American College of Medical Genetics and Genomics (ACMG)

TECHNICAL STANDARDS FOR CLINICAL GENETICS LABORATORIES

(2021 Revision)

(For a General Overview of these Technical Standards, including Purpose and Disclaimer, see **Section A**)

E: CLINICAL CYTOGENETICS

Table of Contents

Note: This document contains hyperlinks between the table of content items on the first page and each section. It also contains hyperlinks to the original articles published in *Genetics in Medicine* journal, which are also included in appendices 1 to 8.

E1 Cell Culture

E1.1 Biosafety containment cabinets (Class IIA or IIB) must be used for all cell cultures and for the handling of viable tissues and/or fluids.

E1.2 Incubators

E1.2.1 Two incubators on separate electrical circuits (if no emergency backup power is available) are required for all amniotic fluid and chorionic villi cell cultures. These should have separate CO_2 lines and filters and should have emergency temperature alarms.

E1.2.2 Incubators must be cleaned regularly, and incubators must be monitored for 1) temperature each working day, 2) gas weekly, and 3) humidity, as needed. Maximum- minimum control thermometers are recommended. Appropriate operating ranges for equipment should be established and posted. Protocols should outline steps to be taken when readings are outside of appropriate ranges.

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 **chromosomal microarray data**, see section E10 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 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 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.

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, 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, 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.

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.

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.

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 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.

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 Chromosomal Microarray (CMA) Analysis
Chromosomal microarray analysis, including constitutional and neoplastic disease applications, 2021 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG) **[See Genet Med 2021;23(10):1818-1829 OR Appendix 5]**

This also includes:

- **Interpretation standards for constitutional copy number variants (CNVs):**
  Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen) **[See Genet Med 2020;22(2):245-257 OR Appendix 6]**

- **Interpretation and reporting of large regions of homozygosity and suspected consanguinity:**
  Interpretation and reporting of large regions of homozygosity and suspected consanguinity/uniparental disomy, 2021 revision: A technical standard of the American College of Medical Genetics and Genomics (ACMG) **[See Genet Med 2022;24(2):255-261 OR Appendix 7]**

- **Interpretation standards for acquired copy number abnormalities (CNAs) and copy-neutral loss of heterozygosity (CN-LOH) in neoplastic disorders:**
  Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC) **[See Genet Med 2019;21(9):1903-16 OR Appendix 8]**

E11 This section has been incorporated into Section E10 (see above)

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):845-50.

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 260-1, 319-30, 331-5.

Gardner RJM, Sutherland GR, Shaffer LG (eds). *Chromosome Abnormalities and Genetic Counseling*, 4th Edition 2012. Oxford University Press, pp 260-8.

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.

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, 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.

Mikhail FM, Biegel JA, Cooley LD, Dubuc AM, Hirsch B, Horner VL, Newman S, Shao L, Wolff DJ, Raca G. Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC). *Genet Med* 2019;21(9):1903-1916.

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.

Riggs ER, Andersen EF, Cherry AM, Kantarci S, Kearney H, Patel A, Raca G, Ritter DI, South ST, Thorland EC, Pineda-Alvarez D, Aradhya S, Martin CL. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). *Genet Med* 2020;22(2):245-257.

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.

Shao L, Akkari Y, Cooley LD, Miller DT, Seifert BA, Wolff DJ, Mikhail FM; ACMG Laboratory Quality Assurance Committee. Chromosomal microarray analysis, including constitutional and neoplastic disease applications, 2021 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG). Genet Med 2021;23(10):1818-1829.

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)

APPENDIX 1

Diagnostic cytogenetic testing following positive noninvasive prenatal screening (NIPS) results

(See following page)

ACMG PRACTICE RESOURCES | Genetics inMedicine

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

Athena M. Cherry, PhD[1], Yassmine M. Akkari, PhD[2], Kimberly M. Barr, MS[3], Hutton M. Kearney, PhD[4], Nancy C. Rose, MD[5], Sarah T. South, PhD[6], James H. Tepperberg, PhD[7] and Jeanne M. Meck, PhD[8]; on behalf of the ACMG Laboratory Quality Assurance Committee

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.

Genet Med advance online publication 20 July 2017

Key Words: cell-free DNA; chromosome analysis; chromosomal microarray (CMA); noninvasive prenatal screening (NIPS); noninvasive 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 microdeletion 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

[1]Department of Pathology, Stanford University School of Medicine, Stanford Health Care, Stanford, California, USA; [2]Cytogenetics and Molecular Pathology, Legacy Laboratory Sciences, Legacy Health, Portland, Oregon, USA; [3]Genetics Department, Kaiser Permanente, San Francisco, California, USA; [4]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA; [5]Department of Obstetrics and Gynecology, University of Utah, Intermountain Healthcare University of Utah, Salt Lake City, Utah, USA; [6]Ancestry DNA, Lehi, Utah, USA; [7]Clinical Cytogenetics Laboratory, Laboratory Corporation of America, Research Triangle Park, North Carolina, USA; [8]Cytogenomics, GeneDx, Gaithersburg, Maryland, USA. Correspondence: Athena M. Cherry (acherry@stanfordhealthcare.org)

The Board of Directors of the American College of Medical Genetics and Genomics approved this clinical laboratory practice resource on 24 April 2017.

Submitted 11 May 2017; accepted 11 May 2017; advance online publication 20 July 2017. doi:10.1038/gim.2017.91

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.

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

A.M.C., Y.M.A., H.M.K., J.H.T. and J.M.M. are clinical laboratory directors at their respective institutions and perform the assays described herein as a clinical service. The other authors declare no conflict of interest.

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

ACMG technical standards and guidelines: chromosome studies of neoplastic blood and bone marrow–acquired chromosomal abnormalities

(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

Fady M. Mikhail, MD, PhD[1], Nyla A. Heerema, PhD[2], Kathleen W. Rao, PhD[3,4,8], Rachel D. Burnside, PhD[5], Athena M. Cherry, PhD[6] and Linda D. Cooley, MD, MBA[7]; on behalf of the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee

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.

