should gain experience with the instrumentation, platform design, software, reagents, methodology, technological limitations, workflows, DNA quality parameters, etc., by experimental sample runs. Similarly, the laboratory should become familiar with the features of each sample type the laboratory will process, as different sample types may have unique considerations for microarray data quality and clinical applicability. The laboratory must also be familiar with the potential imbalances and rearrangements associated with the clinical indications.

The use of samples well characterized as "normal" and "abnormal" by another method is valuable during the familiarization process to gain experience in the recognition of CNVs that may represent true biological variation or a probe/platform performance issue. It is suggested that laboratories use a

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combination of data from well-characterized cases processed and run on their platform(s), data from other laboratories, and/or data available from online databases to gain and broaden their experience. Data sharing should involve a spectrum of array results and data quality.

Laboratories need to be able to recognize nonperforming (or nonresponsive) probes, technically induced artifacts, and other issues affecting data quality. Laboratories should become familiar with CNVs that are benign and/or common and resources to aid in the recognition and interpretation of CNVs.[3-8]

VERIFICATION AND VALIDATION

Verification of a Food and Drug Administration–approved/cleared platform

At the time of the publication of these guidelines, there are no commercially available Food and Drug Administration (FDA)-approved or FDA-cleared microarrays for this application. However, laboratories are advised to keep abreast of new developments in this rapidly developing technology.

For any FDA-approved or FDA-cleared microarrays where the laboratory plans to claim the test as FDA-approved/cleared, the approved protocol and intended use (usually included in the package insert) must be followed. The laboratory must verify that it can obtain comparable performance specifications as those established by the manufacturer with regard to accuracy, precision, and reportable range of results.

At the onset of verification, pass/fail criteria for the verification protocol should be established. If the prespecified acceptance criteria are not met, and a repeat or evaluation of the reasons for the failure does not resolve the concern, the laboratory should consider whether or not the array is appropriate for clinical testing.

Accuracy testing will measure the ability of the platform and software to detect known abnormalities. The accuracy evaluation is accomplished by running a series of previously characterized abnormal cases (this may be accomplished through sharing samples with an established laboratory). A minimum of 15 cases is recommended. To the extent possible, the laboratory should use abnormal samples that represent abnormalities that the array is designed to detect. This evaluation should include both a comparison of the findings from the region(s) expected to be abnormal as well as a comparison of the rest of the genome analyzed by the platform. The laboratory must document the concordance of the expected results and any unexpected findings. Because this technology may detect true alterations not previously identified, any unexpected findings that fall within the determined reportable range (as defined in "Validation of a new platform for the laboratory" section) should be further investigated to determine whether the finding represents true biological variation. This will involve the use of an alternative technology or microarray platform for correlation of the unexpected finding.

Precision testing should measure the reproducibility of repeated tests for the same result. The precision of the platform is established by running a minimum of two abnormal samples, each run multiple times in separate experiments. The concordance of the repeated runs should be documented, and any alterations should be considered (variability of breakpoints, calls, and potential reasons for variation, i.e., segmental duplication-rich region) as they pertain to the reportable range, functional resolution, and potential variability around breakpoints. Some variability around breakpoints may be expected due to segmental duplications and individual probe performance. Precision testing can allow for an assessment of breakpoints and potential impact on the clinical interpretation. Breakpoint variability that does not alter the clinical interpretation would be less concerning than variability that does alter the interpretation. Samples with multiple abnormalities are preferable as they maximize the number of findings for the precision study.

Any modification to the FDA-approved use of the product (as specified in the package insert) will be considered as off-label use, and therefore the microarray must then be validated as a non-FDA-approved platform.

Validation of a non-FDA-approved platform

All platforms intended for clinical testing must be either FDA-approved/cleared and verified or must be validated by the performing laboratory. Validation is the process by which the laboratory measures the efficacy of the test in question by determining its performance characteristics when used as intended. This is necessary to demonstrate that it performs as expected and achieves the intended result. Validation is required when using laboratory-developed tests or modified FDA tests. The method and scope of the validation must be documented.

At the onset of validation, pass/fail criteria for the validation protocol should be established. If the prespecified acceptance criteria were not met, and a repeat or evaluation of the reasons for the failure does not resolve the concern, the laboratory should consider whether or not the array is appropriate for clinical testing.

The extent of work necessary for a validation can depend in part on whether the laboratory is validating a new microarray platform for the laboratory, validating a modified design of a previously validated platform, or adding additional sample types or intended uses to a previously validated platform. A new platform is defined as any new methodology or array introduced to the laboratory. A single microarray vendor may produce multiple similar platforms, but each must be validated independently. A modified design may include either minor modification to probe coverage, either through manufacturing of the array or by *in silico* probe filtering.

Validation of a new microarray platform for the laboratory

Through the validation process, the laboratory must establish the performance characteristics of the microarray platform and accompanying software. The performance characteristics that must be established include the accuracy and precision of results, the analytical sensitivity and specificity, and the reportable ranges. Validations should be documented for each array

platform used for clinical testing, regardless of whether the laboratory has prior experience with a different platform.

The reportable range of results includes criteria to identify a CNV and criteria to report a CNV. Laboratories, with consideration of the manufacturer's recommendations, should identify the parameters specific to their platform (number of consecutive probes, $\log_2$ ratios, SNP allele ratios, QC metrics, etc.) that are necessary to conclude that a copy number call represents a true CNV. As the functional resolution is a combination of probe density and number of probes necessary to identify a true CNV, the reportable range should be at or above the functional resolution of the platform. The reportable range should be determined before the evaluation of the validation set, and data from the familiarization process should be utilized. The reportable range may exclude well-characterized benign CNVs. If the reportable range is altered for the laboratory, the validation data should be re-evaluated with the new reportable range. However, if the previously identified validation samples do not contain abnormalities that challenge the altered reportable range, additional samples should be evaluated.

The accuracy evaluation is accomplished by running a minimum of 30 previously characterized abnormal controls. To the extent possible, the laboratory should use abnormal controls that represent abnormalities that the array is designed to detect. This should include both autosomal and sex chromosome abnormalities as duplications and deletions on the sex chromosomes may behave differently in each sex. Furthermore, blinding the evaluators to the expected abnormalities has the additional benefit of validating the settings, evaluation of data, and reportable range. Samples used for validation should represent a variety of findings with various sizes of abnormalities, combinations of gains and losses, various regions of the genome, and some aberrations that challenge the technical limits of detection for reportable DNA gains and losses.

Sample exchanges with a laboratory that is proficient with a similar microarray platform can provide a good source of samples for validation. Exchange of validated data sets (e.g., array files) between laboratories is recommended for additional experience in data analysis.

This evaluation should initially include a full review of the data to identify aberrations that meet the reportable range while blinded to the expected abnormality (as would fit the clinical workflow), followed by an unblinded comparison of the findings from the region(s) expected to be abnormal, as well as an evaluation of the rest of the genome analyzed by the platform. An evaluation of the regions expected to be normal is also important in assessing the probe behavior across the genome. The laboratory must document the concordance of the expected results and any unexpected findings. Evaluation should also include breakpoint evaluation with regard to gene content and genomic architecture. The laboratory should also recognize nonresponsive probes in a region expected to show loss or gain (this may be due to either poor performance probes or underlying genomic architecture). As this technology may detect true alterations not previously identified, any unexpected

CNVs that fall within your laboratory-determined reportable range should be further investigated to determine whether the finding represents true biological variation. This may involve the use of an alternative technology or microarray platform for correlation of the unexpected finding. As both expected and unexpected findings are evaluated, careful selection of the 30 samples is important and the ability to evaluate unexpected findings in the 30 samples should be considered.

Sensitivity and specificity are determined by the number of true-positive, true-negative, false-positive, and false-negative results in a validation data set that meet reporting criteria. However, for a whole-genome assay, all true positives and true negatives are not known. Therefore, specificity and sensitivity for genome-wide array platforms cannot be calculated as traditionally defined.

Sensitivity is evaluated by comparison of expected versus observed abnormalities, and this is then extrapolated to the rest of the genome. Rather than a traditional calculation of specificity, an evaluation of the positive predictive value of the assay is desirable. Determination of the positive predictive value will involve the identification of copy number calls that fall within the laboratory's determined reportable range and a determination of the proportion of those calls that are true. To improve the specificity of the platform, if certain probes are recognized to repeatedly act as false positives, these probes should be removed from future analyses. The identification of false-positive probes may be due to technical or biological variables considering that not all regions of the genome are amenable to accurate locus-specific evaluation of copy number with this technology. If probe content is masked by the laboratory, these changes should be documented. If the changes are sufficient to alter the performance of the platform, an evaluation of the validation data with the altered probe content is required.

The precision testing should measure the closeness of repeated test results to one another. The precision of the platform is established by running a minimum of two abnormal samples, each run multiple times in separate experiments. The concordance of the repeated runs should be documented, and any alterations should be considered (variability of breakpoints, calls, and potential reasons for variation, i.e., segmental duplication-rich region) as they pertain to the reportable range, functional resolution, and potential variability around breakpoints. Some variability around breakpoints may be expected due to genomic architecture and individual probe performance. The precision testing can allow for an assessment of breakpoints and potential impact on the clinical interpretation. Breakpoint variability that does not alter the clinical interpretation would be less concerning than variability that does alter the interpretation. Samples with multiple abnormalities are preferable as they maximize the number of findings for the precision study.

Validation of a new version of a previously established platform

The definition of a new version should be limited to those situations in which a minimal number of probes are removed,

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added, and/or replaced for the purpose of improved performance, and/or coverage is enhanced over a limited number of genomic regions. This would likely involve <10% of the total probe coverage, with no more than 5% probe removal. It should be recognized that these types of changes to an established platform are likely a rare event and most changes in platforms will require a full validation.

In the laboratory that is proficient with microarray technologies, a new version of a platform in use by the laboratory from the same manufacturer should be validated with a minimum of five abnormal samples.

Known abnormal samples from the previous version should be run using the new platform version for comparison to ensure that the performance meets the laboratory standards and to assess performance of probes added into higher-resolution platforms.

New content on an upgraded version should be assessed, if possible, using known abnormal sample(s) with variation in the region of the new content to determine performance.

The evaluation of this validation set of at least five samples should include data analyzed to determine whether the platform and software detected the expected abnormality. If other abnormalities are detected that meet the laboratory-reporting range, the validation should determine whether the findings represent true biological variation.

Validation of additional sample types on an established platform

It is expected that the initial validation will involve the most common sample type for the expected intended use. For example, if the intended use is postnatal evaluation, the sample type may be DNA extracted from peripheral blood. Because the quality of the DNA may vary from alternative tissue sources and this may add interference factors to the microarray analysis, use of DNA from alternative sample types requires an evaluation of the potential for interference.[9]

For a new sample type, an evaluation of the impact of the new sample type on data quality is necessary. The DNA extraction process should be part of the validation process. If there will be minimal changes to the processing or analysis, then a validation of the new sample type can involve equivalency of data quality with the new sample type. If alterations are made in the processing of the array or analysis (e.g., change of reference set), then a new validation is required. In addition, if the new sample type requires a different reportable range, then a new validation is required.

Validation of the allelic differentiation potential of SNP-detecting platforms

The detection of AOH is not in and of itself diagnostic but can identify a concern that would require additional testing such as sequence-based mutation analysis or uniparental disomy testing. However, as AOH may be reported by the laboratory, evaluation of the performance of the SNP-detecting probes to define regions of AOH should be included in the validation.

Reports of these findings must clearly state that the finding is not diagnostic.

Given sufficient probe density, there should be a correlation between copy number state and SNP allele state.

A minimum of five samples need to contain expected copy neutral AOH in addition to CNVs. Interlaboratory comparisons of samples that contain known uniparental isodisomy or regions identical by descent are recommended. This comparison should address the data types that would be included in a report, such as approximate regions of AOH and approximate percentage of genome identical by descent. The detection and accurate size assessment of AOH by SNP-based arrays depend on the density of SNP probes. If the validation method does not address accuracy of breakpoints in AOH calls, reports should reflect this uncertainty. Inaccurate size estimation for regions of AOH could lead to unwarranted follow-up testing for uniparental isodisomy, somatic loss of heterozygosity, and/or autozygosity mapping.[10]

Mosaicism detection

Low-level mosaicism for unbalanced rearrangements and aneuploidy may not be detected by microarray analysis. In addition, the level of detectable mosaicism will vary by size, region of genome, copy number state, DNA quality and data quality. Therefore, it is not likely that a specific level of mosaicism can always be identified uniformly throughout the genome, and this limitation should be recognized.

Without extensive validation to determine specific levels of mosaic detection for a wide variety of CNV sizes and genomic regions, it is not recommended that this technology be used to rule out mosaicism. However, experience in mosaicism identification is desirable to maximize opportunity for detection. Methods for determining detectable levels of mosaicism include dilution studies and analysis of the sample by other quantitative methods. Fluorescence *in situ* hybridization analysis of fresh (uncultured) samples provides a reliable means to establish the level(s) of mosaicism detectable by microarray. Conventional cytogenetic analysis of metaphase cells provides information about mosaicism but may not accurately reflect levels of mosaicism. The laboratory director should determine the method used by the laboratory. More than one method is recommended.

For cells in suspension, dilution studies using samples with known CNVs may help to determine detectable levels of mosaicism. This method can provide an effective means to establish thresholds; however, it may have limitations as an artificial method. Dilution studies for SNP-detecting arrays may not be possible because they may introduce additional genotypes that complicate the analysis.

The detection of mosaicism may include information from both the $\log_2$ ratio and the SNP allele pattern as applicable for each platform.

Be aware that microarray analysis gives a relative level of copy number across the cells within the sample but does not provide a cell-by-cell determination of copy number (e.g., trisomy in 60% vs. tetrasomy in 30% of cells).

Special considerations for validation of prenatal specimens

Experience with postnatal arrays and with common and rare CNVs is important for the processing and interpretation of array results for prenatal specimens. For validation, a distinction should be made between cultured amniocytes and chorionic villus sampling (CVS) cells and uncultured (direct) amniocytes and villi. The validation performed depends on whether the platform has been previously validated for postnatal use or is new to the laboratory and whether both cultured and uncultured cells will be used.

For cultured amniocytes and CVS cells, if prenatal array analysis is performed on an array platform new to the laboratory, the issues and process discussed in the "Validation of a new platform for the laboratory" section apply, and a minimum of 30 previously characterized cases should be processed. Due to the difficulty of obtaining abnormal prenatal specimens, this collection of 30 samples will likely include some previously characterized as normal cases. Therefore, additional experience with abnormal array findings through additional tissue types and data exchanges should occur, to ensure that a wide variety of abnormalities have been evaluated both in-house and *in silico*.

For a previously validated platform for postnatal use, the addition of prenatal specimens requires an understanding of the potential issues that these samples can present regarding data quality. The DNA extraction process should be part of the validation process. If the laboratory will perform analysis on cultured amniocytes and CVS, both sample types should be represented in the validation.

Prenatal samples (including products of conception). Healthy cultures established from amniocytes, villi, and fetal tissue yield an adequate quantity and quality of DNA and can be viewed as essentially equivalent for validation purposes. However, the laboratory should be aware of factors that can affect DNA yield and data quality including culture age, growth rate, confluency, and shipping conditions.

Because uncultured cells may yield different amounts and quality of DNA, additional validation is required to become familiar with potential differences as compared with cultured cells. Parameters to consider for uncultured amniocytes include method of DNA extraction, volume, and gestational age given that these parameters influence the amount and quality of DNA. For example, uncultured amniocytes yield less DNA than cultured cells; however, the quality of the DNA is generally higher from uncultured cells.

Because villi represent a more complicated tissue with different cell types/layers (syncytiotrophoblast, cytotrophoblast, and mesenchymal core), DNA may be extracted from all cell types, or the laboratory may eliminate or concentrate on different cell layers for DNA extraction.[11]

Special quality assurance requirements for prenatal specimens. Back-up cultures of all prenatal samples undergoing array analysis should be established and maintained for the purposes of (i) possible array failures on direct extractions, (ii)

evaluation of possible mosaicism on an independent culture, and (iii) the need to perform metaphase chromosome or fluorescence in situ hybridization analysis to investigate CNVs.

Maternal cell contamination (MCC) analysis should be performed on all prenatal samples, unless contamination is otherwise excluded. MCC can result from direct samples of amniocytes with blood admixture, CVS samples not successfully cleaned of maternal decidua (a more frequent problem with products of conception), and cell cultures undergoing extensive subculturing resulting in expansion of maternal cells. When undetected, MCC can result in missed detection or misinterpretation of copy number changes, even in the context of a male result. Laboratories should understand that MCC can be detected by array software (i.e., SNP-based platforms) or, in the case of male fetuses, by a shift of the sex chromosome plots (mimicking mosaicism). Laboratories should also understand how the presence of MCC can affect detection of CNVs, including different types (gains and losses) and different sizes (small versus large gains and losses).