This updated Section E6.1–6.4 has been incorporated into and supersedes the previous Section E6 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.

Genet Med advance online publication 28 April 2016

Key Words: bone marrow; cancer cytogenetics; clonal chromosomal abnormalities; cytogenetic analysis; hematological malignancies

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

[1]Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA; [2]Department of Pathology, The Ohio State University, Columbus, Ohio, USA; [3]Department of Pediatrics, University of North Carolina, Chapel Hill, North Carolina, USA; [4]Department of Pathology, University of North Carolina, Chapel Hill, North Carolina, USA; [5]Laboratory Corporation of America Holdings, Center for Molecular Biology and Pathology, Research Triangle Park, North Carolina, USA; [6]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA; [7]Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, University of Missouri Kansas City Medical School, Kansas City, Missouri, USA. [8]Deceased. Correspondence: Fady M. Mikhail (fmikhail@uab.edu)

These technical standards and guidelines were approved by the ACMG Board of Directors on 25 January 2016.

Submitted 1 March 2016; accepted 1 March 2016; advance online publication 28 April 2016. doi:10.1038/gim.2016.50

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

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 preferably 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 suboptimal 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 translocations 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 following 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 addition, regions of cnLOH are more often detected in patients with normal karyotypes than with abnormal 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 simultaneously 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 (intrachromosomal 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 cytometry, chromosome analysis, and FISH results, additional 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; however, it provides a clonal target for future monitoring 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 cytometry results are suggestive of B-cell ALL (Burkitt leukemia 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

b. 13q14.2q14.3 probes (including *RB1*): 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

Fady M. Mikhail MD, PhD, Nyla A. Heerema PhD, Kathleen W. Rao PhD, Rachel D. Burnside PhD, Athena M. Cherry PhD and Linda D. Cooley MD, MBA ; on behalf of the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee

Genet Med advance online publication, April 28, 2016; doi:10.1038/gim.2016.50

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

ACMG technical standards and guidelines: chromosome studies of lymph node and solid tumor–acquired chromosomal abnormalities

(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

Linda D. Cooley, MD, MBA[1], Cynthia C. Morton, PhD[2,3], Warren G. Sanger, PhD[4,7], Debra F. Saxe, PhD[5] and Fady M. Mikhail, MD, PhD[6]; on behalf of the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee

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.

Genet Med advance online publication 28 April 2016

Key Words: cancer cytogenetics; chromosome; guidelines; lymph node; solid tumor

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.

[1]Department of Pathology & Laboratory Medicine, Children's Mercy Hospital, University of Missouri Kansas City Medical School, Kansas City, Missouri, USA; [2]Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; [3]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; [4]Department of Pathology, University of Nebraska Medical Center, Omaha, Nebraska, USA; [5]Department of Pathology, Emory University School of Medicine, Atlanta, Georgia, USA; [6]Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA. [7]Deceased. Correspondence: Linda D. Cooley (lcooley@cmh.edu)

Approved by the ACMG Board of Directors on 25 January 2016.

Submitted 1 March 2016; accepted 1 March 2016; advance online publication 28 April 2016. doi:10.1038/gim.2016.51

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 cm^3. 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

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

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

The ACMG Working Group acknowledges Marilu Nelson, Laboratory Supervisor at the University of Nebraska Human Genetics Laboratory in Omaha, NE, for her extensive contribution to Supplementary Table S5 online (lymphomas), Felix Mitelman at the University of Lund in Lund, Sweden, for his review and helpful comments on Supplementary Tables S1–5, and Matthew Meredith, postdoctoral fellow at the Harvard Medical School in Boston, MA, for his help with Supplementary Table S2 (genitourinary tumors). These technical standards and guidelines were approved by the ACMG Board of Directors on 25 January 2016.

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.

APPENDIX 4
ACMG technical standards and guidelines: Fluorescence in situ hybridization
(See following page)

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

James T. Mascarello, PhD[1], Betsy Hirsch, PhD[2], Hutton M. Kearney, PhD[3], Rhett P. Ketterling, MD[4], Susan B. Olson, PhD[5], Denise I. Quigley, PhD[6], Kathleen W. Rao, PhD[7], James H. Tepperberg, PhD[8], Karen D. Tsuchiya, MD[9], and Anne E. Wiktor, BS[4], A Working Group of the American College of Medical Genetics (ACMG) Laboratory Quality Assurance Committee

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 metaphase 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.

From the [1]Genzyme Genetics, Santa Fe, New Mexico; [2]University of Minnesota Medical School, Minneapolis, Minnesota; [3]Fullerton Genetics Center, Asheville, North Carolina, [4]Mayo Clinic College of Medicine, Rochester, Minnesota, [5]Oregon Health and Science University, Portland, Oregon; [6]Kaiser Permanente, NW, Portland, Oregon; [7]University of North Carolina, Chapel Hill, North Carolina; [8]Laboratory Corp America, Research Triangle Park, North Carolina; and [9]Seattle Children's Hospital and University of Washington Medical Center, Seattle, Washington.

James T. Mascarello, PhD, Genzyme Genetics, 2000 Vivigen Way, Santa Fe, NM 87505. E-mail: MascarJ@LabCorp.com.

Disclosure: The authors declare no conflict of interest.

DOI: 10.1097/GIM.0b013e3182227295

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 $\pm$ 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

Chromosomal microarray analysis, including constitutional and neoplastic disease applications, 2021 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

(See following page)

www.nature.com/gim

Genetics inMedicine

Check for updates

ACMG TECHNICAL STANDARD

Chromosomal microarray analysis, including constitutional and neoplastic disease applications, 2021 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

Lina Shao[1], Yassmine Akkari[2], Linda D. Cooley[3,4], David T. Miller[5], Bryce A. Seifert[6], Daynna J. Wolff[7], Fady M. Mikhail[8] and ACMG Laboratory Quality Assurance Committee[9]*

Disclaimer: This technical standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this technical standard is voluntary and does not necessarily assure a successful medical outcome. This technical standard 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 this technical standard. They also are advised to take notice of the date any particular technical standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It would also be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Chromosomal microarray technologies, including array comparative genomic hybridization and single-nucleotide polymorphism array, are widely applied in the diagnostic evaluation for both constitutional and neoplastic disorders. In a constitutional setting, this technology is accepted as the first-tier test for the evaluation of chromosomal imbalances associated with intellectual disability, autism, and/or multiple congenital anomalies. Furthermore, chromosomal microarray analysis is recommended for patients undergoing invasive prenatal diagnosis with one or more major fetal structural abnormalities identified by ultrasonographic examination, and in the evaluation of intrauterine fetal demise or stillbirth when further cytogenetic analysis is desired. This technology also provides important genomic data in the diagnosis, prognosis, and therapy of neoplastic disorders, including both hematologic malignancies and solid tumors. To assist clinical laboratories in the validation of chromosomal microarray methodologies for constitutional and neoplastic applications, the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee has developed these updated technical laboratory standards, which replace the ACMG technical standards and guidelines for microarray analysis in constitutional and neoplastic disorders previously published in 2013.

Genetics in Medicine (2021) 23:1818–1829; https://doi.org/10.1038/s41436-021-01214-w

GENERAL CONSIDERATIONS

Purpose of chromosomal microarray testing

Cytogenetic abnormalities include numerical abnormalities (aneuploidy, hypodiploidy, hyperdiploidy, and polyploidy) and structural abnormalities (deletion, duplication, triplication, amplification, translocation, inversion, insertion, marker chromosome, etc.). The chromosomal microarray (CMA) platforms discussed in these technical standards are those designed for the detection of DNA copy-number gains and losses associated with unbalanced chromosomal aberrations. In addition, regions of homozygosity (ROH), also referred to as copy-neutral loss of heterozygosity (CN-LOH), regions with absence of heterozygosity (AOH), or long continuous stretches of homozygosity (LCSH), may also be detected by platforms that include single-nucleotide polymorphism (SNP)-detecting probes.

The CMA diagnostic yield for detection of germline copy-number changes in patients with developmental delay, intellectual disability, autism, and/or multiple congenital anomalies has

[1]Department of Pathology, University of Michigan, Ann Arbor, MI, USA. [2]Legacy Laboratory Services, Legacy Health, Portland, OR, USA. [3]University of Missouri Kansas City Medical School, Kansas City, MO, USA. [4]Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, Kansas City, MO, USA. [5]Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA, USA. [6]National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA. [7]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, USA. [8]Department of Genetics, University of Alabama at Birmingham, Birmingham, AL, USA. [9]American College of Medical Genetics and Genomics, Bethesda, MD, USA. *The Board of Directors of the American College of Medical Genetics and Genomics approved this technical standard on 12 April 2021. ✉email: documents@acmg.net

been well documented, and CMA is recommended by the American College of Medical Genetics and Genomics (ACMG) as the first-tier test for these indications.[1,2] Similarly, CMA is recommended by the American College of Obstetricians and Gynecologists (ACOG) and the Society for Maternal–Fetal Medicine (SMFM) as the first-tier test in a prenatal setting in patients undergoing invasive prenatal diagnosis with one or more major fetal structural abnormalities identified by ultrasonographic examination, and for the evaluation of intrauterine fetal demise or stillbirth when further cytogenetic analysis is desired.[3] In addition, CMA is recommended as a follow-up test for small copy-number changes that are reported by noninvasive prenatal screening (NIPS).[4]

In a neoplastic setting, cytogenetic analysis plays important roles in the diagnosis, prognosis, and therapy of many neoplastic disorders. G-banded chromosome and/or fluorescence in situ hybridization (FISH) analyses are the gold standard for detection of clinically significant chromosomal abnormalities in many neoplasms.[5] Published clinically applicable data now show the clinical utility of CMA in the assessment of multiple neoplastic disorders, both hematologic malignancies and solid tumors.[6–11] Examples of the clinical utility of CMA in both hematologic malignancies and solid tumors can be found in the ACMG/Cancer Genomics Consortium (CGC) technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and CN-LOH in neoplastic disorders.[12]

The current technical standards serve as an update of the ACMG technical standards and guidelines for CMA analysis in constitutional disorders, including postnatal and prenatal applications and neoplastic disorders, that were published in 2013.[13,14] In an attempt to standardize the terminology used to communicate clinical CMA results, the workgroup reached consensus on the definitions of specific terms shown below:

- Copy-number variant (CNV): This term is used to describe germline copy-number gain and/or loss of chromosomal material.
- Copy-number abnormality (CNA): This term is used to describe acquired copy-number gain and/or loss of chromosomal material in neoplastic disorders.
- Copy-number change: This term is used to describe germline and acquired copy-number gain and/or loss of chromosomal material (both CNV and CNA).
- Region of homozygosity (ROH): This term is used to describe a region with germline allelic imbalance (i.e., homozygosity) without an associated copy-number change.
- Copy-neutral loss of heterozygosity (CN-LOH): This term is used to describe a region with acquired allelic imbalance (i.e., homozygosity) without an associated copy-number change in neoplastic disorders.

Advantages of CMA

The advantages of the use of CMA include the ability to:

- Use any sample that yields DNA of sufficient quality and quantity.
- Detect abnormalities that are cytogenetically cryptic by standard G-banded chromosome analysis.
- Better define and characterize abnormalities detected by standard G-banded chromosome analysis.
- Customize the CMA platform to concentrate probes in areas of interest.
- Interpret objective data, rather than a subjective visual assessment of band intensities.
- Detect ROH and CN-LOH using CMA platforms incorporating SNP probes.
- Interface the data with genome browsers and databases.