Mosaicism may be detected in prenatal samples and may represent culture artifact (pseudomosaicism), true fetal mosaicism, or, for CVS, confined placental mosaicism. Careful investigation may be required to determine the fetal genotype. For traditional chromosome analysis, algorithms have been developed to deal with confined placental mosaicism and pseudomosaicism. These algorithms will also need to be developed for microarray analysis and will depend on whether the analysis used direct or cultured cells, and if the mosaicism can be confirmed on an independent culture.

ESTABLISHING A REFERENCE DNA SET

Depending on the platform used, the reference DNA set may come from a single individual or multiple individuals and may be sex matched or mismatched, and may be used *in silico* or as a direct competitive hybridization. The laboratory should understand the benefits and limitations of each scenario. The laboratory should also consider how the data quality is affected by the source and components of the reference DNA set. For example, data quality is likely improved when the conditions used for data acquisition from the reference set closely match the experimental conditions used for the test.[12] Any changes to the reference DNA set require a verification of the quality and accuracy of results obtained with the new reference DNA set as compared with the previous reference DNA set, especially because changes to the control can result in variation of results, particularly within polymorphic regions. For arrays that use *in silico* controls, versioning should be documented.

SOFTWARE CONSIDERATIONS

The laboratory should recognize software limitations and the need for manual and visual inspection of the data for aberration and mosaicism detection because the software may not flag all relevant calls that may be identifiable by a visual inspection of the data. To verify that the method for result generation (including software calls and manual inspection) detects known

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aberrations at certain mosaicism levels, the system should be challenged with different types of aberrations. During the familiarization phase, the settings should be explored and optimized for aberration detection and then established parameters should be used consistently throughout the validation process. Algorithmic parameter settings may be different for various sample types.

Changes to the software settings from those used during the validation may require a re-analysis of at least a subset of the validation data using the new settings to identify any changes to the performance characteristics of the microarray platform. Such changes may include, but are not limited to, new annotation libraries, changes to any *in silico* reference set, or any changes to the aberration-calling algorithm.

The laboratory should understand that most normalization algorithms assume a primarily diploid state, which may obscure the detection of polyploidy. In some situations, the allele pattern may assist with the detection of triploidy but may not identify tetraploidy. Both situations are likely rare in the postnatal constitutional population but may be present as mosaic findings as well as in prenatal settings.

The laboratory should document the software, parameters, and rules used in the analysis of the microarray, as well as all limitations of the analysis program.

QUALITY CONTROL

Identification
For each array, the slide identification number, sample sex, control sex, and sample-tracking control (if applicable) should be verified. Discrepancies in the documentation from the physical sample should be investigated and resolved before processing.

DNA requirements
The laboratory should establish the minimum DNA requirements to perform testing. Each laboratory should have established parameters for the determination of the sample quality and quantity and criteria for adequacy of each. If a sample does not meet these minimum requirements and is deemed suboptimal, the recommended action is to reject the specimen and request a repeat specimen.

Equipment calibration, maintenance, and QC
Equipment, instrumentation, and methodologies employed during the validation and use of microarray platforms should be calibrated, monitored for QC, and regularly maintained as appropriate. Quality metrics should be established whenever possible throughout the assay. Laboratories should ensure that documentation and safeguards are provided by the software manufacturer and that data are processed and summarized in a consistent fashion for every clinical analysis. Most analysis software provides a hierarchy of users with customizable permissions, which enable the laboratory director or supervisor to prevent modification of analysis settings so that all specimens are analyzed consistently. Any changes to data processing should be validated and documented.

General QC metrics
Every microarray platform has defined quality metric values, e.g., adequate dye incorporation and/or amplification, fluorescence intensities, signal-to-background noise ratio, and standard deviation or standard error. Standard cutoff values and acceptable limits should be established for these metrics to ensure that the generated results are reliable and precise enough to be used for a clinical assessment. Quality metrics should be monitored for DNA labeling, hybridization efficiency, data generation and analysis, and other platform-specific parameters. The QC metrics should be incorporated into the laboratory quality assurance and quality improvement programs to monitor analytical variables.

Data quality
The quality of the data will affect the ability to detect genomic aberrations. Therefore, it is absolutely necessary for the laboratory to understand the within-array metrics provided by the analysis software and how each of these metrics reflects the quality of the data. There are a number of metrics that provide a measurement of signal to noise (i.e., artifactual random variance unrelated to genomic location) in the data, such as the difference between the log ratio values of consecutive probes and the spread of the derivative log ratio values after outlier rejection. Similar metrics of variance exist for each platform.

The laboratory should establish acceptable ranges for each QC metric chosen to represent data quality by the laboratory. These ranges are often provided by the manufacturer. However, the laboratory may want to modify these ranges on the basis of their experience with the arrays during the validation process. The laboratory should establish criteria for next steps, should the data fall outside of these established ranges.

Annotation/databases
An integral part of the data analysis is accessibility and use of private and public annotations/databases during the analysis process. Because these annotations are critical for interpretation, it is important that these tools are carefully constructed and applied by the laboratory or software manufacturer. All critical annotations should be thoroughly vetted, and the source(s) should be verified. For all reportable calls, the genomic content should be verified by an independent database source. The manufacturers should provide mechanism(s) for updates to these annotations. Documentation of resources and databases accessed for interpretation is recommended.

Verification of new lots of microarrays/reagents
Verification should ensure that new lots of microarray slides and/or reagents perform in the same manner as the previous lot. The manufacturer should supply documentation of the QC comparison between lots (e.g., oligonucleotide synthesis verification, accuracy of SNP calls or other defined control parameters). New lots of reagents (e.g., new labeling kits, consumables) should have documented equivalency between runs. This may be accomplished by documenting that the QC metrics meet certain set parameters for the new lot of reagents.

Confirmation of specific CNVs

With proper technical performance and analytical validation, it should not be necessary for the performing laboratory to further confirm a CNV called with the laboratory-validated parameters, after the validation stage. Each laboratory should establish a threshold (number of probes and/or genomic size, as well as other QC metrics) for declaring what constitutes a reportable abnormality with their assay. Features to keep in mind when assessing copy number changes are the appropriate log ratio difference between data, the presence of uniform contiguous probe behavior within and adjacent to call, sharp copy number state transitions at breakpoint boundaries, supportive SNP allele states (when applicable), and evaluation of least processed $\log_2$ ratio data (e.g., weighted versus not weighted). Any call-specific quality score provided by the software may be considered.

Given that it is desirable to maximize detection of aberrations of clinically important genes and of aberrations in mosaic form (which may not generate a robust copy number call), it is acceptable and appropriate at the discretion of the performing laboratory to evaluate calls that do not meet the laboratory-validated parameters. These calls may be flagged for review and correlated with the patient's clinical indication, but should be confirmed by an independent methodology if reported.

USE OF ALTERNATIVE TECHNOLOGIES FOR MECHANISM DETERMINATION

Determination of the mechanism leading to the detected CNV may be considered on a case-by-case basis because this may lead to better determination of recurrence risk. Some mechanisms can be identified through the combination of both the CNV and recognition of the genomic location of the altered material, or the genomic structure surrounding the alteration. Examples include both terminal and insertional translocations and ring or marker chromosomes. The appropriate alternative technology may depend on the size, type, and location of the identified CNV and the likely mechanism of formation. Therefore, use of these alternative technologies should be considered as separate testing and should use validated technologies performed and interpreted by appropriately trained personnel.

INTERPRETATION AND REPORTING

For further guidance on interpretation and reporting, refer to recently published guidelines from the American College of Medical Genetics and Genomics for interpretation and reporting of postnatal constitutional copy number variants[13] and for reporting suspected consanguinity as an incidental finding of genomic testing.[14]

METHODOLOGY AND DISCLAIMERS

All reports should include a brief description of methodology, including platform specifics and reporting criteria. Disclaimers should be included as appropriate and required.

Example: testing limitations

Current microarray analysis technologies will detect only gains and losses of genomic segments. Therefore, a normal microarray result does not exclude mutations (nucleotide base-pair changes) in any gene represented on the microarray, gains and losses below the level of resolution of the platform, a balanced rearrangement, or epigenetic events. Additional testing may be appropriate for certain syndromes or conditions when the microarray analysis yields normal results.

Alternative example

This microarray platform will not detect truly balanced chromosomal rearrangements, point mutations, or imbalances of regions not represented on the microarray, and may not detect mosaicism. Failure to detect an alteration at any locus does not exclude all anomalies at that locus.

Example: disclaimer for a non-FDA-approved microarray platform

This test was developed and its performance characteristics determined by (your laboratory name here) as required by Clinical Laboratory Improvement Act (CLIA)1988 regulations. It has not been cleared or approved for specific uses by the US Food and Drug Administration. Pursuant to the 1988 CLIA requirements, this laboratory has established and verified the test's accuracy and precision.

PROFICIENCY TESTING

The laboratory should participate in an external proficiency testing program through an appropriate deemed organization (e.g., the College of American Pathologists). The laboratory should also establish internal proficiency testing of normal and abnormal samples as part of the laboratory internal quality assurance program and ongoing quality improvement program. Correlation between microarray results run in parallel on different array platforms or correlation of microarray results with conventional cytogenetic and/or fluorescence *in situ* hybridization results may be sufficient to provide ongoing proficiency. Proficiency testing should be performed according to 1988 CLIA guidelines.

Documentation of participation and the performance results of all internal and external proficiency tests must be retained by the laboratory and made available to all accreditation agency inspectors.

LABORATORY ACCREDITATION AND PERSONNEL QUALIFICATIONS

Laboratory personnel must have documentation of education, degrees, and certifications as appropriate for level of testing, as well as training, competency assessments, and continuing education as required by appropriate regulatory bodies, e.g., College of American Pathologists, CLIA, Centers for Medicare & Medicaid Services. The testing laboratory must have CLIA certification and state certifications as required to provide clinical testing. College of American Pathologists accreditation is strongly encouraged.

ACMG STANDARDS AND GUIDELINES

RETENTION OF FILES AND DOCUMENTATION

Laboratories should make explicit in their policies which file types and what length of time each type will be retained and that data retention policy must be in accordance with local, state, and federal requirements. CLIA regulations (Sec. 493.1105) require storage of analytic systems records and test reports for at least 2 years. For more specific suggestions for microarray technologies, we recommend that the laboratory consider a minimum of 2-year storage of a file type that would allow regeneration of the primary results as well as re-analysis with improved analytic pipelines. In addition, laboratories should consider retention of the aberrations identified in the analysis, along with the final clinical test report interpreting the subset of clinically relevant variants, for as long as possible, given the likelihood of a future request for reinterpretation of variant significance.

CONCLUSIONS

Each new technological development in the field of genetics brings with it the desire to apply the technology to improve medical care. The transition of a new technology from the research bench into the clinical realm of diagnostic testing must be accompanied by extensive validation to ensure that the results reported to the health-care provider are accurate and reliable for use in patient-care decision making.

Microarray technologies provide a high-resolution view of the whole genome. Medical laboratory professionals must be prepared to identify, interpret, and report the results with clinical relevance, while keeping in mind the social, ethical, and legal responsibilities of reporting genetic information. The interpretation of the data from microarray analysis into clinically relevant information is a difficult and complex undertaking and is the practice of medicine. No algorithm for CNV interpretation can substitute for adequate training and knowledge in the field of genetics. We recommend that genomic microarray analysis be performed in laboratories overseen by individuals with appropriate professional training (American Board of Medical Genetics–certified clinical cytogeneticists or clinical molecular geneticists, or American Board of Medical Genetics/American Board of Pathology–certified molecular genetic pathologists) and that the interpretation and reporting of clinical genomic microarray findings be performed by these same certified individuals.

ACKNOWLEDGMENTS

DISCLOSURE

All members of this working group are directors of clinical laboratories that use genomic microarray technologies (please see affiliations for locations of clinical laboratories). S.T.S. has received honoraria from Affymetrix, a manufacturer of genomic microarray platforms, for speaking engagements. Also, S.T.S. is a consultant to Lineagen, a provider of genomic microarray testing services. The other authors declare no conflict of interest.

REFERENCES

1. Manning M, Hudgins L; Professional Practice and Guidelines Committee. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. Genet Med 2010;12:742–745.
2. Miller DT, Adam MP, Aradhya S, et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. Am J Hum Genet 2010;86:749–764.
3. Kearney HM, South ST, Wolff DJ, Lamb A, Hamosh A, Rao KW; Working Group of the American College of Medical Genetics. American College of Medical Genetics recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities. Genet Med 2011;13:676–679.
4. Lee C, Iafrate AJ, Brothman AR. Copy number variations and clinical cytogenetic diagnosis of constitutional disorders. Nat Genet 2007;39(suppl 7):S48–S54.
5. Firth HV, Richards SM, Bevan AP, et al. DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources. Am J Hum Genet 2009;84:524–533.
6. Riggs ER, Church DM, Hanson K, et al. Towards an evidence-based process for the clinical interpretation of copy number variation. Clin Genet 2012;81:403–412.
7. Sayers EW, Barrett T, Benson DA, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 2012;40(Database issue):D13–D25.
8. Zhang J, Feuk L, Duggan GE, Khaja R, Scherer SW. Development of bioinformatics resources for display and analysis of copy number and other structural variants in the human genome. Cytogenet Genome Res 2006;115:205–214.
9. van Heesch S, Mokry M, Boskova V, et al. Systematic biases in DNA copy number originate from isolation procedures. Genome Biol 2013;14(4):R33.
10. Mason-Suares H, Kim W, Grimmett L, et al. Density matters: comparison of array platforms for detection of copy-number variation and copy-neutral abnormalities. Genet Med 2013;15:706–712.
11. Cytogenetics TAfC. Professional Guidelines for Clinical Cytogenetics and Clinical Molecular Genetics: QF-PCR for the Diagnosis of Aneuploidy, Best Practice Guidelines. In: Genetics BSfH (ed), v3.01. Clinical Genetics Unit, Birmingham Women's Hospital: Birmingham, England, B 15 2TG: ACC Heads of Department; 2012:1–16.
12. Pinto D, Darvishi K, Shi X, et al. Comprehensive assessment of array-based platforms and calling algorithms for detection of copy number variants. Nat Biotechnol 2011;29:512–520.
13. Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. Genet Med 2011;13:680–685.
14. Rehder CW, David KL, Hirsch B, Toriello HV, Wilson CM, Kearney HM. American College of Medical Genetics and Genomics: standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing. Genet Med 2013;15:150–152.

APPENDIX 6
(See following page)

American College of Medical Genetics recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities

Hutton M. Kearney, PhD[1], Sarah T. South, PhD[2], Daynna J. Wolff, PhD[3], Allen Lamb, PhD[4], Ada Hamosh, MD[5], and Kathleen W. Rao, PhD[6], A Working Group of the American College of Medical Genetics

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Disclaimer: These ACMG Standards and Guidelines are developed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to these standards and guidelines is voluntary and does not necessarily assure a successful medical outcome. These Standards and Guidelines should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific circumstances presented by the individual patient or specimen. Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with these Standards and Guidelines. They also are advised to take notice of the date any particular standard or guidelines was adopted and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Abstract: Genomic copy number microarrays have significantly increased the diagnostic yield over a karyotype for clinically significant imbalances in individuals with developmental delay, intellectual disability, multiple congenital anomalies, and autism, and they are now accepted as a first tier diagnostic test for these indications. As it is not feasible to validate microarray technology that targets the entire genome in the same manner as an assay that targets a specific gene or syndromic region, a new paradigm of validation and regulation is needed to regulate this important diagnostic technology. We suggest that these microarray platforms be evaluated and manufacturers regulated for the ability to accurately measure copy number gains or losses in DNA (analytical validation) and that the subsequent interpretation of the findings and assignment of clinical significance be determined by medical professionals with appropriate training and certification. To this end, the American College of Medical Genetics, as the professional organization of board-certified clinical laboratory geneticists, herein outlines recommendations for the design and performance expectations for clinical genomic copy number microarrays and associated software intended for use in the postnatal setting for detection of constitutional abnormalities. *Genet Med* 2011:13(7):676–679.

Key Words: *microarray, aCGH, CMA, guideline*

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Searching for a genetic etiology for intellectual disabilities, developmental delays, and congenital anomalies is often difficult due to the high frequency of nonspecific features shared among numerous potential syndromes. Cytogenetic examination of banding patterns on metaphase chromosomes, the traditional karyotype analysis, has proven a useful diagnostic tool over the past 4 decades for the detection of these conditions. The goal of traditional cytogenetic analyses is to identify a specific genetic cause for the patient's symptoms by examining the genome in as much detail as possible, searching for an alteration of the typical chromosomal number or banding pattern. In many studies, the result might be easily anticipated (e.g. trisomy 21). However, in some cases, a novel or complex abnormality is discovered, even when the phenotype of the patient is highly suggestive of a different etiology. It is for this reason that, even when a newborn presents with features of a classic syndrome such as Down syndrome, the cytogeneticist does not simply analyze the region of interest in the case but rather examines the entire genome for unexpected significant imbalances (losses or gains) that may be present.