Limitations of CMA

The limitations of the use of CMA include the inability to detect:

- Genetic events that do not affect the relative copy-number of DNA sequences (e.g., molecularly balanced chromosomal rearrangements); however, CMA may reveal copy-number changes in apparently "balanced" chromosomal rearrangements (i.e., gains or losses at or near the chromosomal breakpoint sites).
- Low-level mosaicism for unbalanced rearrangements and aneuploidy in a constitutional setting, and inability to detect tumor-specific changes (acquired clonality) in a small percentage of cells. CMA analysis is neither established nor recommended as a method for post-therapy follow-up or for minimal residual disease detection in a neoplastic setting, unless an aberration is only detected by CMA (e.g., CN-LOH). The sensitivity of CMA for detection of mosaicism and acquired clonality will be influenced by the platform, sample type, copy-number state, DNA quality, data quality, and size of imbalance. A discussion about the detection of mosaicism and acquired clonality by CMA is outlined in more detail later in these technical standards.
- The mechanism of some genetic imbalances (e.g., tandem duplication versus unbalanced insertion versus marker chromosome), which may necessitate the use of conventional cytogenetic and/or FISH studies.
- Tetraploidy or other ploidy levels; although, CMA platforms incorporating SNP probes may facilitate detection of these abnormalities.
- Copy-number changes of genomic regions not represented on the CMA platform.
- Duplications and deletions below the detection level according to probe coverage and performance, single-nucleotide variants (SNVs) or insertions/deletions (indels) not covered by the platform, gene expression, or epigenetic modifications.
- All variants associated with a given disorder. Therefore, it must be understood that failure to detect a copy-number change at any locus does not exclude the diagnosis of a disorder associated with that locus.
- All significant clonal and subclonal cell populations; although, clonal diversity can be characterized.

Because of these limitations in a neoplastic setting, results using CMA technologies at diagnosis may need to be correlated with other established methodologies (G-banded chromosome and/or FISH analyses) whenever it is warranted.

CMA platform design and manufacture

CMA platforms currently available for clinical testing use oligonucleotide-based DNA probes. The oligonucleotide-based DNA probes may be designed to detect only copy-number changes 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 (i.e., array comparative genomic hybridization [aCGH]) or comparison of the intensity of the labeled patient DNA to an in silico reference set (i.e., SNP array). The copy-number data are plotted as a log2 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 ($\log2 > 0$), and relative DNA losses having less intensity ($\log2 < 0$). For SNP array platforms, the copy-number changes should also correlate with the allelic information assuming sufficient coverage of the copy-number changes with SNP-detecting probes. For example, a region present in one copy should only have single SNP alleles identified in the region.

CMA platform designs may have probes (1) targeted to specific regions of the genome for detection of imbalances known to be associated with the disease of interest, (2) distributed in a genome-wide manner with a specified distribution and spacing, or (3) 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 a CMA will be determined by both the intermarker probe spacing and the number of consecutive probes necessary to confidently identify a true copy-number change. The functional resolution may be different across different regions of the genome for a given platform due to probe density and may vary for copy-number gains and losses as reflected by the log2 ratio.

Manufacturers of CMA platforms should verify the identity of each 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, including the genome build. All probe descriptions and annotations should be openly accessible to the performing laboratory. Details regarding the CMA design, the synthesis verification, and all quality control (QC) steps taken to validate and assess the performance and reproducibility of the CMA should be documented and provided by the manufacturer. Additional information may be found in the ACMG recommendations for the design and performance expectations for clinical genomic copy-number microarray devices.[15]

CMA 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 germline disorder or cancer gene or feature) should provide the sensitivity needed for detection of a copy-number change.

METHODS

These technical laboratory standards were informed by a review of the literature and current guidelines. Resources consulted included PubMed; relevant ACMG, ACOG, and SMFM guidelines; and current World Health Organization (WHO) guidelines. The workgroup members also used their expert opinion and empirical data to inform their recommendations. Any conflicts of interests for workgroup members are listed at the end of the paper. The ACMG Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was presented to the ACMG Board of Directors for review and approval to post on the ACMG website for member comment. Upon posting to the ACMG website, an email and link were sent to all ACMG members inviting participation in the 30-day open comment process. All members' comments and additional evidence received were assessed by the authors, and these recommendations were incorporated into the document as deemed appropriate. Member comments and author responses were reviewed by representatives of the ACMG Laboratory Quality Assurance Committee and the ACMG Board of Directors. The final document was approved for publication by the ACMG Board of Directors.

FAMILIARIZATION WITH A NEW TECHNOLOGY FOR THE LABORATORY BEFORE VALIDATION

The laboratory with little or no experience with CMA 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 of 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 CMA data quality and clinical applicability. The laboratory should demonstrate expertise in technical performance of the CMA, reproducibility of results, and data analysis and interpretation. Expertise should be documented for each CMA platform used for clinical testing, regardless of whether the laboratory has prior experience with a different platform. The laboratory must also be familiar with the potential imbalances and rearrangements associated with the clinical indications.

It is strongly suggested that laboratories use data from well-characterized samples to gain and broaden their experience. Sample exchanges with a laboratory proficient with CMA technology 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 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 or CNAs.[15–21]

VERIFICATION AND VALIDATION

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 (QA) 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? Verification is required when using Food and Drug Administration (FDA)–cleared/approved tests. For the purposes of these technical standards, the terms "FDA-cleared tests" and "FDA-approved tests" will be interchangeably used and denoted as "FDA-cleared/approved tests."

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? Validation is required when using laboratory-developed tests or modified FDA tests.

New platform. 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.

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

All platforms intended for clinical testing must be either FDA-cleared/approved and verified or must be validated by the

performing laboratory. 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 version, or adding additional sample types or intended uses to a previously validated platform. The scope of the verification, and method and scope of the validation must be documented.

Verification of an FDA-cleared/approved test

For any FDA-cleared/approved microarrays where the laboratory plans to claim the test as FDA-cleared/approved, 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 regards to accuracy, precision, and reportable range of results. Any modification to the FDA-cleared/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-cleared/approved platform.

At the onset of verification, pass/fail criteria for the verification protocol should be established. Each laboratory should define the pass/fail criteria for quality control metrics at various steps of the assay. 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 samples (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 the "Validation of a new CMA test for the laboratory" section) should be further investigated to determine whether the finding represents true biological variation. This may involve the use of an alternative technology, e.g., multiplex ligation-dependent probe amplification (MLPA), quantitative polymerase chain reaction (qPCR), FISH, or a different 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.