We now have new analytical tools, including genomic copy number microarrays (also known as cytogenetic microarrays or chromosomal microarrays), that detect genomic gains and losses with unprecedented resolution. As genomic microarrays have sig-

nificantly increased the diagnostic yield over a karyotype for clinically significant imbalances in individuals with developmental delay, intellectual disability, multiple congenital anomalies, and autism, they are now accepted as a first tier diagnostic test for these indications.[1–4] The introduction of genomic microarrays into clinical laboratories has presented unique validation and regulatory challenges. It is not feasible to validate a molecular technology that targets the entire genome in the same manner as an assay that targets a specific gene or syndromic region. Because this technology represents such a tremendous advance in diagnostic utility and patient care, we must accept these limitations and seek a new paradigm of validation and regulation.

We suggest that these microarray platforms be evaluated and manufacturers regulated for the ability to accurately measure copy number gains or losses in DNA (analytical validation) and that the subsequent interpretation of these findings and assignment of clinical significance be determined by medical professionals with appropriate training and certification. To this end, the American College of Medical Genetics, as the professional organization of board certified clinical laboratory geneticists, has outlined recommendations for the design and performance expectations for clinical genomic copy number microarrays and associated software intended for use in the postnatal setting for detection of constitutional abnormalities.

RECOMMENDATIONS FOR MICROARRAY DESIGN, PROBE SELECTION, AND GENOMIC COVERAGE

1. Probes should be placed throughout the genome at regular intervals, such that the microarray will detect genomic copy number variants (CNVs), both gains and losses, of 400 kb or larger, genome wide. This will allow for a broad genomic screen for novel imbalances and reliably detect all currently described syndromic microdeletions and microduplications mediated by segmental duplication architecture. Experience gained from the use of high-density genomic microarrays has shown that a significant proportion of patients with a diagnosis of a common deletion or duplication syndrome have atypical or complex rearrangements with clinical significance. High-density, whole-genome coverage allows for accurate delineation of breakpoints, which can allow for increased diagnostic certainty of the clinical consequence of the CNV, particularly if the boundary regions contain relevant genes. The number of probes necessary to achieve this resolution will vary dependent on the platform used. The purpose is not to limit the resolution of clinical genomic microarrays; manufacturers are encouraged to exceed the minimum detection size of 400 kb, when feasible through array design and performance.

2. It is desirable to have enrichment of probes targeting dosage-sensitive genes known to result in phenotypes consistent with common indications for a genomic screen (e.g., intellectual disability, developmental delays, autism, and congenital anomalies). Dosage-sensitive genes include those for which a deviation from the normal copy number state (through single copy gain or loss) has clinical implications for the patient. The purpose of this targeting is to maximize serendipitous detection of CNVs smaller than 400 kb in regions of known clinical relevance. The selection of targeted genes should be made in collaboration with medical professionals, preferably with expert consensus review, and consultation of the medical literature. It is

important to clarify that regardless of probe enrichment, no microarray platform will detect all mutations associated with a given syndrome (i.e., will not detect very small deletions and point mutations). Therefore, the manufacturer and performing laboratories must clearly state that failure to detect a copy number alteration at any locus does not exclude the diagnosis of any of the disorders targeted on the microarray.

3. If the microarray platform is meant to replace alternative clinically validated technologies for the detection of gains or losses of particular regions of the genome (subtelomeric regions, syndromic microdeletions, etc.), the manufacturer and performing laboratory should ensure appropriate probe coverage in those genomic regions, such that the analytical sensitivity for the typical imbalances seen in the patient population meets or exceeds that achieved with the alternative technology.

4. Microarrays that also assess genotype at common sites of single-nucleotide polymorphisms (SNPs) can determine copy number state and reveal regions of homozygosity, which may indicate uniparental disomy and/or direct attention to potential candidate genes responsible for recessive conditions. SNP allele ratios can also provide supportive evidence for copy number state and mosaic conditions. Therefore, inclusion of SNP probes is a desirable feature of a clinical genomic microarray, provided that there is appropriate genomic coverage and performance for detection of CNVs, as outlined in this document. SNP analysis is not a requirement at present for clinically appropriate genomic microarrays, as the primary goal of these analyses is to reliably detect copy number alterations.

5. Probes that target repetitive sequences and/or show spurious calls as gains or loss that do not represent true copy number variation in the sample should be avoided (or permanently suppressed from analysis). See section on analytical specificity. It is recognized and expected that this will result in gaps in genome-wide coverage, particularly in regions with many repetitive elements. The manufacturer should clearly state the limitations of the design by outlining which regions of the genome are covered at regular intervals and which regions have no probe coverage.

6. Before a design is finalized, manufacturers should challenge the microarray with a comprehensive set of abnormal samples that in combination survey all regions of the genome represented on the microarray. Those probes that fail to demonstrate appropriate and reliable copy number response should be excluded (or permanently suppressed from analysis). The source of DNA for this purpose may be any well-characterized cell line or control specimen. Artificial (spiked) controls may also be considered, provided there is reasonable assurance that the conditions mimic the relative copy number for biologically relevant gains and losses. If only a deletion control is available, it should be ensured that probes in the interval show appropriate dynamic range for detection of single copy gains.

RECOMMENDATIONS FOR MANUFACTURER'S PREMARKET ANALYTICAL VALIDATION

The goal of the manufacturer's premarket analytical validation process should be (1) to identify the parameters specific to their platform (number of consecutive probes, $\log_2$ ratio, SNP allele ratios, quality control metrics, etc.) and to specify an appropriate software algorithm necessary to achieve ~99%

detection of CNVs >400 kb, (2) to establish a recommended metric of confidence that allows for <1% false-positive calls of any size, and (3) ideally, to provide confidence intervals at copy number transition boundaries, and therefore accurate assessment of genomic content within the detected CNV. Because these considerations are dependent on probe density and performance and software algorithms, the following recommendations apply both to microarray and software manufacturers.

Analytical sensitivity

7. Manufacturers should detail the assay performance, quality parameters, and software algorithm required to achieve a target 99% analytical sensitivity of CNVs >400 kb in the regions covered by the platform. The assessment of the analytical sensitivity may be first made by testing a sufficiently large number (200–300 or more) of well-characterized cases to establish a ~99% analytical sensitivity for CNVs at least 400 kb in size, with a lower limit of the 95% confidence interval >98%. Ideally, these cases should contain unique CNVs with defined copy number alterations throughout the genome, with the majority representing CNVs <1 Mb in size. These samples should be derived from the same tissue source(s) using the DNA extraction procedures recommended in the manufacturer's protocol. A combination of these actual experimental challenges with further *in silico* data modeling approaches (simulation of additional abnormalities) should provide reasonable confidence that >99% of all copy number alterations >400 kb will be detected with the microarray platform.

Analytical specificity

8. It is not feasible to calculate specificity in the usual way, as even phenotypically normal individuals are likely to have one or more CNVs present in the genome. Rather, one can determine the false-positive rate per CNV call. This can be achieved using the same samples from the analytical sensitivity study. Manufacturers should detail the parameters specific to their platform (number of consecutive probes, $\log_2$ ratio, SNP allele ratios, quality control metrics, etc) that are necessary to conclude that a copy number call represents true copy number variation. Any CNV call in the sample set meeting these parameters, regardless of pathogenicity, should be confirmed by an independent methodology with a target false-positive rate <1%. The 95% confidence interval around the false-positive rate should also be determined and will be based on the number of CNVs identified in this study. It is expected that the parameters (e.g., number of consecutive probes) necessary to achieve this low false-positive rate will likely be more stringent for gains versus losses, as the expected probe ratios for single copy gains are more similar to the normal copy number state.
   a. At the manufacturer's discretion, CNVs <400 kb may also be called as long as this does not increase the false-positive rate. With proper technical performance and analytical validation, the performing laboratory should not be required to confirm a CNV, regardless of size, called with the manufacturer's recommended parameters.
   b. In addition to the overall confidence with which a CNV is called, it is recommended that confidence measures also be assigned to probes/regions at the boundaries of a CNV to define the breakpoints as-

signed and provide appropriate minimum/maximum CNV intervals.
   c. Given that it is desirable to maximize detection of aberrations below the 400 kb threshold that result in deletion or loss of function of clinically important genes and of aberrations in mosaic form (both of which may not generate a robust copy number call), it is acceptable and appropriate for the software to highlight certain calls that do not meet the stringent confidence parameters. At the discretion of the performing laboratory, such low confidence calls may be flagged for review and correlation with the patient's clinical indication but should be confirmed by an independent methodology if reported.

CONSIDERATIONS REGARDING ANALYSIS AND INTERPRETATION OF GENOMIC MICROARRAY FINDINGS

9. For the same reasons that we do not recommend microarrays targeted only to well-characterized syndromic regions, we strongly discourage manufacturers' use of in silico data filters that blind the performing laboratory to all data outside the targeted regions. These filters have been suggested to allow for interrogation of only those regions of the genome that are well characterized in the medical literature. We believe that blinding the interpreting geneticist to parts of the genome can lead to oversimplification and misinterpretation of complex and atypical abnormalities and may in turn result in missed or incorrect diagnoses. For this reason, even an array designed to target known pathogenic regions should be analyzed and interpreted using the same parameters outlined in this document, and in the context of a whole-genome analysis, rather than through the use of overly simplified reporting software.

10. Performing laboratories may wish to establish size restrictions for their CNV calls, and these parameters may be different for deletions versus duplications or syndromic regions versus uncharacterized regions of the genome. Manufacturers are encouraged to provide software that enables the performing laboratory to customize their analysis with such tools and additionally allows them to custom annotate regions on the microarray according to the growing evidence base. The selection and application of analysis and interpretation tools should be at the discretion of the (appropriately board certified) interpreting geneticist.

11. It is recognized that with increased genomic coverage, there will be both increased detection of pathogenic CNVs and increased detection of CNVs of uncertain clinical significance and CNVs that are likely benign. The interpretation and appropriate clinical reporting of these findings are complex, and are the practice of medicine.[5] We recommend that the reporting of clinical genomic microarray findings be performed by individuals with appropriate professional training and certification (American Board of Medical Genetics-certified clinical cytogeneticists, American Board of Medical Genetics-certified clinical molecular geneticists, or American Board of Medical Genetics/American Board of Pathology-certified molecular genetic pathologists). Because many of the abnormalities identified by genomic microarrays result from cytogenetic rearrangements, it is often necessary to characterize the mechanism responsible for the imbalance for appropriate genetic counseling. If the microarray assay is performed in

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a molecular genetics laboratory, this laboratory should establish a close partnership with a cytogenetics laboratory, such that abnormal results have cytogenetic characterization and interpretation, as appropriate, before reporting. Family members should also be provided appropriate follow-up testing. With these considerations in mind, the ideal clinical laboratory setting for genomic copy number microarray analysis is one with both molecular and cytogenetic expertise.

REFERENCES

1. Manning M, Hudgins L; Professional Practice and Guidelines Committee. Array-based technology and recommendations for utilization in medical ge-

netics practice for detection of chromosomal abnormalities. *Genet Med* 2010; 12:742–745.
2. Miller DT, Adam MP, Aradhya S, et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet* 2010;86:749–764.
3. Stavropoulos J. CCMG guidelines for genomic microarray testing. CCMG Cytogenetics Committee. Available at: http://www.ccmg-ccgm.org/pdf/policy/ 2010/(7)%20CCMG_PractGuide_CYTO_Microarray_13July10.pdf. Accessed June 9, 2011.
4. Farndon P, Westwood J. Supporting genetic testing in the NHS—second report of the UKGTN. South East London, England: UKGTN, 2010.
5. Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST; A Working Group of the American College of Medical Genetics (ACMG) Laboratory Quality Assurance Committee. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. *Genet Med* 2011;13:680–685.

APPENDIX 7
(See following page)

American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants

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Disclaimer: These ACMG Standards and Guidelines are developed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to these standards and guidelines is voluntary and does not necessarily assure a successful medical outcome. These Standards and Guidelines should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific circumstances presented by the individual patient or specimen. Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with these Standards and Guidelines. They also are advised to take notice of the date any particular standard or guidelines was adopted and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Abstract: Genomic microarrays used to assess DNA copy number are now recommended as first-tier tests for the postnatal evaluation of individuals with intellectual disability, autism spectrum disorders, and/or multiple congenital anomalies. Application of this technology has resulted in the discovery of widespread copy number variation in the human genome, both polymorphic variation in healthy individuals and novel pathogenic copy number imbalances. To assist clinical laboratories in the evaluation of copy number variants and to promote consistency in interpretation and reporting of genomic microarray results, the American College of Medical Genetics has developed the following professional guidelines for the interpretation and reporting of copy number variation. These guidelines apply primarily to evaluation of constitutional copy number variants detected in the postnatal setting. *Genet Med* 2011:13(7):680–685.

Key Words: *CNV, copy number variant, microarray, aCGH, CMA*

Genomic microarrays used to assess DNA copy number, often referred to as chromosomal or cytogenetic/cytogenomic microarrays, are powerful clinical diagnostic tools now recommended as first-tier tests for the postnatal evaluation of individuals with intellectual disability, autism spectrum disor-

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The authors declare no conflict of interest.

ders, and/or multiple congenital anomalies.[1,2] Most genomic microarrays used in clinical practice, including array-based comparative genomic hybridization and single-nucleotide polymorphism-based platforms, provide genome-wide coverage for detection of chromosomal imbalances at a much higher resolution than a conventional cytogenetic analysis (e.g., G-banded karyotype). The ability to examine the genome at this high resolution has resulted in the discovery of widespread copy number variation in the human genome, both polymorphic variation in healthy individuals and novel pathogenic copy number imbalances.

A copy number variant (CNV) is defined as a segment of DNA at least 1 kb in size that differs in copy number compared with a representative reference genome. The term "CNV" does not imply clinical significance; therefore, a qualifier such as pathogenic CNV or benign CNV is necessary for clear communication of clinical relevance. In addition, the term "CNV" does not imply relative dosage. Copy number loss (deletion) or copy number gain (duplication) must be specified to clarify the nature of a CNV.

Although genomic regions rich in low copy repeat sequences (or segmental duplications) may result in commonly recurring CNVs usually well described in the medical literature, many CNVs represent rare variation. In many cases, the interpreting geneticist can assess the genomic content of the CNV, correlate with established clinical literature, and provide an interpretation that is unambiguous and consistent with the interpretation derived from multiple laboratories.[3] However, given the presence of benign CNVs within the genome and the continual discovery of novel CNVs, assessing the clinical significance of CNVs found in a clinical setting can be challenging.[4,5] Accordingly, when the CNV is extremely rare or limited clinical literature is available, interpretation and reporting practices may vary among laboratories.[6] To assist clinical laboratories in the eval-

uation of CNVs and to promote consistency in interpretation and reporting of genomic microarray results, the American College of Medical Genetics (ACMG) has developed the following professional guidelines for the interpretation and reporting of copy number variation. In addition, these guidelines may serve as a reference for referring clinicians, so they may better understand the complexity of CNV interpretation and communicate these findings to patients and families appropriately.

These guidelines apply primarily to evaluation of constitutional CNVs detected in the postnatal setting. Although these guidelines are relevant for CNVs detected during prenatal testing, interpretation and reporting of prenatal CNVs require additional considerations outside the scope of this document. These guidelines do not apply to CNVs representing acquired mutations in neoplasia. In addition, this document does not address analytical validation; therefore, the recommendations assume that the laboratory is confident the CNV represents true biological copy number variation in the patient. Although these guidelines attempt to cover common issues encountered during evaluation of CNVs, there are many CNVs with unique characteristics, and no algorithm will be applicable to all findings.

RECOMMENDATIONS FOR SYSTEMATIC EVALUATION AND CLINICAL INTERPRETATION OF CNVs

Familiarization with well-established contiguous gene syndromes

Approaching a large segmental deletion or duplication by interrogation of single genes within the interval may not reveal the associated syndromic deletion/duplication. It is necessary that recurrent and clinically characterized deletion/duplication syndromes, any associated low copy repeat sequences, and critical regions be recognized and carefully mapped before offering clinical interpretations of microarray data. The following represent helpful reviews but do not substitute for continuing education and monitoring of the rapidly expanding medical literature: OMIM,[7] GeneReviews,[8] DECIPHER,[9] and recently published reviews.[10,11]

Consideration of CNV size

Although generalizations drawn between CNV size and significance hold true as a general rule, it is clear that very large CNVs can be benign in nature,[12–15] and very small CNVs can be clinically significant. It is, therefore, recommended that any size restriction for inclusion of a CNV in a clinical report be based on the laboratory's consideration of the performance characteristics of the array used and generation of a reasonable amount of clinical follow-up, rather than assumptions regarding clinical significance.