Validation of a non-FDA-cleared/approved test

Validation of a non-FDA-cleared/approved test is specific for each analysis type (constitutional, neoplastic, or formalin-fixed paraffin-embedded [FFPE] tissue), which are considered different tests. 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.

1. Validation of a new CMA test for the laboratory

Validation of a new CMA test includes establishing the performance characteristics of the microarray platform and software, in addition to technical data analysis and interpretation. 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 new clinical CMA test, regardless of whether the laboratory has prior experience with a different platform.

The reportable range of results includes criteria to identify a copy-number change, and criteria to report it. Laboratories, with consideration of the manufacturer's recommendations, should identify the parameters specific to their platform (number of consecutive probes, log2 ratios, SNP allele ratios, QC metrics, etc.) that are necessary to conclude that a copy-number call represents a true copy-number change. As the functional resolution is a combination of probe density and number of probes necessary to identify a true copy-number change, 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 by 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 samples. To the extent possible, the laboratory should use abnormal samples 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 positive results 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 copy-number gains and losses.

Sample exchanges in a blind, split-sample comparison with a laboratory that is proficient with microarray technology can provide a good source of samples for validation. This sample exchange should include abnormal samples and involve comparison of results at the appropriate detection levels declared by the laboratories. Exchange of validated data sets (e.g., array files) between laboratories is recommended for additional experience in data analysis. All validation data for multiple disease and sample types, including discordant results and limitations, should be documented.

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 a 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. 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 detection limits established by the laboratory for the diagnosis and/or sample type. Evaluation should also include breakpoint assessment 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 performing probes or underlying genomic architecture). As this technology may detect true alterations not previously identified, any unexpected copy-number changes that fall within the 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, e.g., MLPA, qPCR, FISH, or a different 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 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, sensitivity and specificity for genome-wide array tests 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.

2. Validation of a new version of a previously established platform

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 version for comparison to ensure that the performance meets the laboratory standards and to assess performance of probes added into a higher-resolution version. 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.

3. Validation of additional sample/tumor types on an established platform

It is understood that the CMA platform employed by the laboratory may be used to analyze multiple sample types and, in a neoplastic setting, multiple neoplastic disorders. 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 constitutional evaluation, the sample type will likely be DNA extracted from peripheral blood, whereas if the intended use is neoplastic hematologic malignancy evaluation, the sample type will likely be DNA extracted from bone marrow or peripheral blood.

Because the quality of the DNA may vary from alternative tissue/tumor sources and this may add interference factors to the CMA analysis, use of DNA from alternative sample types requires an evaluation of the potential for interference.[22] Inherent differences in results obtained from different biological materials require that the laboratory determines the performance characteristics of the CMA for each sample type to be used for clinical testing. Examples include constitutional or neoplastic blood, neoplastic bone marrow, fresh or frozen tissue/tumor, and FFPE tumor.

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 plan. Evaluation of the array QC metrics of the new sample type is critical to ensure that they are within the established acceptable range. If there are only 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 significant alterations are made in the processing of the sample or CMA analysis (e.g., change of reference DNA), then a new validation is required.

In a neoplastic setting, laboratories often offer clinical testing for different neoplastic disorders using different sample types. In this case, the laboratory should process and analyze a sufficient number of each type to establish proficiency. Tumor-specific sample types for which clinical testing will be offered should be included in the validation. Each laboratory should use professional judgment and experience to determine the number of samples of a particular neoplastic disorder to include in their preclinical testing validation. Laboratories will also need to use professional judgment and experience to determine differences in processing various sample types and adjust sample numbers of each type accordingly, with the goal of optimizing quality and analytic interpretation of results.

4. Validation of the allelic differentiation potential of SNP-detecting platforms

In postnatal constitutional CMA, the detection of ROH is not in and of itself diagnostic but can identify a concern that may require additional testing such as sequence-based variant analysis or uniparental disomy testing. In the neoplastic setting, the detection of CN-LOH with or without additional CNAs may be diagnostic of

certain types of malignancy, and/or have therapeutic or prognostic implications. Given sufficient probe density, there should be a correlation between the copy-number state and the SNP allele state. Evaluation of the performance of the SNP-detecting probes to define ROH or CN-LOH should be included in the validation.

A minimum of five samples need to contain expected ROH or CN-LOH in addition to copy-number changes. Interlaboratory comparisons of such samples are recommended. This comparison should address the data types that would be included in a report, such as approximate sizes of ROH or CN-LOH, and percentage of the genome demonstrating ROH or CN-LOH, whenever clinically applicable. The detection and accurate size assessment of ROH or CN-LOH by SNP-based CMA depend on the density of SNP probes. If the validation method does not address accuracy of breakpoints in ROH or CN-LOH calls, reports should reflect this uncertainty in the disclaimer section. In constitutional CMA, inaccurate size or breakpoint estimation for ROH may lead to unwarranted follow-up testing for uniparental isodisomy and/or autozygosity mapping,[23] and in neoplastic CMA, inaccurate size or breakpoint estimation for CN-LOH may lead to misinterpretation of a variant as homozygous when a somatic variant is detected in a suspected region of CN-LOH.

5. Determining percentage of cells with abnormality: mosaicism and clonality

Constitutional mosaicism, the admixture of non-neoplastic cells in a tumor sample and clonal diversity can readily be detected by CMA. However, each laboratory needs to perform extensive validation studies to determine the dynamic range and the limit of detection for different cell populations for a wide variety of copy-number changes. For constitutional studies, it is not recommended that this technology be used as the sole method to rule out mosaicism. In cases with apparent low percentages of cells with questionable aberrations, FISH, conventional cytogenetics, or another quantitative method may be needed to fully characterize the genetic lesion(s).

The ability to detect mosaicism or clonal changes can be influenced by several factors including the microarray platform used, sample source, DNA quality and quantity, size and copy-number state of the abnormality, and probe coverage. Noise from poor-quality DNA and mixed chimerism may mask clonal abnormalities. Each laboratory will need to challenge their microarray with various percentages of abnormal cells, different ploidy levels, and clonally diverse samples to gain experience in their detection. It is not likely that a specific percentage of cells with an aberration will always be identified uniformly throughout the genome. This could be due to either poor performing probes, and/or genomic content affecting microarray performance, and this limitation should be recognized.

Methods for determining detectable percentages of cells include dilution series studies from an admixture of normal and abnormal cells from the same individual (if possible), obtaining samples from another laboratory with known abnormal cell percentages, and analysis of the mosaic sample by other quantitative methods. FISH analysis of fresh (uncultured) samples provides a reliable means to establish the percentage of cells with an aberration to compare with the microarray data. Flow cytometric data may also be used to estimate clone size (e.g., blast percentage for acute leukemia). Conventional cytogenetic analysis of metaphase cells provides information about mosaicism but may not accurately reflect levels of mosaicism. Note that methods to evaluate levels of detectable mosaicism/clonality will differ with sample type, e.g., fresh or FFPE tissue.

Dilution studies using samples with known copy-number changes may help to determine detectable levels of mosaicism.[24] This method can provide an effective means of establishing thresholds but may have limitations as a simulated method. For SNP-detecting arrays, dilution studies require non-neoplastic and tumor DNA from the same patient; buccal cells or blood may provide a source of non-neoplastic patient DNA. 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).

Microarray analysis tools were designed primarily for nonmosaic abnormalities; therefore mosaicism/clonality may not be reliably detected by the standard software algorithms. Laboratories should recognize software limitations and the need for manual and visual inspection of the data for mosaic aberration and clone/subclone detection. For validation, clinically relevant calls made by visual/manual inspection and calls made by software should be verified by another method, e.g., interphase FISH, qPCR, and/or replicate array analysis.

The percentage of cells with a specific abnormality in a mosaic or clonal state can be estimated using software parameters, including the log2 ratio, B-allele frequency, and/or allele difference.[24–27] However, the estimate is influenced by factors such as array platform, type of mosaic abnormality (i.e., one-copy loss or gain, two-copy loss or gain, and ploidy changes), and array quality. For example, the log2 ratio of the same mosaic percentage may be different between aCGH and SNP array. Each laboratory should consider these variables and be familiar with the capabilities/limitations of the array analysis software used.

6. Special considerations

6.1. Special considerations for validation of prenatal specimens

Experience with postnatal CMA and with common and rare CNVs is important for the processing and interpretation of array results in the prenatal setting. For validation, a distinction should be made between cultured amniotic fluid and chorionic villus sampling (CVS) cells and uncultured (direct) amniotic fluid and CVS cells. The validation 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. Both cultured and uncultured amniotic fluid and CVS should be included in the test validation, especially if the laboratory plans to perform CMA analysis on all these sample types.

Analysis of DNA extracted from uncultured amniotic fluid or CVS cells is preferable to DNA from cultured amniotic fluid or CVS cells. CMA analysis of uncultured amniotic fluid and CVS cells allows for the great majority of results to be available within one week and avoids the possibility of culture artifacts.[28–31] Maternal cell contamination (MCC) is a possibility when studying uncultured amniotic fluid or CVS cells (see MCC recommendations below). The villi need to be manually cleaned of maternal decidua prior to DNA extraction. Confined placental mosaicism (CPM) is a concern when studying uncultured CVS cells, but only a low frequency of CPM in CMA analysis of uncultured CVS cells has been reported.[32] This could be attributed to the evidence demonstrating that cleaned villi are mostly composed of the mesenchymal core, which is more representative of the fetal genome.[33] Back-up cultures for all prenatal samples undergoing CMA analysis should be established and maintained. This is necessary for the purposes of (1) possible array failures using direct DNA extractions, (2) evaluation of possible mosaicism, and (3) the need to perform metaphase chromosome or FISH analysis to investigate CNVs.

If prenatal CMA is performed on an array platform new to the laboratory, the issues and process discussed in the "Validation of a new CMA test 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, the collection of 30 samples will likely include those cases previously characterized as normal. Additional experience with abnormal array findings through data exchange 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 DNA quantity and data quality. The DNA extraction process should be part of the validation process. A minimum of five samples from each sample type, uncultured amniotic fluid, uncultured CVS, or cultured amniotic fluid/CVS, should be included in the preclinical testing validation.

Uncultured prenatal samples. Because uncultured cells may yield inconsistent DNA quantity and quality, additional validation may be required to become familiar with potential differences as compared with cultured cells. Parameters to consider for uncultured amniocytes include amniotic fluid volume, gestational age, and DNA extraction method. In general, uncultured amniocytes yield less DNA than cultured cells; however, CMA results are obtained faster.

Cultured prenatal samples. Healthy cultures established from amniocytes, CVS, and fetal tissue need to yield an adequate quantity and quality of DNA and provide consistent CMA results. The laboratory needs to be aware of factors that can affect DNA yield and data quality such as culture age, growth rate, confluency, and shipping conditions.

Maternal cell contamination and mosaicism. It is recommended that prenatal samples submitted for CMA be assessed for MCC. MCC may be present in direct samples of amniocytes that contain maternal blood, in CVS samples not adequately cleaned of maternal decidua, and in cell cultures after extensive subculturing with maternal cell expansion. Underlying MCC may affect detection and interpretation of CNVs, including different CNV types (gains and losses) and different CNV sizes (small versus large gains and losses). Low-level mosaicism in fetuses may be missed in the face of a significant level of MCC.

MCC may be detected with different methods including short tandem repeats (STR) analyses and SNP-based CMA platforms. For male fetuses, a shift in the sex chromosome plots mimicking mosaicism can suggest MCC. Each laboratory should validate their method for MCC detection to discern the level of MCC that is acceptable for their particular CMA platform. The acceptable level of MCC in uncultured samples should be determined to assess when cultured cells would be best for obtaining a successful CMA analysis.

Mosaicism detected by CMA should be investigated to confirm its presence and level and may represent a culture artifact (pseudomosaicism), true fetal mosaicism, or for CVS, CPM.[32]

Coverslip colony cultures may be used to investigate mosaicism (see section E4 of the ACMG Technical Laboratory Standards). Depending on the chromosome involved and the type of abnormality, additional studies using a different sample (e.g., amniotic fluid in a CVS sample suspected for CPM) may be considered to confirm or exclude mosaic status. FISH analysis may be used to investigate presence and level of mosaicism but may be unable to distinguish true from pseudomosaicism.