Consideration of genomic content in CNV interval

This is by far the most relevant interpretive consideration and is fairly broad in scope. One should consider whether the CNV contains unique, gene-rich sequence or is void of genes and/or is primarily comprised of repetitive elements or pseudogenes. The gene content should be carefully scrutinized for documented and relevant clinical association.

When considering the potential phenotypic effect due to copy number gain or loss of a gene or group of genes, one should investigate whether the genes in the interval are reported to be dosage sensitive and associated with clinical disorders. It is useful and convenient to review entries in the OMIM database[7];

however, it is also prudent to search for recent publications that may not have been incorporated into the OMIM review.

Given that CNV breakpoints are not precisely mapped due to gaps in probe coverage, it is important to consider all the genes in the maximum CNV interval before presuming a CNV to be clinically benign. Further evaluation may be necessary to clarify the genomic content of the CNV for appropriate clinical interpretation.

Genes with reported pathogenic mutations in the medical literature. The nature of the disease-associated mutations should be carefully interrogated to ensure relevance for the CNV in question. Although this list is not comprehensive, the following examples illustrate the need for a clear understanding of genetic mechanisms: (1) a gene associated with a clinical phenotype due to haploinsufficiency may have no phenotype associated with a copy number gain. (2) Dominant disorders often result from specific gain of function mutations rather than dosage imbalance; therefore, CNVs involving such genes may either have no clinical relevance or result in an entirely different phenotype (e.g., gain of function/activating mutations in *FGFR1* result in skeletal dysplasias, whereas deletions/loss of function mutations are associated with Kallmann syndrome[16]). (3) Copy number gains involving only part of a gene may result in gene disruption or altered coding sequence and should not be dismissed without further investigation when involving genes with reported haploinsufficiency.[17] (4) Single-copy deletions of genes associated with recessive disease may only suggest carrier status for the condition. (5) Small CNVs involving only intronic sequence may have no effect on gene function.

Genes with no reported mutations in the medical literature. Avoid, or use great caution when, inferring a pathogenic role for a gene based solely on predicted gene function or functions characterized in model organisms or in vitro studies. This inference is speculative until well characterized in the human population.

No genes in interval. Generally, it is acceptable to adopt a laboratory policy not to report these CNVs, as there is no relevant literature to interrogate. An exception might be made if the CNV exceeds a size cutoff established by the laboratory or is located in close proximity to a well-characterized region with clear relevance to the reason for referral (e.g., a deletion bordering a holoprosencephaly locus in a patient with a holoprosencephaly indication[18–20]).

Comparison of CNV with internal and external databases

We recommend that laboratories performing array-based assessment of copy number track their experience and document pathogenic CNVs, CNVs of uncertain significance, and CNVs that have been determined to represent benign variation. A CNV that is well documented as a benign variant in the performing laboratory, or in peer reviewed published reports or curated databases, likely needs little additional investigation provided the CNV is periodically reinvestigated to ensure that no new data have emerged contradicting this classification. A CNV with which the laboratory has no prior experience should be carefully compared with publicly available databases of copy number variation in the general population, such as the Database of Genomic Variants.[21,22] Note that we have used the term general population rather than normal population, as it is clear that "normal" is relative to the phenotype in question, and some of the individuals represented in large population databases have no phenotypic data available (e.g., HapMap).

Considerations when comparing patient data with CNVs reported in general population databases include:

Dosage of copy number imbalance reported in the

general population studies. The CNV reported as a benign finding in the general population might exclusively represent a copy number gain. If the CNV in question overlaps the same region, but is a copy number loss, a pathogenic outcome cannot be excluded. The opposite situation also applies. Similarly, a CNV observed commonly as a heterozygous deletion in the general population may have a pathogenic outcome when present in a homozygous state.

Size of the reported benign CNV relative to the CNV in

question. One should ensure that the CNV reported in the general population includes the same gene content as the CNV being interrogated. Different array platforms represented in the public databases can lead to differences in the reported size of identical CNVs. Notably, many of the benign CNVs reported from earlier bacterial artificial chromosome-based microarray studies represent size overestimates.[23]

Sex of individual in database relative to patient sex. This consideration is particularly important for X-linked CNVs in males, as many of the reported benign variants are seen in females who may be nonmanifesting carriers of the condition. In addition, consider that contributors to these databases may have excluded the sex chromosomes from analysis; therefore, CNVs mapping to the X and Y chromosomes may be underrepresented.

Validity of the CNV reported in general population

databases. The majority of CNVs reported from large population studies have not been experimentally validated; therefore, CNVs reported in a single study or through use of a single microarray platform should be interpreted with caution.

Clinical characterization of "normal" individuals. One should consider the extent of clinical characterization of individuals represented in the database. Each population series is selected based on defined criteria, usually outlined in the primary reference. Consider how these individuals were selected for inclusion and how likely it is that the clinical phenotype presented in the patient of interest might be present in the "normal" population. Factors such as incomplete penetrance, variable expressivity, age of onset, and parent of origin imprinting effects need to be considered before classification of a CNV as benign in all instances. CNVs occurring with relatively high frequency in the general population and in multiple publications may be interpreted with more confidence as benign in nature. Of note, many publications use the same reference set (e.g., Hap-Map); therefore, a CNV represented in multiple publications may represent the same individual studied multiple times.

RECOMMENDED CATEGORIES OF CLINICAL SIGNIFICANCE

Using the guidelines outlined earlier for systematic investigation of a CNV for clinical significance, the interpreting laboratory geneticist should assign any CNV reported in the patient to one of three main categories of significance. It is recommended that consistent terminology for these categories be used in clinical reporting to facilitate unambiguous communication of clinical significance throughout the medical community.

Pathogenic

The CNV is documented as clinically significant in multiple peer-reviewed publications, even if penetrance and expressivity of the CNV are known to be variable.

This category includes large CNVs, which may not be described in the medical literature at the size observed in the patient but which overlap a smaller interval with clearly established clinical significance. Although the full clinical effect of the patient's CNV is not known, the pathogenic nature of the CNV is not in question.

With the exception of well-established cytogenetic heteromorphisms, this category will include most cytogenetically visible alterations (>3–5 Mb). In the absence of clearly defined syndromic loci within the interval, this inference should be made with caution.

Uncertain clinical significance

This represents a fairly broad category and will include findings that are later demonstrated to be either clearly pathogenic or clearly benign. However, if at the time of reporting, insufficient evidence is available for unequivocal determination of clinical significance and the CNV meets the reporting criteria established by the laboratory, the CNV should be reported as a CNV of *uncertain clinical significance.*

When warranted, one may provide evidence for the likelihood that the CNV is pathogenic or benign, provided any such speculation is well supported in the report, and the uncertainty of this classification is still communicated. Three categories for classification of uncertain variants are suggested and examples provided below. These examples do not cover all scenarios, as each CNV will have unique considerations requiring clinical judgment.

- *Uncertain clinical significance; likely pathogenic:* for example: (1) The CNV is described in a single case report but with well-defined breakpoints and phenotype, both specific and relevant to the patient findings. (2) A gene within the CNV interval has a very compelling gene function that is relevant and specific to the reason for patient referral. Inferences made from data derived solely from model systems should be made with discretion and in general, are discouraged. Such speculation is strongly discouraged for scenarios with nonspecific indications (e.g., intellectual disability) and/or limited evidence regarding gene function (e.g., only information available for gene is documented neuronal expression).
- *Uncertain clinical significance; likely benign:* for example: (1) The CNV has no genes in interval (but is reported because it exceeds a size criterion that may be established by the laboratory). (2) The CNV is described in a small number of cases in databases of variation in the general population but does not represent a common polymorphism.
- *Uncertain clinical significance (no subclassification):* for example: (1) The CNV contains genes, but it is not known whether the genes in the interval are dosage sensitive. (2) The CNV is described in multiple contradictory publications and/or databases, and firm conclusions regarding clinical significance are not yet established.

Benign

The CNV has been reported in multiple peer-reviewed publications or curated databases as a benign variant, particularly if the nature of the copy number variation has been well characterized (e.g., copy number variation of the salivary amylase gene[24]) and/or the CNV represents a common polymorphism. To qualify as a polymorphism, the CNV should be documented in >1% of the population. It is important to carefully

consider dosage of the CNV documented as a benign variant, given, for example, that duplications of some regions may be benign, whereas deletions of the same interval may have clinical relevance.

REPORTING GUIDELINES FOR GENOMIC COPY NUMBER MICROARRAYS USED IN THE POSTNATAL CONSTITUTIONAL SETTING

The following guidelines describe those elements of the clinical report that are necessary to specify the precise identity of a CNV and clearly communicate the clinical significance of the microarray results. Other required elements of a clinical report (e.g., methodology and relevant disclaimers) are outlined in detail in the ACMG Laboratory Standards and Guidelines.

Reporting criteria

The laboratory report should include a description of the criteria used to review the data (e.g., CNV size restrictions) and the criteria used for inclusion of a CNV in the report. Laboratories may choose not to disclose benign CNVs, especially those that represent common polymorphisms. If benign CNVs are listed in the report, they should meet the same analytical performance criteria used in the laboratory to evaluate suspected pathogenic CNVs.

Description of position, size, and relative gain or loss for each CNV detected

Each CNV included in the laboratory report should be described with the elements below. Current nomenclature from the International System for Cytogenetic Nomenclature should be included in the report but should not serve as a substitute for a clear description of the imbalance for clinical professionals unfamiliar with International System for Cytogenetic Nomenclature conventions.

- Cytogenetic location (chromosome number and cytogenetic band designation).
- Dosage (e.g., copy number gain or loss) with CNV mechanism specified when understood (e.g., single-copy deletion, tandem duplication). Assessment of mechanism will usually require additional testing methodology.
- CNV size and linear coordinates with genome build specified. When applicable, particularly when gene content of the CNV is unclear, minimum/maximum coordinates should be provided.

Clear statement of clinical significance

Each reported CNV should be accompanied by a clear statement of significance as outlined in these recommendations. The evidence in support of this interpretation should be summarized and appropriate references provided.

Designation of genes in CNV interval

To the extent feasible, genes involved in a CNV should be specified in the laboratory report. For large imbalances, particularly those with well-established clinical significance, it is acceptable to provide only the name of the corresponding syndrome and/or the most clinically relevant genes in the interval. For CNVs of uncertain significance, it is suggested that all RefSeq genes in the interval be included in the report, when feasible, to facilitate periodic reviews of relevant medical literature. The incorporation of links to websites that list the genes in a particular interval is not recommended because the links may not faithfully direct the clinician to the appropriate gene

content in the future, particularly when genome builds change. When only one or few representative genes are specified in the report, it is suggested that the total number of genes in the CNV interval be provided to allow for perspective of the total gene content.

Recommendation for appropriate clinical follow-up

When pathogenic CNVs or CNVs of uncertain clinical significance are found, the laboratory report should include recommendations for genetic consultation/counseling, any necessary cytogenetic characterization of the CNV, and evaluation of relevant family members. In addition, when a CNV is determined to have uncertain clinical significance, the report may include a recommendation for continued surveillance of the medical literature for new information, which could later clarify the clinical significance of the finding. The responsibility for continuing monitoring of the medical literature for a specific patient lies primarily with the physician with an ongoing patient relationship.[25]

SPECIAL CONSIDERATIONS REGARDING REPORTING OF UNANTICIPATED CLINICALLY SIGNIFICANT FINDINGS UNRELATED TO THE REASON FOR REFERRAL

In rare cases, CNVs may be found in a patient which (1) reveal carrier status for a recessive condition, (2) are diagnostic or predictive of a presymptomatic condition or a symptomatic condition with unrecognized clinical presentation, or (3) are associated with an increased risk of neoplasia. In general, these findings are unanticipated and unrelated to the patient's reason for referral for a genomic screen. It is not possible to construct a whole genome microarray platform that purposefully avoids interrogation of any loci associated with the aforementioned cases, especially as many of the findings will be part of a large CNV involving multiple contiguous genes. Therefore, referring clinicians must have a clear understanding of the potential for these discoveries, and patients/families should be duly informed before test ordering. A formal informed consent process is strongly recommended. In rare situations, creation of unique CNV-reporting categories (e.g., carrier-presumed unaffected) may be necessary for unambiguous reporting.

Reporting carrier status for recessive conditions

It is recognized that detection of some CNVs, particularly deletions, will reveal carrier status for recessive disorders in the deletion interval. Comprehensive reporting of heterozygous recessive mutations is outside the scope of the intended use of these tests and, in general, is not recommended. Reports should clearly state that recessive carrier status may not be disclosed, and that any clinical concern for recessive disorders should be communicated to the reporting laboratory for appropriate consideration. Individual laboratories may choose to adopt specific disclosure policies for recessive conditions. There are some situations when disclosure of recessive mutations may be considered.

1. Well-characterized recessive disorders, for which carrier frequency is reasonably high in the patient population and/or carrier screening is commonly available (e.g., cystic fibrosis). In such cases, there may be justification for reporting carrier status to provide opportunity for reproductive counseling and potential additional testing in the proband or relevant family members. It should be recognized that these disclosures will represent serendipitous

findings, and no claim should be made to the ordering clinician or patient that this test will detect carrier status for any condition.

2. Recessive disorders with clinical features consistent with the patient's reason for referral. In such cases, recommendation for further molecular testing for this disorder may be warranted. This should be restricted to well-described recessive disorders with clear clinical consequence. The report should clearly state the recessive nature of the condition and that the CNV is not diagnostic of affected status without confirmation of a second mutation.

Reporting mutation status for adult onset/presymptomatic or undiagnosed conditions

Some CNVs, although unrelated to the patient's reason for referral, may be clearly diagnostic of a presymptomatic or clinically undetected condition (e.g., male infertility due to deletions involving the AZF region on the Y chromosome[26]). Given that it is impossible to construct a predefined list of all possible diagnoses to allow the patient to consent specifically to the interrogation/reporting of each disorder, it is our general recommendation that CNVs associated with presymptomatic conditions be reported to facilitate early access to medical care. Individual laboratories may wish to adopt nondisclosure policies for specific conditions. Any such policies should be stated in the laboratory report.

Reporting CNVs associated with risk of neoplasia

Deletions that include a known or putative tumor suppressor gene should be carefully considered. Tumor suppressor genes with clearly pathogenic germline mutations and information on penetrance, lifetime risk, tumor spectrum, and clinical management (e.g., *RB1*, *TP53*, and *APC*) should be discussed in the report, regardless of the indication for study.[27] Speculation regarding putative tumor suppressor genes should be avoided, particularly in the absence of well-characterized germline mutations in humans and in the case of genes where tumor suppressor functions have only been shown in animal or in vitro models.

SPECIAL CONSIDERATIONS FOR REEVALUATION OF SIGNIFICANCE BASED ON DATA FROM OTHER FAMILY MEMBERS

When CNVs are found to have uncertain clinical significance, it may be informative to determine whether the CNV was inherited or represents a de novo mutation. Although this is a very relevant line of evidence by which to gauge clinical significance, it is important to stress that it is difficult, and often imprudent, to attribute clinical significance based on the inheritance pattern of a CNV in a single family. It is only through ascertainment of significantly large families with multiple affected and unaffected family members segregating a given CNV or ascertainment of multiple individuals with the same CNV that a true measure of clinical significance can be confidently assessed. For this reason, the ACMG strongly supports efforts to collect and curate deidentified patient data from clinical studies to facilitate rapid and unambiguous assessment of the clinical significance of CNVs.[2,9,28]

In the absence of a large data series, a cautious inference should be made based on the limited family information available. Addendums to the original interpretation, based on information from evaluation of family members, should be appropriately communicated with disclaimers summarizing the following points. Each CNV and each family will have unique considerations, and these investigations require significant communication between laboratorians and referring clinicians.

De novo CNVs

When a CNV is shown to represent a de novo mutation in the proband, this is generally taken as evidence supporting pathogenicity, particularly if the CNV was suspected to have clinical significance based on other lines of evidence, such as gene content. Nonpaternity may complicate this interpretation and, as such, should be disclaimed in the report. Specific testing to confirm paternity is not recommended unless there is a compelling clinical reason to make this assessment and explicit informed consent is obtained.

Because many regions of the genome have significantly elevated mutation rates, some CNVs may indeed be de novo mutations yet represent findings with no associated clinical consequence.[29]

If only one parent is available for follow-up, and the CNV is not found in the parent available for study, no additional inference regarding clinical significance can be made.

Inherited CNVs

When a CNV is found in a parent or other relevant family member, there are numerous caveats one should consider. Rarely can a conclusive inference be made based on the inheritance pattern observed in a single family. The carrier parent and other relevant family members should have a thorough medical evaluation for the presence or absence of the clinical features present in the proband. When this information is not provided to the laboratory, this should be disclaimed in the report and a recommendation for correlation with parental clinical features made.

Parent is affected. In general, this may be cautiously taken as evidence that supports the CNV as the cause of the clinical features. This observation may be coincidental, however, as the CNV and clinical trait may be inherited independently by chance. When available, other family members may be evaluated to determine whether the CNV continues to segregate in concordance with the clinical phenotype.

Parent is unaffected. In general, this may be taken as evidence that supports the CNV as unrelated to the clinical features and likely benign. Special considerations that preclude confident inference and may only be well defined after ascertainment of multiple families include the following:

- Incomplete penetrance: The CNV may be pathogenic but nonpenetrant in the carrier parent.
- Variable expressivity: The carrier parent may have subclinical features that will later be shown to be in the spectrum of the disorder caused by the CNV.
- Parent of origin imprinting effects: The CNV region may be imprinted, such that the disorder only manifests when inherited from a particular sex (and the carrier parent is not manifesting the disorder because of chance inheritance).
- Second mutation not detectable by microarray: The proband may be manifesting a recessive disorder (e.g., a deletion may be inherited from an unaffected carrier parent and an undetectable mutation inherited in a gene within the interval from the other parent). Alternatively, the proband may have one or more "modifier" genes/DNA elements not present in the unaffected parent.
- Mosaic CNV in parent: The CNV may not be present in all tissues of the parent, and therefore, the parent may not manifest all clinical features associated with the CNV.

- CNV in proband is not identical in size to that seen in parent: Rarely, CNVs have been found to undergo further modification (e.g., expansion of deletion) when transmitted from carrier parent to affected child.[30] When the parental studies are performed by an alternative methodology such as fluorescence in situ hybridization, this rare possibility cannot be excluded.
- Special consideration for X-linked CNVs: When an X-linked CNV in a male is found in an unaffected carrier mother, one should consider whether the mother is a non-manifesting carrier. Studies of X-inactivation may be informative, but not all X-linked disorders exhibit skewed X-inactivation in carrier females. It may be more informative to seek other male relatives in the maternal family.

CONCLUDING REMARKS

With the expanding availability of whole-genome testing methodologies, clinical genetics professionals must be prepared to interpret unexpected findings and report them appropriately, not only with consideration of clinical relevance but also with consideration of social, ethical, and legal responsibilities. The interpretation of the clinical relevance of copy number variation is complex and is the practice of medicine. As evident from the numerous considerations outlined in this document, there is no one formula or algorithm for CNV interpretation that will substitute for adequate training in clinical genetics and sound clinical judgment. We, therefore, recommend that the reporting of clinical genomic microarrays be performed by individuals with appropriate professional training and certification (American Board of Medical Genetics-certified clinical cytogeneticists, American Board of Medical Genetics-certified clinical molecular geneticists, or American Board of Medical Genetics/American Board of Pathology-certified molecular genetic pathologists). In addition, given the complexity of this interpretation and the different laboratory methodologies necessary for CNV characterization and evaluation of additional family members, the ideal laboratory setting for this testing is one with both cytogenetic and molecular genetic expertise.

REFERENCES

1. Manning M, Hudgins L; Professional Practice and Guidelines Committee. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet Med* 2010;12:742–745.
2. Miller DT, Adam MP, Aradhya S, et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet* 2010;86:749–764.
3. Brothman AR, Dolan MM, Goodman BK, Park JP, Persons DL, Saxe DF, et al. College of American Pathologists/American College of Medical Genetics proficiency testing for constitutional cytogenomic microarray analysis. [published online ahead of print May 30, 2011] *Genet Med* doi: 10.1097/GIM.0b013e31821d3165
4. Lee C, Iafrate AJ, Brothman AR. Copy number variations and clinical cytogenetic diagnosis of constitutional disorders. *Nat Genet* 2007;39(suppl 7): S48–S54.
5. Sharp AJ. Emerging themes and new challenges in defining the role of structural variation in human disease. *Hum Mutat* 2009;30:135–144.
6. Tsuchiya KD, Shaffer LG, Aradhya S, et al. Variability in interpreting and reporting copy number changes detected by array-based technology in clinical laboratories. *Genet Med* 2009;11:866–873.
7. McKusick VA. National Center for Biotechnology Information (U.S.). OMIM. Bethesda, MD: The Center, 1987. Available at: http://www.ncbi nlm.nih.gov/Omim/. Accessed April 24, 2011.
8. Pagon RA. GeneReviews. Seattle: University of Washington. Available at: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call_bv.View.ShowTOC&rid_gene.TOC. Accessed April 24, 2011.
9. Firth HV, Richards SM, Bevan AP, et al. DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources. *Am J Hum Genet* 2009;84:524–533.
10. Slavotinek AM. Novel microdeletion syndromes detected by chromosome microarrays. *Hum Genet* 2008;124:1–17.
11. Mefford HC, Eichler EE. Duplication hotspots, rare genomic disorders, and common disease. *Curr Opin Genet Dev* 2009;19:196–204.
12. Bateman MS, Mehta SG, Willatt L, et al. A de novo 4q34 interstitial deletion of at least 9.3 Mb with no discernible phenotypic effect. *Am J Med Genet A* 2010;152A:1764–1769.
13. Itsara A, Cooper GM, Baker C, et al. Population analysis of large copy number variants and hotspots of human genetic disease. *Am J Hum Genet* 2009;84:148–161.
14. Filges I, Röthlisberger B, Noppen C, et al. Familial 14.5 Mb interstitial deletion 13q21.1–13q21.33: clinical and array-CGH study of a benign phenotype in a three-generation family. *Am J Med Genet A* 2009;149A:237–241.
15. Barber JC. Directly transmitted unbalanced chromosome abnormalities and euchromatic variants. *J Med Genet* 2005;42:609–629.
16. Wilkie AO. Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. *Cytokine Growth Factor Rev* 2005;16:187–203.
17. Swensen JJ, Keyser J, Coffin CM, Biegel JA, Viskochil DH, Williams MS. Familial occurrence of schwannomas and malignant rhabdoid tumour associated with a duplication in SMARCB1. *J Med Genet* 2009;46:68–72.
18. Cooper DN, Chen JM, Ball EV, et al. Genes, mutations, and human inherited disease at the dawn of the age of personalized genomics. *Hum Mutat* 2010;31:631–655.
19. Kleinjan D, van Heyningen V. Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am J Hum Genet* 2005;76: 8–32.
20. Beysen D, Raes J, Leroy BP, et al. Deletions involving long-range conserved nongenic sequences upstream and downstream of FOXL2 as a novel disease-causing mechanism in blepharophimosis syndrome. *Am J Hum Genet* 2005; 77:205–218.
21. Iafrate AJ, Feuk L, Rivera MN, et al. Detection of large-scale variation in the human genome. *Nat Genet* 2004;36:949–951.
22. Shaikh TH, Gai X, Perin JC, et al. High-resolution mapping and analysis of copy number variations in the human genome: a data resource for clinical and research applications. *Genome Res* 2009;19:1682–1690.
23. Perry GH, Ben-Dor A, Tsalenko A, et al. The fine-scale and complex architecture of human copy-number variation. *Am J Hum Genet* 2008;82:685–695.
24. Perry GH, Dominy NJ, Claw KG, et al. Diet and the evolution of human amylase gene copy number variation. *Nat Genet* 2007;39:1256–1260.
25. Hirschhorn K, Fleisher LD, Godmilow L, et al. Duty to re-contact. *Genet Med* 1999;1:171–172.
26. Vogt PH. Genomic heterogeneity and instability of the AZF locus on the human Y chromosome. *Mol Cell Endocrinol* 2004;224:1–9.
27. Adams SA, Coppinger J, Saitta SC, et al. Impact of genotype-first diagnosis: the detection of microdeletion and microduplication syndromes with cancer predisposition by aCGH. *Genet Med* 2009;11:314–322.
28. Church DM, Lappalainen I, Sneddon TP, et al. Public data archives for genomic structural variation. *Nat Genet* 2010;42:813–814.
29. Bradley WE, Raelson JV, Dubois DY, et al. Hotspots of large rare deletions in the human genome. *PLoS One* 2010;5:e9401.
30. South ST, Rope AF, Lamb AN, et al. Expansion in size of a terminal deletion: a paradigm shift for parental follow-up studies. *J Med Genet* 2008;45:391–395.

APPENDIX 8
(See following page)

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American College of Medical Genetics and Genomics: standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing

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Genomic testing, including single-nucleotide polymorphism–based microarrays and whole-genome sequencing, can detect long stretches of the genome that display homozygosity. The presence of these segments, when distributed across multiple chromosomes, can indicate a familial relationship between the proband's parents. This article describes the detection of possible consanguinity by genomic testing and the factors confounding the inference of a specific parental relationship. It is designed to guide the documentation of suspected consanguinity by clinical laboratory professionals and to alert laboratories to the need to establish a reporting policy in conjunction with their ethics review committee and legal counsel.

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Many genomic microarray platforms use a combination of probes designed to assess copy number and probes to genotype single-nucleotide polymorphisms. In addition to copy-number changes (i.e., deletions, duplications), these array platforms can identify genomic regions that display an absence of heterozygosity, often in the form of one or more long contiguous stretch of homozygosity. Large regions of homozygosity, when observed on a single chromosome, can be indicative of uniparental disomy;[1] however, when these regions are distributed throughout the genome, they usually represent segments of autozygosity or regions that are identical by descent (IBD). These autozygous segments originate from a common ancestor and can indicate a consanguineous relationship between the proband's parents. The health impact of consanguinity has been recently reviewed.[2] Whole-genome and potentially whole-exome sequencing strategies can also detect long contiguous stretches of homozygosity. Because the results obtained using any of these technologies can point to a familial relationship or consanguinity between parents, these technologies could reveal situations suspicious for potential abuse, especially, but not limited to, situations when the mother is disabled or a minor.[3]

The guidelines presented here are designed to assist clinical laboratories in the management of microarray and exome/genome sequencing findings that suggest parental consanguinity, with a primary focus on detection and reporting results back to the ordering clinician.

DETECTION OF CONSANGUINITY

Genomic regions that are IBD originate from a common ancestor, with the proportion of the genome that is autozygous increasing as the parental relationship becomes closer. The average proportion of the autosomal genome that is IBD in the offspring of related parents is given by the coefficient of inbreeding (F).[4] For example, on average, 6.25% or 1/16th of the genome of offspring of first cousins ($F = 1/16$) is IBD. Although the coefficient of inbreeding provides a theoretical value, significant deviations from the expected values do occur.

Because smaller stretches of homozygosity (<3 Mb) spread throughout the genome are common even in outbred populations, laboratories typically set a size threshold, below which segments are not considered significant. In theory, in the offspring of a second-cousin mating, an average of four 12.5 Mb stretches of homozygosity per genome will be present, although both the number and the size of homozygous segments are known to be highly variable.[5] When long contiguous stretches of homozygosity involving multiple chromosomes are present, the percentage of the genome that is IBD can be estimated by the sum of the sizes of the homozygous segments divided by the total autosomal genomic length (~2,881 Mb for GRCh37/hg19). The sex chromosomes are typically excluded from the calculation because males have only a single X and Y chromosome and therefore cannot have homozygosity at any locus outside of the pseudoautosomal regions. This calculation is likely an underestimation of the actual percentage of the genome that is IBD because only those segments of homozygosity meeting the threshold set by the laboratory will be flagged for inclusion in the calculation.[6] This percentage can then be compared with the theoretical value derived from the coefficient of inbreeding for any given parental relationship. These theoretical values are found in many genetics texts and resources.[5]

Because recombination during meiosis is a somewhat random process, the variation from the theoretical value increases with each meiosis[7] such that third cousins may share more DNA sequences than second cousins. Even among the progeny of first cousins, in whom the average percentage of the genome that is IBD is 6.25%, the SD is 2.43%.[5] The expected percentages are based on a single common ancestor; however, multiple loops of consanguinity or multiple generations of breeding within a relatively closed community could complicate the estimation of the degree of relationship. These variations from the expected or theoretical values are more pronounced for more distantly related individuals[5] and may be caused by stochastic events, multiple loops of consanguinity, small gene pools, and unknown family structures (e.g., adoptions, nonpaternity). Because of these variables, the specific familial relationship or degree of relatedness between the parents cannot always be extrapolated from the percentage of the genome that is IBD. Single-nucleotide polymorphism array analysis is not designed to be a paternity test, nor should it be used to definitively assign a specific relationship between the parents of the proband.

Concerns for abuse arise when IBD proportions suggest that the parents of the proband are first- or second-degree relatives, particularly when the mother is a minor or intellectually disabled. Among the progeny of first- ($F = 1/4$) and second-degree ($F = 1/8$) relative matings, the number of meioses separating the parents is sufficiently low such that the SD is relatively low. Therefore, when high percentages of the genome ($\geq 10\%$) are IBD and several large segments of absence of heterozygosity are present, it is reasonable to suspect a close parental relationship.

RECOMMENDATIONS FOR PRETEST COUNSELING

It is recommended that each patient/family undergoing microarray and exome/genome testing receive pretest counseling.

RECOMMENDATIONS FOR REPORTING FINDINGS OF CONSANGUINITY TO THE ORDERING CLINICIAN

It is important to recognize that the detection of one or more long contiguous stretch of homozygosity, in and of itself, is not abnormal. However, the detection of segments that are homozygous does increase the likelihood that the proband has inherited two copies of a deleterious allele for an autosomal recessive disorder. Clinicians may find utility in this knowledge if the patient's phenotype matches that of an autosomal recessive disorder for which one or more candidate genes are located within one of these segments. Because there is clinical utility in the detection of excessive homozygosity, even when the percentage of the genome that is IBD is quite low (<3%), many laboratories may choose to report this finding back to the ordering clinician to encourage consideration of recessive mechanisms and facilitate autozygosity mapping. Laboratories may choose to include a percentage or proportion of the genome that is homozygous in their reports. In general, caution should be exercised when using an automated calculation of the percentage of the genome

that is IBD. Some analysis programs generate this calculation using all segments displaying absence of heterozygosity, regardless of size or mechanism, which can include deletions. This automated calculation is also typically inflated by small regions of homozygosity that are more likely representative of regions of suppressed recombination or linkage disequilibrium (identity by state). Limiting this calculation to segments >2–5 Mb is more likely to result in the inclusion of segments that are truly IBD. Each laboratory should establish parameters for calculating the percentage of the genome that is IBD and determine a threshold for reporting back the results.

In general, laboratories have very limited information regarding the structure of the proband's family (e.g., maternal age, adoptions, multiple loops of consanguinity, other familial relationships). Therefore, speculation of a specific relationship in written reports is strongly discouraged. An example of suggested language is as follows:

"Several large regions of homozygosity (_ Mb or larger) were detected, encompassing >_% of the genome. Although this result is not diagnostic of a specific condition, it raises the possibility of a recessive disorder with a causative gene located within one of these regions. A genetics consultation is recommended."

SPECIAL CONSIDERATIONS

The observation of a possible first- or second-degree parental relationship, particularly when the mother of the proband is known to be a minor or has an intellectual disability, raises a suspicion for abuse involving the mother of the proband. Laboratories do not typically have information regarding the mother's age, intellectual status, or family structure; therefore, they do not have adequate information to communicate a suspicion for abuse to any authoritative agency. Therefore, when the percentage of homozygosity reaches a level that could be consistent with a first- or second-degree parental relationship (>10% with multiple regions of homozygosity 2–5 Mb or larger), laboratory reports should indicate that the results could be associated with possible consanguinity to ensure that the ordering clinician (geneticist or nongeneticist) understands the implications of the results. An example of suggested language is as follows:

"Several large regions of homozygosity (_ Mb or larger) were detected, encompassing >_% of the genome. Although this result is not diagnostic of a specific condition, it raises the possibility of a recessive disorder with a causative gene located within one of these regions. Additionally, these results could indicate a familial relationship (first or second degree) between this individual's parents. A genetics consultation is recommended."

Laboratories are encouraged to engage the ordering clinician when a first- or second-degree mating is suspected based on the results of the analysis. The clinician is the most appropriate

person to correlate laboratory results with family history and cultural traditions and to investigate any concern for abuse. Clinicians should be aware that many states have mandatory reporting statutes requiring that anyone with cause to suspect that a child, juvenile, or disabled adult has been the victim of abuse, including rape or sexual assault, report his/her concern to the appropriate governmental authorities.[8,9] These same statutes provide protection for the reporting individual as long as the concern is raised in good faith. It is advised that each laboratory or hospital consult with its ethics review committee and legal counsel for policy development concerning the requirements for and manner of reporting.