6.2. Special considerations for validation of oncology specimens

The validation of oncology specimens will follow the "Validation of a new CMA test for the laboratory" section regardless if the laboratory has experience with postnatal CMA, and a minimum of 30 previously characterized cases should be processed. Experience with postnatal CMA and with common and rare CNVs is helpful for the processing and interpretation of array results for oncology specimens. If multiple tumor types from different tissue sources will be processed using CMA, the DNA extraction process should be validated following the "Validation of additional sample/tumor types on an established platform" section.

Clonal diversity. CMA uses an averaged DNA pool of all cells in the sample; thus, clonal and subclonal populations may not be accurately defined from CMA data. However, combinations of multiple abnormalities with the same estimates of cell percentages can be used to infer information about clones, including delineating clonal diversity of the tumor. Clonal diversity, common to neoplastic disorders, is observed when the cell populations of different clones reach the threshold for detection. Additionally, independent clonal populations can occur in the same tumor tissue and CMA alone will not be able to distinguish unrelated clones or subclones. Correlation with conventional cytogenetic and FISH analyses can aid in the interpretation of the tumor clonal and subclonal composition. CMA platforms in use or in the process of being validated should be assessed for the capability of detecting clinically significant CNAs and CN-LOH (i.e., tiers 1 and 2) within clonally diverse cell populations.[12]

Germline abnormalities. CMA testing in neoplasia may uncover germline abnormalities in patients. The interpretation and reporting of unanticipated clinically significant germline variants should be performed in accordance with "Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders"[12] and "Points to consider for reporting of germline variation in patients undergoing tumor testing."[34]

6.3. Determination of ploidy

Hypodiploidy, hyperdiploidy, and polyploidy can be detected by CMA but may be challenging to appreciate and interpret. The allelic states of SNP probes can assist in determining ploidy levels; allele differences and/or B-allele frequency and log2 ratio together assist in determining the diploid baseline. The validation process should include samples with varying levels of ploidy to gain experience in analysis and recognition of different ploidies. Correlation with FISH and karyotype can help to determine the ploidy level and the potential need to readjust the diploid baseline. If FISH and karyotype are not available, the triallelic region (balanced genotypes with homozygous A and B and heterozygous AB alleles) with the lowest log2 ratio often represents the diploid baseline, and the rest of the genome may be normalized accordingly. It may be necessary to confirm that CNAs detected by this approach are reported as recurrent abnormalities in the neoplastic disorder under investigation. 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.

6.4. Chimerism

A mixture of genotypes may occur with constitutional samples (e.g., MCC in prenatal samples) or cancer samples (e.g., following stem cell/bone marrow transplantation) and can also be detected when clinical samples are inadvertently mixed. It is important to recognize the specific pattern that is generated from chimerism. When two samples are mixed together, the SNP allele tracks become increasingly complex. Thus, it may not be possible to determine the origin of the major and minor contributions without known copy-number changes or ROH/CN-LOH from at least one of the individuals (e.g., germline CNVs or previously reported patient abnormalities). Adjunct tests to assess the levels of chimerism, such as STR analysis, can assist with estimation of the contribution of individuals. When chimerism is present, the ability to detect low levels of cells with abnormalities and small aberrations will be impacted and data interpretation may be compromised. In addition, the report should indicate the limited analysis, given the complexity of the results from chimerism.

REFERENCE SET CONSIDERATIONS

Depending on the array platform used, the reference set may come from a single individual or multiple individuals, may be sex matched or mismatched, and may be used in silico (with SNP-

based microarray) or as a direct competitive hybridization (with aCGH). The laboratory should understand the benefits and limitations of each scenario. The laboratory should be aware of how the data quality may be affected by the source and components of the reference set. For example, data quality is likely improved when the assay conditions used for the reference set closely match those for the test.[35] Any changes to the reference set could alter the results, and therefore require a verification of the quality and accuracy of results obtained with the new set as compared with the previous one.

Array comparative genomic hybridization analysis

aCGH analysis requires comparison of sample DNA to reference (control) DNA in the assay, thus the selection of an appropriate reference DNA is essential. Laboratories may establish their own reference DNA or use reference DNA provided by the manufacturer. The laboratory should characterize any reference DNA to identify germline CNVs that may have an effect on the interpretation of patient data. During the familiarization phase, it is important to optimize the reference DNA to ensure high quality data. This includes the DNA extraction process, purity, and concentration. Fluorometric quantitation of the DNA is recommended to ensure that equal quantities of sample and reference DNA are used in the assay.

Laboratory policies should detail how reference DNA will be used, i.e., 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. The QC metrics of each new lot of purchased reference DNA should be compared with the previous lot to ensure that they are within the expected range and to verify the accuracy and reproducibility of results.

In an oncology setting, constitutional DNA from blood or normal tissue from the same individual may be used as the reference DNA. While constitutional patient DNA will mask germline CNVs and reduce the complexity of postanalytic interpretation, novel underlying germline abnormalities that could contribute to disease will not be detected.

SNP-based microarray analysis

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 an in silico reference that mimics the typical study population. In creating the internal reference file (sometimes referred to as the "cluster" file), the laboratory minimizes interlaboratory variation resulting from varied equipment and other external conditions, and normalizes the data to minimize population variation. The laboratory should also consider variables that may differ between reference sample and test sample, such as DNA extraction methodology and sample type (e.g., fresh tissue versus FFPE sample). The laboratory should follow the manufacturer's recommendations for the minimum number of male and female controls used by the analysis software.

Laboratory policies should document the rationale for the use of an internal reference file and detail how reference files will be used. Reference files may be updated by adding, removing, or replacing samples. A new reference file should be established for new SNP-based array designs.