CONCLUDING REMARKS

The ability to detect regions of homozygosity is an important clinical tool with clear utility in the context of the detection of autosomal recessive conditions and uniparental disomy. A secondary consequence of the observation of regions of homozygosity is the possible discovery of a consanguineous relationship between the proband's parents. Although a specific relationship cannot be determined using the currently available technologies, this information may be useful to the clinician caring for the patient and family. It is the responsibility of the clinician, not the laboratorian, to perform clinical correlation and investigate any concern for abuse. The laboratorian's duty is to effectively communicate the possibility of a familial relationship between the parents to the ordering clinician when a first- or second-degree relationship is suspected based on the results of the analysis. Laboratories are encouraged to develop a reporting policy in conjunction with their ethics review committee and legal counsel.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Papenhausen P, et al., UPD detection using homozygosity profiling with a SNP genotyping microarray. *Am J Med Genet Part A* 2011;155A:757–768.
2. Hamamy H, Antonarakis SE, Cavalli-Sforza LL, et al. Consanguineous marriages, pearls and perils: Geneva International Consanguinity Workshop Report. *Genet Med* 2011;13:841–847.
3. Schaaf CP, Scott DA, Wiszniewska J, Beaudet AL. Identification of incestuous parental relationships by SNP-based DNA microarrays. *Lancet* 2011;377:555–556.
4. Hartl DL, Clark AG. *Principles of Population Genetics.* Sinauer Associates: Sunderland, MA, 1997.
5. Carothers AD, Rudan I, Kolcic I, *et al.* Estimating human inbreeding coefficients: comparison of genealogical and marker heterozygosity approaches. *Ann Hum Genet* 2006;70(Pt 5):666–676.
6. Kearney HM, Kearney JB, Conlin LK. Diagnostic implications of excessive homozygosity detected by SNP-based microarrays: consanguinity, uniparental disomy, and recessive single-gene mutations. *Clin Lab Med* 2011;31:595–613, ix.
7. McQuillan R, Leutenegger AL, Abdel-Rahman R, et al. Runs of homozygosity in European populations. *Am J Hum Genet* 2008;83:359–372.
8. Burgess AW. How many red flags does it take? *Am J Nurs* 2007;107:28–31.
9. West JC. Case law update: a review of recent rulings of interest to risk managers. Anonymous Hospital v A.K., 920 N.E.2d 704 (Ct. App. Ind., January 26, 2010). *J Healthc Risk Manag* 2010;30:36–37.

APPENDIX 9
(See following page)

American College of Medical Genetics and Genomics technical standards and guidelines: microarray analysis for chromosome abnormalities in neoplastic disorders

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Microarray methodologies, to include array comparative genomic hybridization and single-nucleotide polymorphism–based arrays, are innovative methods that provide genomic data. These data should be correlated with the results from the standard methods, chromosome and/or fluorescence *in situ* hybridization, to ascertain and characterize the genomic aberrations of neoplastic disorders, both liquid and solid tumors. Over the past several decades, standard methods have led to an accumulation of genetic information specific to many neoplasms. This specificity is now used for the diagnosis and classification of neoplasms. Cooperative studies have revealed numerous correlations between particular genetic aberrations and therapeutic outcomes. Molecular investigation of chromosomal abnormalities identified by standard methods has led to discovery of genes, and

gene function and dysfunction. This knowledge has led to improved therapeutics and, in some disorders, targeted therapies. Data gained from the higher-resolution microarray methodologies will enhance our knowledge of the genomics of specific disorders, leading to more effective therapeutic strategies. To assist clinical laboratories in validation of the methods, their consistent use, and interpretation and reporting of results from these microarray methodologies, the American College of Medical Genetics and Genomics has developed the following professional standard and guidelines.

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Neoplastic processes are a complex group of disorders that develop as a result of the accumulation of genetic alterations including gene mutations, chromosomal rearrangements, gain and loss of genetic material, epigenetic changes, loss of heterozygosity (LOH), and various other genetic changes. Defining and understanding the genetic alterations of specific neoplastic disorders influences the diagnoses, prognoses, and therapeutic choices for patients with both malignant and benign neoplasms.[1–7]

Published clinically applicable data now show the utility of DNA microarray analysis in the assessment of multiple neoplastic disorders.[8–13] Data indicate that microarray technologies provide information about gain and loss of genetic material in neoplastic disorders, including hematologic malignancies and solid

tumors.[14–17] These gains and losses, represented as an increase or decrease in the proportion of genetic material as compared with a reference genome, are collectively referred to as copy-number variants (CNVs). Microarray methodologies are appropriate complementary methods to standard methods of chromosome and fluorescence *in situ* hybridization (FISH) analyses for detection of genetic anomalies in neoplastic disorders.

DNA microarray technologies should confirm genetic imbalances identified by conventional and molecular cytogenetic or FISH analyses and provide further detail of the aberrations.[15–17] However, additional important information about the genetics of specific disorders may be revealed, e.g., leukemia with normal cytogenetic and FISH analyses.[18–23]

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DNA MICROARRAY PLATFORMS

Different types of DNA microarray platforms currently available for clinical testing include bacterial artificial chromosome–based array comparative genomic hybridization, oligonucleotide-based array comparative genomic hybridization, oligonucleotide plus single-nucleotide polymorphism (SNP)-based arrays that contain both copy-number (intensity-only) and SNP (allele-differentiating) probes, as well as SNP-only–based arrays.[9,24–26]

For comparative genomic hybridization–based microarrays, patient DNA and reference DNA are labeled with different fluorochromes and hybridized to probes on the microarray. SNP-based arrays use a single color dye compared with an *in silico* reference. A scanner measures differences in the intensities of the fluorochromes, and the data are expressed as having more or less signal as compared with the reference. For genomic regions with two copies of the DNA sequence, copy-number data are graphed as a $\log_2$ ratio with the expected normal copy number equaling "0." Duplications will have signals of greater intensity ($\log_2 > 0$) and deletions less intensity ($\log_2 < 0$). Microarrays that incorporate SNP probes allow simultaneous detection of DNA copy-number changes and absence of heterozygosity (AOH) by providing information about the intensity of the signals at the loci. AOH may be due to LOH, hemizygosity, or homozygosity.

Advantages of DNA microarray analysis
Advantages of DNA microarray analyses include:

- The ability to use any sample that yields DNA of sufficient quality,
- Assessment of the genome at very high resolution,
- Interpretation of raw data using objective biostatistical algorithms,
- The ability to detect copy-number-neutral runs or regions of homozygosity (ROHs or AOH) with SNP-array technology, and
- A ready interface of the digital data with genome browsers and Web-based genome-annotated databases.

Limitations of DNA microarray analysis
Limitations of DNA microarray analyses include:

- Inability to detect molecularly balanced chromosomal rearrangements,[27]
- Inability to detect tumor-specific changes (acquired clonality) with a low ratio of tumor cells to normal cells,
- Inability to determine the chromosomal mechanisms of the genetic imbalance, e.g., insertion, tandem duplication; chromosome and/or FISH studies may be needed,
- Inability or difficulty in detection of tetraploidy or other ploidy levels; platforms that include SNP probes may facilitate detection, and

- Inability to characterize clonal and subclonal populations; the $\log_2$ ratio may provide an indication of clonal heterogeneity.

Because of these limitations, results using microarray technologies at diagnosis should be correlated with other established methodologies (chromosome analysis, FISH). Microarray analysis is neither established nor recommended as a method for posttherapy follow-up or for minimal residual disease detection.

It should be understood that the current copy-number genomic microarray technologies are not designed to detect point mutations, gene expression levels, methylation anomalies, and microRNA anomalies, all of which may contribute to tumorigenesis. Detection of a "small" insertion or deletion, e.g., intragenic, will be affected by platform resolution, probe spacing, gene coverage, laboratory software parameters, and sample DNA quality.

Microarray platform design and verification
The laboratory should choose a microarray design with probe coverage suitable for detection of known copy-number aberrations associated with the neoplasm of interest. Microarray platform design may be (i) targeted to specific regions of the genome for detection of known cancer-associated unbalanced genomic alterations, (ii) genome-wide with a specified distribution and spacing of probes, or (iii) both targeted and genome-wide, with varying distribution and spacing of probes in specific regions and across the entire genome.

Manufacturers of microarrays should verify the identity of each clone or probe on the platform used for clinical testing. Probes selected from the public domain should be listed with their physical and cytogenetic positions on the human genome. All probe descriptions and annotations should be openly accessible. Details regarding the microarray design, the synthesis verification, and all quality control (QC) steps taken to validate and assess the performance and reproducibility of the microarray should be documented and provided by the manufacturer. Additional information may be found in the American College of Medical Genetics and Genomics recommendations for the design and performance expectations for clinical genomic copy-number microarray devices.[28]

Microarrays should be designed with consideration of the statistical algorithms to be used for determining abnormal thresholds. The number and density of probes within a given region of interest, i.e., within a region known to be associated with a cancer gene or feature, should provide the sensitivity needed for detection of a copy-number variation.

VERIFICATION AND VALIDATION OF HARDWARE, SOFTWARE, REAGENTS, AND PROCESSES
Definitions
Verification. Verification is a confirmation, through provision of objective evidence, that specified requirements have been fulfilled. This is a one-time process completed to determine or

confirm test performance characteristics before the test system is used for patient testing. Verification is a quality assurance process to determine that instruments, software, and associated data are accurate per the manufacturer's description and specifications, i.e., does the system (hardware, software, probes) function as described by the vendor/manufacturer?

Validation. Validation is a confirmation through the provision of objective evidence that requirements for a specific intended use or application have been fulfilled. Validation is a QC process to determine that the data from test samples are accurate for the intended use when compared with a validated method, i.e., does the system (processes) provide the correct (accurate, reproducible) result(s) when test samples or test data are analyzed?

Platform
Initiation of microarray technologies requires the laboratory verify that the instrumentation, software, and probes perform as specified by the vendor. All platforms intended for clinical testing must be verified and validated. The method and scope of the verification and validation must be documented. A new platform is defined as any new methodology or microarray type introduced into the laboratory. A single microarray vendor may produce multiple similar platforms, but each must be assessed independently. A new version is defined as a minor modification to probe coverage, either through manufacturing of the microarray or by *in silico* probe filtering.

Laboratory with little or no experience with microarray technologies
The laboratory with little or no experience with microarray technology should become familiar with all aspects of the new technology through the verification process, consultation with vendor support, and if possible, other laboratories with demonstrated proficiency using the same platform before beginning the validation process. Familiarization includes understanding the processes, features, and capabilities of the technology selected. The laboratory should gain experience with the instrumentation, platform design, software, reagents, methodologies, technological limitations, workflows, and DNA quality parameters by experimental sample runs. Similarly, the laboratory should become familiar with the features of each sample type the laboratory will process.

It is strongly suggested that laboratories use a combination of data from well-characterized controls and/or data from public databases to gain and broaden their experience. Sample exchanges with a laboratory proficient with a similar microarray platform can provide a good source of samples for validation. Exchange of validated data sets between laboratories provides additional experience in data analysis. Samples chosen for validation studies should have aberrations that challenge the technical limits of detection for reportable deletions and duplications.

Laboratories must be able to recognize nonperforming probes, technically induced artifact and quality issues.

Laboratories should become familiar with benign and/or common CNVs and resources to aid in recognition and interpretation of CNVs, whether in a constitutional or neoplastic setting.

The laboratory should demonstrate expertise in technical aspects of the processing of sample types to be used for clinical testing, technical performance of the microarray, reproducibility of results, and data analysis and interpretation. Expertise should be documented for each microarray platform used for clinical testing, regardless of whether the laboratory has prior experience with a different platform.

New platform
A minimum of 30 samples should be processed and interpreted by the laboratory to verify and validate any new platform. This includes changing to a platform of the same type from a different manufacturer or a different platform type, e.g., array comparative genomic hybridization to SNP. Samples with known abnormalities should be used to gain expertise with the new methodology and assess performance.

New/different version of an established platform
Analysis of a minimum of five known abnormal samples should be run on a new platform version. Data from a new version should be compared with data from the established version to determine if the platform and software perform as expected to detect known CNVs. New probe additions for enhanced coverage or improved performance should be investigated with samples known to have variation in the region of new content (when possible).

New versions of established platforms will vary with the manufacturer and platform type. A manufacturer may define minor upgrades as new versions. There are no definitive criteria for a new version; however, a different version should be limited to minimal probe changes, e.g., removal and/or replacement of probes to improve performance and/or coverage over a limited number of genomic regions. These types of changes to an established platform are likely to be rare, with most changes of platforms requiring a full validation.

Validation of a new clinical test or assay
Any assay intended for clinical diagnosis must be verified and validated before offering as a clinical test. Proficiency in test performance, analysis, and interpretation must be demonstrated.

It is understood that the microarray platform employed by the laboratory may be used to analyze multiple sample types and multiple neoplastic disorders. Inherent differences in obtainable results from different biological materials require that the laboratory determine the performance characteristics of the microarray for each sample type, e.g., bone marrow/blood, fresh or frozen tissue/tumor, formalin-fixed paraffin-embedded (FFPE) tumor, to be used for clinical testing. A surgical pathologist should be involved in the collection of optimal solid tumor samples to ensure a minimum of 25% tumor in the sample.

Laboratories that plan to offer clinical testing for different neoplastic disorders using different sample types should

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prepare by processing and analyzing a sufficient number of each type to establish proficiency. Disease-specific samples for which clinical testing will be offered should be included in the validation sample collection. The laboratory should run technical replicates of multiple samples during the validation process to ensure that the assay results are accurate and reproducible. Discrepancies between replicates should be investigated and documented.

Each laboratory should use judgment and experience to determine the number of samples of a particular type of neoplastic disorder to include in their preclinical testing validation. Laboratories will also need to use judgment and experience to determine differences and issues of processing various sample types and adjust sample numbers of each type accordingly, with the goal of optimizing quality and analytic interpretation of results.

Sample assays for a specific diagnosis may be validated by comparison of results with those obtained by other methods, e.g., conventional cytogenetics, FISH, or another validated microarray assay. During the validation process, all genomic imbalances identified by standard method(s) should be detected by the microarray within the limits of clonality detection levels established by the laboratory for the diagnosis or sample type. Reportable abnormalities, e.g., CNVs or LOH detected by microarray but not by cytogenetic analysis, should be confirmed by another method, e.g., multiplex ligation-dependent probe amplification (MLPA), quantitative PCR (qPCR), FISH, or a different microarray platform, during the validation process to gain sufficient expertise and confidence in data interpretation.

Exchanging samples with another laboratory conducting similar assays in a blind, split-sample comparison using both normal and abnormal samples and comparing results at the appropriate detection levels declared by the laboratories can provide valuable feedback during the validation process. After the validation period, sample sharing can be used for external proficiency testing (PT). All validation data for each disease and sample type, including discordant results and limitations, should be documented.

Clonality detection and limits

Samples from neoplastic disorders can be expected to have varying amounts of nonneoplastic cells admixed with neoplastic cells. The proportion of clonal and nonclonal cells may or may not be clinically relevant but will affect assay sensitivity. Detectable clonality can be influenced by several factors including microarray platform used, sample source, DNA quality, size and copy-number state of the abnormality, and probe coverage. Noise from poor-quality DNA may mask clonality. Each laboratory will need to challenge their microarray with mosaic, aneuploid, and clonally diverse samples to gain experience in their detection. The various factors should be considered with data analysis.

Visual inspection and manual review of the data should be employed to detect clonality and gain experience with data interpretation. The software may not flag low-level clonality. A

call made by visual/manual inspection, when the call was not made by the software, should be verified by another method, e.g., interphase FISH, qPCR.

Determination of levels of detectable clonality

Methods to evaluate levels of detectable clonality will differ with sample type, e.g., fresh, fixed, or FFPE. Dilution studies are one method that may be used to create different levels of clonality for test purposes.[29] Flow cytometric analysis and interphase FISH analysis of fresh (uncultured) samples provide reliable methods for confirmation of clonality level(s). Conventional cytogenetic analysis of metaphase cells provides information about clonal populations but does not reliably reflect levels of clonality.

Dilution studies for SNP arrays require nonneoplastic and tumor DNA from the same patient. Buccal cells or blood may provide a source of nonneoplastic patient DNA.

Assessment of levels of neoplastic to nonneoplastic cells or sizes of different clonal populations in fresh or fixed (FFPE) tissue samples is more difficult. Dissection of fresh tumor with an inverted microscope can reduce the amount of nonneoplastic tissues. Microdissection of FFPE tumors can enrich the DNA sample for tumor. Estimation of clonality in tumor tissue samples can be useful when analyzing data from these tumor types.[11,29]

Determination of ploidy

Polyploidy may be detected by microarray analysis but may be difficult to appreciate. The allelic states of SNP probes can assist in determining ploidy levels. The validation process should include samples with varying levels of ploidy to gain experience in analysis and recognition of different ploidies. The manufacturer should provide the method used for normalization. The laboratory must understand the effect that normalization may have on polyploidy detection and subsequent interpretation of gains and losses in the context of polyploidy.

Clonal diversity

Clonal diversity, common to neoplastic disorders, should be visible by microarray when the cell populations of different clones reach the threshold for detection. However, determination of the composition of clones or the sequence of progression of clonal evolution will not be possible. Correlation with conventional cytogenetic analysis may facilitate interpretation of the microarray results.

Software experience and evaluation

Software may not be specifically designed for analysis of cancer specimens. Laboratories may choose to design their own software programs or modify parameters of the platform's standard software program. The laboratory should recognize software limitations and the need for manual and visual inspection of the data for aberration and clonality detection.

A comprehensive evaluation of any software to be used to analyze microarray data should be performed. The laboratory

must determine and document the ability of the software to define accurately the limits of copy-number variations, i.e., deletions, duplications, and/or amplifications, according to software rules and parameters. When applicable, the laboratory should also determine the ability of the assay to define the end points of copy-neutral ROHs according to the software settings. Limits should be reestablished whenever the microarray platform, probes, software, or analysis rules change. The laboratory should challenge the software with a variety of aberrations, especially copy-number variations that help define the limits of detection. The limits, rules, and parameters for detection of clonality should be determined. The laboratory should document the software parameters and rules used in the analysis of the microarray, as well as all limitations of the analysis program.

REFERENCE DNA

Comparative genomic hybridization–based microarray analysis requires comparison of sample DNA to reference DNA. Selection of an appropriate reference DNA is essential. Constitutional DNA from blood or normal tissue from the same individual may be used. Constitutional patient DNA will mask constitutional CNVs and reduce the complexity of postanalytic interpretation. However, novel underlying germline abnormalities that could contribute to disease will not be detected.

Laboratories may establish their own reference DNAs. Reference DNA may be from a set of normal individuals with common CNVs identified for a specific type of microarray. The laboratory should characterize any reference DNA to identify CNVs that may have an effect on the interpretation of patient data.

Male and female controls should be established. Laboratory policies should detail how reference DNAs will be used, i.e., for mismatched opposite-sex or same-sex comparisons, as single male or single female references, or as pools from multiple male or multiple female DNA samples. The laboratory should document the rationale for the use of reference DNA types and have provisions for use in different situations. The advantages and limitations of different approaches should be understood and considered during interpretation of data.

Each new reference DNA or new lot of purchased reference DNA for array comparative genomic hybridization should be compared by microarray analysis to the previous lot of reference DNA.

SNP-based microarray analysis requires comparison of the sample result with established references or an *in silico* reference library. If sufficient data are available for a control population, a laboratory may establish its own *in silico* reference that mimics the typical study population. New reference data should be established for new SNP-based array designs.

QUALITY CONTROL

Identification

For each microarray, the slide ID, sample sex, control sex (when appropriate), and sample-tracking control (for multiplex microarrays) should be verified. Discrepancies in the documentation from the physical sample should be investigated and resolved before processing.

Sample requirements

The laboratory should establish parameters for the minimum DNA quality and quantity requirements for each sample type used for clinical testing. The laboratory should demonstrate proficiency in sample preparation, DNA extraction, and DNA purification for each sample type. Fresh or frozen tumor tissue is preferable to fixed tumor tissue for quality. FFPE tumor samples should be evaluated by a surgical pathologist to assess the quality and quantity of tumor in the sample used for microarray analysis. A minimum of 25% tumor is recommended to prevent masking of clonal changes by normal tissue DNA.

DNA extraction, purification, measurement, and amplification with different sample types

DNA extraction methods should ensure the highest-quality DNA possible from the sample type(s) tested by the laboratory. Samples from neoplastic disorders present unique challenges for generating high-quality, tumor-specific DNA. Written protocols should be available in the laboratory procedure manual and/or quality management program for optimizing DNA extraction and labeling, DNA quantification (e.g., fluorometer, spectrophotometer), DNA quality and concentration (e.g., examination by gel electrophoresis), DNA fragmentation (e.g., via sonication or digestion), fluorescent labeling (e.g., examination by gel electrophoresis, visual inspection, ultraviolet/visible spectroscopy), and amplification (e.g., significant increase in product). For any labeling method, acceptable ranges should be determined for proper dye incorporation. Protocols for optimization, e.g., reextraction, repurification, tumor cell enrichment for hematological samples (cell sorting or concentration), and/or microdissection for paraffin-embedded tumor, should be available as appropriate. Laboratories should be aware that fixatives other than formalin may influence DNA quality and that decalcification of bony tumors may adversely affect DNA quality.

Suboptimal samples

The laboratory should establish sample adequacy requirements. Samples that do not meet the laboratory requirements should be rejected with a repeat sample requested from the referring physician.

When a repeat sample is not available, whole-genome amplification may be a reasonable alternative if the laboratory has expertise with the method and if potential biases inherent in the technique are detailed in the report. Laboratory policies and protocols should describe when and how whole-genome amplification is performed.

Equipment calibration, maintenance, and QC

Equipment, instrumentation, and methodologies employed during the validation and use of microarray platforms should be calibrated, receive regular maintenance, and be monitored for QC. Quality metrics should be established for each step of the assay.

QC metrics

Every microarray platform has defined quality metric values, e.g., adequate dye incorporation and/or amplification, fluorescence intensities variance, signal-to-background-noise ratio, and SD or error. Standard cutoff values and acceptable limits should be established for these metrics to ensure that the generated results are reliable and sufficiently precise to be used for a clinical assessment. Quality metrics should be monitored for DNA labeling, hybridization efficiency, data generation and analysis, and other platform-specific parameters. QC metrics should be incorporated into the laboratory quality assurance and quality improvement programs to monitor analytical variables.

Microarray content

It is not feasible for a laboratory to validate the identity and copy-number responsiveness of every probe on a microarray. The laboratory should obtain documentation from the microarray manufacturer that the probes on each microarray are the intended sequence, located appropriately by the software, empirically selected for appropriate copy-number responsiveness and/or SNP allele specificity, and stable for these assessments from lot to lot.

Data quality

Detection of genomic aberrations is dependent on the size of the DNA targets, the probe density, the probe performance, and the distance between the sequences naturally located on the chromosome. The quality of the data will affect the ability to detect genomic aberrations; thus, the laboratory needs to understand the within-array metrics provided by the analysis software and how each metric reflects the quality of the data. One metric that provides a measurement of noise or random variance unrelated to genomic location in the data is the derivative log ratio. The derivative log ratio is the difference between the log ratio values of consecutive probes (derivative log ratio spread), i.e., the spread of the derivative log ratio values after outlier rejection. For SNP arrays, quality may be assessed using data from such parameters as call rates and variability (spread) of allele frequency.

Other features to be kept in mind when assessing copy-number changes are the appropriate log ratio difference between patient and control samples, presence of nonrandom contiguous probe behavior, sharp copy-number state transitions, and supportive SNP allele states (when applicable). The software manufacturer should provide confidence metrics for a copy-number call or SNP allele state/genotype based on the algorithms used by the software for aberration calls.

The laboratory should establish acceptable ranges for each QC metric chosen to assess data quality. The manufacturer often provides these ranges; however, the laboratory may want to modify these ranges based on their experience with the microarrays during the validation process. The laboratory should establish criteria for next steps should the data fall outside of these established ranges.

Custom and public annotations/databases are integral to data analysis. Because these annotations are critical for interpretation, it is important that these tools are carefully constructed and applied by the software manufacturer. Manufacturers should provide updates to these annotations as they become available. The laboratory should check any inconsistencies with an additional data source, e.g., compare results from the University of California, Santa Cruz (UCSC) genome browser with those from the Ensembl browser. Custom annotations from the laboratory should be verified.

Laboratories should ensure that the software manufacturer provides documentation and safeguards such that data are processed and summarized in a consistent manner for every clinical analysis. Most analysis software provides a hierarchy of users with customizable permissions, which enables the laboratory to prevent modification of analysis settings so that all specimens are analyzed consistently. Any changes to data processing should be validated and documented.

Verification of new lots of microarrays and/or reagents

Verification should ensure that new lots of microarray slides and/or reagents perform in the same manner as the previous lot. The manufacturer should supply documentation of the QC comparison between lots of microarray slides, e.g., oligo synthesis verification, accuracy of SNP calls, or other defined control parameters. A new lot of microarray slides should be tested to ensure equivalency by testing, either before or concurrently with new patient specimens, preferably using a patient specimen with an abnormal result that has been tested on a previous lot. Manufacturers may include a normal control and request that it be run. New lots of reagents, e.g., new labeling kits and consumables, should have documented equivalency between runs. This may be accomplished by documenting that the QC metrics meet certain set parameters for the new lot of reagents.

QUALITY ASSURANCE

Laboratory accreditation and personnel qualifications

Laboratory personnel must have documentation of education, degrees, and certifications as appropriate for the level of testing, as well as training, competency assessments, and continuing education as required by appropriate regulatory bodies, e.g., College of American Pathologists (CAP), Clinical Laboratory Improvement Amendments (CLIA), Center for Medicare and Medicaid Services (CMS). The testing laboratory must have CLIA certification and state certifications as required to provide clinical testing. CAP accreditation is strongly encouraged.

Indications and ordering for microarray analysis of neoplastic disorders

Microarray analysis of tumors should be limited to specimens that contain ample tumor, e.g., diagnostic or relapse. The sample should be accompanied by an appropriate indication for the test. Clinical testing should be limited to neoplastic disorders for which unbalanced genomic anomalies are well documented to have diagnostic, prognostic, and/or therapeutic implication(s).

Microarray analysis is not indicated for tumor surveillance or detection of minimal or residual disease because of insensitivity of the test for low levels of disease. Alternative methods should be recommended to monitor patient response to treatment and for residual disease detection, e.g., FISH, qPCR. A clonal abnormality identified and confirmed at diagnosis may be used for follow-up. The same method used for confirmation, e.g., qPCR or FISH, is recommended for use in follow-up studies. Alternatively, DNA or cells may be saved and used as a control when follow-up samples are assessed for residual disease.

Laboratories may facilitate appropriate ordering by providing a directive or disease-specific testing menu. The test requisition should provide sufficient clinical and/or pathological information for the laboratory to assess the appropriateness of the test order.

Proficiency testing (PT)
The laboratory should participate in PT for sample types and tumor types that are included in the laboratory test menu by participating in an external PT program when available through an appropriate-deemed organization, e.g., CAP. In addition, the laboratory may establish external PT of normal and abnormal specimens by the exchange of DNAs, in a blinded manner, with another laboratory performing microarray testing for neoplastic disorders.

The laboratory should also establish internal PT of normal and abnormal samples as part of the laboratory internal quality assurance program and ongoing quality improvement program. Correlation between microarray results run in parallel on different microarray platforms or correlation of microarray results with conventional cytogenetic and/ or FISH results may be sufficient to provide ongoing proficiency. PT should be performed according to the CLIA '88 guidelines.

Documentation of participation and the performance results of internal and external PT must be retained by the laboratory and made available to all accreditation agency inspectors.

Failure to achieve agreement on external or internal proficiency tests should be documented and followed by investigation of the discrepancy with resolution. If indicated, appropriate remediation should be undertaken.

Turnaround time
Laboratory policies should define acceptable standards for microarray analysis test prioritization and turnaround times. Turnaround time should be clinically appropriate so the results are available for patient care management decisions.

It is suggested that 90% of cases should have a final written report by 21 calendar days. A longer turnaround time is acceptable when custom probes, oligos, or primer sequences must be designed, ordered, validated, and used. Normal or preliminary abnormal results should be available within 14 calendar days.

Documentation of problems
A logbook, database, or sample processing form should be created and used to track problems that may occur throughout the processing of samples for neoplasia, from sample intake to final report, e.g., sample adequacy and/or errors. Data from the QC metrics program can provide information for oversight of all processes. Ongoing collection of sample or process variances allows patterns or trends to be recognized and promptly addressed.

ANALYSIS OF DATA INCLUDING ANALYTICAL SOFTWARE ALGORITHMS
Analytical software algorithms differ between platforms. Microarray software is designed to determine gain, loss, or long ROHs in a chromosomal region. Most software manufacturers provide standard algorithms to set cutoff values for calls. However, each laboratory should thoroughly test the rules or filters during the validation process and determine the parameters for cutoff values, e.g., the number of consecutive probes deleted or amplified and the log_2 ratio to call a CNV, depending on probe density. It may be necessary to set different parameters for different chromosome regions or specific genes of interest while keeping in mind the potential for a false-positive call.

Ratio values for mosaic cases will be less than expected for nonmosaic cases and may fall below the standard cutoff value. Clonality may be apparent by visualization or by examination of the moving averages across the chromosomes. The sensitivity of the microarray for detection of clonality should be determined during the platform validation process.

The laboratory must be familiar with the principles of the software program for any platform used. However, the laboratory should never depend solely on the software for analysis. A visual inspection of the moving average across each chromosome and a review of the allele frequency for SNP arrays should be done to identify appropriate and inappropriate results for the disorder being tested. Analysis should be continued until all inconsistencies are resolved.

RESULTS EVALUATION AND INTERPRETATION
The laboratory should be consistent in the analysis, interpretation, and reporting of microarray results. The laboratory should have a record of and be familiar with the microarray coverage, including known cancer-associated genes and regions, benign and/or common population CNVs, and common genetic disorders caused by genomic CNVs and/or LOH.

Systematic evaluation and interpretation of DNA microarrays
The laboratory should establish the methods for microarray result analysis and interpretation using the following recommendations.

Disease-associated genetic aberrations
Analysis and interpretation of microarray data from a neoplastic disorder should take into account the working diagnosis, the clinical information provided, and other disorders in the

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differential diagnosis. The indication or working diagnosis may prove to be incorrect after the diagnostic workup is complete; thus, the laboratory should be aware of other disorders that may be in the differential.

The laboratory should be familiar with recurrent, clonal aberrations associated with particular diagnoses. In addition, the laboratory should be familiar with specific genes known to be pathogenic or to contribute to the pathogenesis of a particular disorder. The medical literature should be used to stay abreast of current disease-specific genetic aberrations, as well as the diagnostic, prognostic, and therapeutic significance of aberrations.

CNV interval size and cancer-associated genes
The size of a CNV is relevant, as larger CNVs encompassing multiple genes are more likely to have a clinical impact; however, very small CNVs that interrupt or delete an established cancer-associated gene may be clinically significant. A single laboratory-established CNV size cutoff or threshold for determination of inclusion of a CNV in a clinical report should not be used as the sole determinant of a call. The laboratory should establish methods for detection of clinically significant CNVs that fall below laboratory-established thresholds, particularly in regions of known cancer-associated genes.

Genomic content in CNV interval
The genomic content of the CNV should be carefully examined for genes relevant to disorders in the differential diagnosis, gene-rich sequences, or genes known to have a clinical association. CNVs encompassing known oncogenes or tumor suppressor genes may have significance, although the implications of the CNV for the particular disorder or patient being studied may not be clear based on current literature.

Copy-number-neutral ROHs detected by SNP analysis
Thresholds or minimal criteria to identify clinically important ROHs consistent with LOH (LOH or AOH) should be established. ROHs associated with parental consanguinity or uniparental disomy should be distinguished from acquired LOH. Distinction of acquired versus constitutional AOH may be facilitated by detection of the clonal aberration in affected tissue (acquired LOH) and/or detection (or not) of the aberration in unaffected tissue (constitutional LOH). Homozygosity in a region that contains a tumor suppressor gene may be associated with an inherited cancer predisposition syndrome. Constitutional analysis should be recommended as appropriate.

Comparison of CNV to internal and external databases
Public databases and the medical literature should be used in determining the significance of CNVs. Available databases include (all last accessed 26 January 2013) the following:

- Database of Genomic Variants (http://projects.tcag.ca/variation/),

- Online Mendelian Inheritance in Man (http:www.ncbi.nlm.nih.gov/omim/),
- DECIPHER (http:www.sanger.ac.uk/research/areas/),
- dbVar—database of Structural Variation (http:www.ncbi.nlm.nih.gov/dbvar),
- dbGaP—database of Genotypes and Phenotypes (http:www.ncbi.nlm.nih.gov/gap),
- Memorial Sloan-Kettering Cancer Center (http://cbio.mskcc.org/CancerGenes),
- The Cancer Genome Anatomy Project (http:www.ncbi.nlm.nih.gov/ncicgap/),
- UCSC Genome Bioinformatics (http://genome.ucsc.edu/cgi-bin/hgGateway),
- The Cancer Genome Atlas (http://cancergenome.nih.gov/),
- Ensembl (http://uswest.ensembl.org/Homo_sapiens/Gene/Summary),
- The International Standards for Cytogenomics Arrays Consortium (https://www.iscaconsortium.org/), and
- Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Laboratories should document pathogenic CNVs, CNVs of uncertain significance, benign CNVs, and CNVs thought or determined to be constitutional. The intralaboratory data should be used along with external data as a reference for interpretation of data from new studies.

Categories of clinical significance
Using the guidelines outlined above for systematic investigation of a CNV for clinical significance, it is recommended that the interpreting laboratory geneticist use the following categories for reporting. Consistent terminology will facilitate unambiguous communication of clinical significance. Taking into account that tumors may be genetically complex, it may not be feasible to provide a detailed interpretation of every CNV and/or AOH region detected. In such cases, a narrative to describe variants and their clinical significance and interpretation should be provided to communicate the desired information. When feasible, the laboratory should provide details of specific CNV and AOH anomalies.

Pathogenic
Acquired. The CNV is a documented clinically significant and/or disease-associated clonal genetic aberration.

Constitutional. Microarray analysis will inevitably reveal common benign and rare constitutional CNVs. Rare constitutional CNVs should be noted and investigated for clinical significance, e.g., cancer-predisposing gene aberration and/or deletion or duplication associated with a known constitutional syndrome. Evaluation and reporting of constitutional CNVs should follow the guidelines set forth in the American College of Medical Genetics and Genomics Standards and Guidelines for interpretation and reporting of constitutional CNVs.[30]

Uncertain clinical significance

This category may include CNVs that are not known to be associated with disease but meet the reporting criteria established by the laboratory. A CNV in this category is not clearly pathogenic, and there is insufficient evidence for an unequivocal determination of clinical significance. The laboratory should interpret novel CNVs in light of the available clinical and/or pathological information and current literature. Reporting CNVs of uncertain significance is at the discretion of the laboratory. If reported, they may be categorized as follows:

Uncertain clinical significance, acquired, likely pathogenic. Many neoplastic disorders have well-recognized and/or well-characterized aberrations. However, microarray resolution may reveal uncharacterized CNVs. If reported, the discussion should avoid speculation as to the pathogenicity or clinical significance of the CNV without supporting evidence.

Examples: (i) A CNV described in a single case report of a similar neoplasm. (ii) A CNV with a gene in the interval that has potential or relevant function as an oncogene or tumor suppressor gene or that belongs to another known gene family that has an association with neoplastic processes but not the neoplastic process being studied. (iii) A CNV that appears related to the clonal neoplastic process being studied by having a similar $\log_2$ ratio as the clonal process being studied but is not a recognized aberration. Evolution and heterogeneity of a clonal neoplastic process is common. Microarray is likely to reveal new, but uncharacterized, aberrations that may be reported in this category.

Collection of the data of CNVs of uncertain significance is encouraged to build a database for intralaboratory reference, for correlation with clinical parameters, and for sharing in publications. The eventual understanding of the clinical significance will depend on accumulation of sufficient information and correlation with clinical features.

Uncertain clinical significance, likely constitutional. Refer to the American College of Medical Genetics and Genomics Standards and Guidelines for interpretation and reporting of constitutional CNVs.[30]

Uncertain clinical significance, not otherwise specified. A CNV that meets the laboratory parameters for reporting but has no features to categorize it further.

Benign. Reporting of benign CNVs is at the discretion of the laboratory. The laboratory should be familiar with common benign CNVs, stay current with the literature, and interpret results with this knowledge. This category will include: (i) CNVs reported in multiple peer-reviewed publications or curated databases as a benign variant and (ii) CNVs without relevant genetic content that meet criteria for reporting. It should be recognized, however, that cancer-associated anomalies that occur in known variant regions might not be benign.

REPORTING GUIDELINES FOR MICROARRAY ANALYSIS OF NEOPLASTIC DISORDERS

The following guidelines describe the elements of the clinical report that are necessary to communicate clearly and completely the clinical significance of microarray analysis results.

Reporting criteria

Microarray reports should be written so the result is understandable to a nongeneticist health-care provider and so that the clinical significance of the result for patient management is clear.

Care and special consideration should be given to reporting of certain results in children, e.g., disease-predisposing genes and adult-onset disease–associated genes.

To the extent possible, the current International System for Human Cytogenetic Nomenclature should be used to describe known recurrent, disease-associated, or clinically significant aberrations. FISH and chromosome studies used for confirmation analysis should be described using this nomenclature, which provides a format to report microarray results with the nucleotide boundaries for copy-number gains or losses. Breakpoints should be given to the extent possible given the employed technology.

The laboratory may choose to list relevant genes within the altered region. The specific genome-build nucleotide numbering should be specified, e.g., February 2009 assembly, NCBI37/hg19.

Verbal discussion of microarray results with the health-care provider is encouraged to facilitate communication and understanding of microarray results and clinical significance.

Written report

The written report should include the following:

1. Case identification with at least two unique patient identifiers: patient name, date of birth, or other unique identifier, e.g., medical record number.
2. Laboratory accession number(s), date of collection and/or receipt of specimen, specimen type, and name(s) of physician(s) or authorized persons to whom the report is to be provided.
3. Indication for the study, e.g., clinical information or diagnosis and/or pathological diagnosis.
4. List of specific CNVs with the following information when relevant:
   - Chromosome location (chromosome number and band designation),
   - LOH and CNVs with linear coordinates and genome build,
   - Genes of potential significance within interval(s), when indicated,
   - Dosage (copy-number loss, gain, amplification with confirmed ploidy/normalization), and
   - Clonality or ploidy, if applicable.
5. Confirmation testing method(s) and results, when applicable, and a statement of additional analyses performed to resolve questions of clonality, as appropriate.

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6. Narrative interpretation to correlate the microarray result with patient-specific clinical or laboratory information, e.g., histopathology, immunophenotype, and/or flow cytometric data. The discussion should include the clinical significance of the results for the diagnosis, prognosis, and/or therapeutic management of the patient with reference to current literature. A note/disclaimer should be included to encourage clinicians to consider the results/data along with other laboratory tests, clinical findings, and recent literature.
7. Clinically significant constitutional CNVs should be discussed with recommendations for further testing as appropriate.
8. If a CNV of uncertain clinical significance is reported, a discussion of the possible relationship or significance to the diagnosis with supporting literature should be provided.
9. References as appropriate for the interpretation and that provide helpful information for the health-care provider.
10. Documentation of date of verbal communication of preliminary or final results to health-care provider(s) with notes regarding discussion of acquired and/or constitutional CNVs or abnormalities and the clinical significance, as appropriate.
11. Recommendation(s) for additional testing as appropriate.
12. Recommendation(s) for genetic counseling as appropriate.
13. Technical information for the testing platform and software, e.g., commercial source, coverage, version, and National Center for Biotechnology Information (NCBI) build used for data analysis. Limitations of the testing platform, e.g., detection of LOH, balanced rearrangements, ploidy, and/or low-level clonality. Biases and limitations of whole-genome amplification when appropriate. Methods summary including criteria for calls, e.g., minimum number of consecutive probes and/or length of area of LOH.
14. Qualified individuals must sign all final reports. Password-protected electronic signatures can be used fulfill this requirement.
15. Date of final report.
16. Disclaimers as appropriate, e.g., when and what investigational procedures are employed. Disclaimers as required.

CONCLUSIONS

Each new technological development in the field of genetics brings with it the desire to apply the technology to improve medical care. The transition of a new technology from the research bench into the clinical realm of diagnostic testing must be accompanied by extensive clinical validation to ensure the results reported to the health-care provider are accurate and reliable for use in patient-care decision making. The validation involves extensive comparison to the existing trusted methodologies to demonstrate that the new method has reliable and consistent results and interpretation. Sufficient comparative data must be accumulated and evaluated before the new method becomes a first-tier method. When the new

technology provides additional information that is unattainable by the existing method, data accumulation and correlation with clinical parameters can expand the benefit provided by the new technology.

Microarray technologies provide a high-resolution view of the whole genome, which may yield massive amounts of new information. Medical laboratory professionals must be prepared to identify, interpret, and report results with clinical relevance while being mindful of the social, ethical, and legal responsibilities of reporting genetic information. Interpretation of the data from microarrays into clinically relevant information is a difficult and complex undertaking and is the practice of medicine. No algorithm for CNV interpretation can substitute for adequate training and knowledge in the fields of oncology, pathology, and medical genetics. Individuals with appropriate professional training and board certification, i.e., American Board of Medical Genetics clinical cytogenetics, clinical molecular genetics, or molecular genetic pathology should provide the interpretation of genomic microarrays for the clinical investigation of neoplastic disorders.

DISCLOSURE

All authors direct clinical testing laboratories that use the technologies and/or perform tests related to those described in this guideline.

REFERENCES

1. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th edn, International Agency for Research on Cancer: Lyon, France, 2008.
2. Astbury C. *Clinical Cytogenetics in Clinics in Laboratory Medicine*, vol. 31(4). Elsevier Saunders: Philadelphia, PA, 2011.
3. Byrd JC, Mrózek K, Dodge RK, et al.; Cancer and Leukemia Group B (CALGB 8461). Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* 2002;100:4325–4336.
4. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000;343:1910–1916.
5. Helm S, Mitelman F. *Cancer Cytogenetics, Chromosomal and Molecular Genetic Aberrations of Tumor Cells*, 3rd edn. Wiley: Hoboken, New Jersey, 2009.
6. Moorman AV, Harrison CJ, Buck GA, et al.; Adult Leukaemia Working Party, Medical Research Council/National Cancer Research Institute. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. *Blood* 2007;109:3189–3197.
7. Mrózek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev* 2004;18:115–136.
8. Armengol G, Canellas A, Alvarez Y, et al. Genetic changes including gene copy number alterations and their relation to prognosis in childhood acute myeloid leukemia. *Leuk Lymphoma* 2010;51:114–124.
9. Gunnarsson R, Staaf J, Jansson M, et al. Screening for copy-number alterations and loss of heterozygosity in chronic lymphocytic leukemia–a comparative study of four differently designed, high resolution microarray platforms. *Genes Chromosomes Cancer* 2008;47:697–711.
10. Okamoto R, Ogawa S, Nowak D, et al. Genomic profiling of adult acute lymphoblastic leukemia by single nucleotide polymorphism oligonucleotide microarray and comparison to pediatric acute lymphoblastic leukemia. *Haematologica* 2010;95:1481–1488.
11. Slovak ML, Bedell V, Hsu YH, et al. Genomic alterations in Hodgkin and Reed/Sternberg (HRS) cells at disease onset reveals distinct signatures for chemo-sensitive and primary refractory Hodgkin lymphoma. *Clin Cancer Res* 2011;17:3443–3454.

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12. Walter MJ, Payton JE, Ries RE, et al. Acquired copy number alterations in adult acute myeloid leukemia genomes. *Proc Natl Acad Sci USA* 2009;106:12950–12955.
13. Yu L, Slovak ML, Mannoor K, et al. Microarray detection of multiple recurring submicroscopic chromosomal aberrations in pediatric T-cell acute lymphoblastic leukemia. *Leukemia* 2011;25:1042–1046.
14. Bungaro S, Dell'Orto MC, Zangrando A, et al. Integration of genomic and gene expression data of childhood ALL without known aberrations identifies subgroups with specific genetic hallmarks. *Genes Chromosomes Cancer* 2009;48:22–38.
15. Carrasco DR, Tonon G, Huang Y, et al. High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. *Cancer Cell* 2006;9:313–325.
16. Gunn SR, Mohammed MS, Gorre ME, et al. Whole-genome scanning by array comparative genomic hybridization as a clinical tool for risk assessment in chronic lymphocytic leukemia. *J Mol Diagn* 2008;10:442–451.
17. Hagenkord JM, Gatalica Z, Jonasch E, Monzon FA. Clinical genomics of renal epithelial tumors. *Cancer Genet* 2011;204:285–297.
18. Paulsson K, Forestier E, Lilljebjörn H, et al. Genetic landscape of high hyperdiploid childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 2010;107:21719–21724.
19. Rücker FG, Bullinger L, Schwaenen C, et al. Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. *J Clin Oncol* 2006;24:3887–3894.
20. Usvasalo A, Elonen E, Saarinen-Pihkala UM, et al. Prognostic classification of patients with acute lymphoblastic leukemia by using gene copy number profiles identified from array-based comparative genomic hybridization data. *Leuk Res* 2010;34:1476–1482.
21. Gorletta TA, Gasparini P, D'Elios MM, Trubia M, Pelicci PG, Di Fiore PP. Frequent loss of heterozygosity without loss of genetic material in acute myeloid leukemia with a normal karyotype. *Genes Chromosomes Cancer* 2005;44:334–337.
22. O'Keefe C, McDevitt MA, Maciejewski JP. Copy neutral loss of heterozygosity: a novel chromosomal lesion in myeloid malignancies. *Blood* 2010;115:2731–2739.
23. Kawamata N, Ogawa S, Zimmermann M, et al. Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray. *Blood* 2008;111:776–784.
24. Hagenkord JM, Monzon FA, Kash SF, Lilleberg S, Xie Q, Kant JA. Array-based karyotyping for prognostic assessment in chronic lymphocytic leukemia: performance comparison of Affymetrix 10K2.0, 250K Nsp, and SNP6.0 arrays. *J Mol Diagn* 2010;12:184–196.
25. Kallioniemi A. CGH microarrays and cancer. *Curr Opin Biotechnol* 2008;19:36–40.
26. Maciejewski JP, Tiu RV, O'Keefe C. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. *Br J Haematol* 2009;146:479–488.
27. Watson SK, deLeeuw RJ, Horsman DE, Squire JA, Lam WL. Cytogenetically balanced translocations are associated with focal copy number alterations. *Hum Genet* 2007;120:795–805.
28. Kearney HM, South ST, Wolff DJ, Lamb A, Hamosh A, Rao KW; Working Group of the American College of Medical Genetics. American College of Medical Genetics recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities. *Genet Med* 2011;13:676–679.
29. Nowak NJ, Miecznikowski J, Moore SR, et al. Challenges in array CGH for the analysis of cancer samples. *Genet Med* 2007;9(9):585–595.
30. Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. *Genet Med* 2011;13:680–685.

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Prenatal Screening

<center>**DOCUMENTS RELIED UPON**</center>

The following documents were relied upon in developing these regulations:

American Academy of Pediatrics and American College of Obstetricians and Gynecologists, 2007. *Guidelines for Perinatal Care (6th ed.)*, Elk Grove Village, American Academy of Pediatrics.*

American College of Obstetricians and Gynecologists, "Screening for Fetal Chromosomal Abnormalities," *ACOG Practice Bulletin – Clinical Management Guidelines for Obstetrician-Gynecologists*, Number 77, January 2007.*

American College of Obstetricians and Gynecologists Committee on Genetics and The Society for Maternal-Fetal Medicine Publications Committee, "Noninvasive Prenatal Testing for Fetal Aneuploidy," *Committee Opinion,* Number 545, December 2012.

Available at:

http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Genetics/Noninvasive-Prenatal-Testing-for-Fetal-Aneuploidy

American College of Obstetricians and Gynecologists Committee on Genetics, "The Use of Chromosomal Microarray Analysis in Prenatal Diagnosis," *Committee Opinion*, Number 581, December 2013.

Available at:

http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Genetics/The-Use-of-Chromosomal-Microarray-Analysis-in-Prenatal-Diagnosis

American College of Obstetricians and Gynecologists Committee on Genetics and The Society for Maternal-Fetal Medicine, "Cell-free DNA Screening for Fetal Aneuploidy," Committee Opinion, Number 640, September 2015.

Available at:

http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Genetics/Cell-free-DNA-Screening-for-Fetal-Aneuploidy

Driscoll, D.A., and Gross, S.J., "First trimester diagnosis and screening for fetal aneuploidy," American College of Medical Genetics Practice Guidelines, *Genetics IN Medicine*, January 2008, Vol. 10, No. 1: 73-75.

Available at:

http://www.acmg.net/StaticContent/SGs/First_trimester_diagnosis_and_screening_for_f etal.10.pdf

Gregg, A.R., Gross, S.J., Best, R.G., Monaghan, K.G., Bajaj, K., Skotko, B.G., Thompson, B.H., and Watson, M.S.; The Noninvasive Prenatal Screening Work Group of the American College of Medical Genetics and Genomics, "ACMG statement on noninvasive prenatal screening for fetal aneuploidy," *Genetics in Medicine*, May 2013;15(5): 395-8.

Available at:

https://www.acmg.net/docs/nips-GiM_galley_text_130301.pdf

*Viewing and Copies:

Upon request, the Department's Office of Regulations will have these documents available for viewing during the public comment period(s).

Prenatal Diagnosis Center Standards and Definitions 2018

American College of Medical Genetics and Genomics, Standards and Guidelines for Clinical Genetics Laboratories, 2018 Edition, Revised January 2018