SOFTWARE CONSIDERATIONS

The laboratory should recognize software limitations and the need for visual inspection of the data. Manual calls are often necessary during validation and clinical testing, for example, to combine calls that are interrupted by poor performing probes, to separate calls that are interrupted by a normal region, to add calls for low-level mosaic aberrations that are not flagged by software, or to revise breakpoints not assigned accurately. To verify that the method for result generation (including software and manual calls) detects known aberrations accurately, the laboratory should test a variety of copy-number changes (i.e., deletions, duplications, and amplifications), CN-LOH, ROH, and aberrations at different mosaicism/clonality levels. During the familiarization phase, the software settings should be optimized for aberration detection and then established parameters should be used consistently throughout the validation process. These include thresholds for size/number of probes, log2 ratio thresholds, and mathematical algorithms used by the software to make calls. The software parameter settings may be different for various sample types.

The laboratory must determine and document the ability and accuracy of the software to detect copy-number changes according to software rules and parameters. When applicable, the laboratory should determine the ability of the software to accurately define the endpoints of CN-LOH and ROH according to the software settings within the resolution of the array design. Limits should be verified whenever the microarray platform, version, software, or analysis rules change. The laboratory should challenge the software with copy-number changes that help define the limits of detection.

Changes to the software settings from those used during the validation may require a reanalysis 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. The allelic states of SNP probes may assist with the detection of hypodiploidy, hyperdiploidy, and polyploidy. These situations are rare in the postnatal constitutional samples but are relatively common in products of conception and oncology samples.

Any upgrade to the software that offers a change or improvement over previous versions is deemed a new version of the software. Laboratories should validate a new version of the software from the same manufacturer with a minimum of five abnormal samples generally representative of the different types of aberrations that arise in clinical testing. Known abnormal samples from the previous version should be analyzed using the new version of software to confirm that the new version detects the expected abnormality. If other abnormalities are detected that meet the laboratory-reporting criteria, the laboratory should determine whether the findings represent true biological variation. A new function in an upgraded version should be assessed, if possible, using known abnormal sample(s) with abnormalities that can test the new function and determine its performance. For example, samples with mosaic triploidy can be used to evaluate the new function of ploidy adjustment.

The laboratory should document the software parameters and rules used for the microarray analysis, and all limitations of the analysis program. The limits, rules, and parameters for detection of mosaicism/clonality should be determined.

QUALITY CONTROL

Identification

For each microarray, the slide identification number, subarray position (when applicable), sample sex, control sex (when appropriate), and sample-tracking control (for multiplex

microarrays) should be verified and a system developed to ensure sample identification throughout the process. Discrepancies in the documentation from the physical sample should be investigated and resolved before processing.

Sample and DNA requirements

The laboratory should establish sample adequacy requirements and 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. Blood samples that do not meet the laboratory requirements can be rejected with a repeat sample requested from the referring physician. For prenatal and oncology samples where a repeat sample may not be available, the laboratory should attempt DNA re-extraction and/or purification and perform the array assay. Improvement approaches may include increasing the DNA input in the assay for certain sample types (e.g., FFPE samples) or whole-genome amplification, provided that the laboratory has expertise with this method and 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.

A surgical pathologist should evaluate FFPE samples to assess tissue quality and select an area from the tissue block that contains ample (suggested minimum of 25%) tumor or villi from products of conception to avoid masking copy-number changes in the tissue of interest by DNA from the normal tissue or maternal decidua, respectively.

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. FFPE samples present unique challenges for generating high quality DNA from the tissue of interest. 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, DNA fragmentation (e.g., via sonication or enzymatic digestion), fluorescent labeling (e.g., examination by gel electrophoresis, visual inspection, ultraviolet/visible spectroscopy), and amplification. For any labeling method, acceptable ranges should be determined for proper dye incorporation. Protocols for optimization, e.g., re-extraction, re-purification, tumor cell enrichment for hematological samples (cell sorting or magnetic bead enrichment), and/or microdissection for paraffin-embedded tissue, 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.

Equipment calibration, maintenance, and quality control

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. Laboratories should ensure 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 enables the laboratory to prevent modification of analysis settings so that sample analysis is consistent. Any changes to data processing should be validated and documented.

Quality control 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 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 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 QA and quality improvement programs to monitor analytical variables.

Microarray content

It is not feasible for a laboratory to validate the identity and copy-number performance of every probe on a microarray. The laboratory should obtain documentation from the microarray manufacturer that the probes on each microarray are the intended sequences, 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

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 log2 ratio. The derivative log2 ratio is the difference between the log2 ratio values of consecutive probes. Similar metrics of variance exist for each platform. Data quality may be assessed using platform-specific parameters.

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 ranges may differ for different sample types. The laboratory policies should describe the appropriate follow-up procedure, 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. Critical annotations should be versioned and updated regularly. The manufacturers should provide mechanism(s) for updates to these annotations. For all reportable calls, the genomic content may need to be verified by an independent database source (e.g., UCSC Genome Browser). Documentation of resources and databases accessed for interpretation is recommended.

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 laboratory should have documentation of the microarray slides manufacturing QC (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, preferably using a patient specimen with an abnormal result that has been tested on a previous lot. 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.

Confirmation of specific copy-number changes

With proper technical performance and analytical validation, it should not be necessary for the performing laboratory to further confirm a copy-number change 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 log2 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 log2 ratio data (e.g., weighted versus not weighted). Any call-specific quality score provided by the software may be considered.

Since it is desirable to maximize detection of aberrations involving clinically significant 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, and when appropriate, should be confirmed by an independent methodology if reported.

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), CLIA, and 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. The sample should be accompanied by an appropriate indication for the test. Clinical testing may be limited to neoplastic disorders for which unbalanced genomic abnormalities and/or CN-LOH are well documented to have diagnostic, prognostic, and/or therapeutic implication(s). Microarray analysis may not be optimal for tumor surveillance or detection of minimal residual disease depending on the limit of detection of the aberration and the ability to use more sensitive methods for monitoring. When applicable, alternative methods should be recommended to monitor patient response to treatment and for residual disease detection (e.g., FISH and/or 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.

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

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

The laboratory should also establish internal PT of normal and abnormal samples as part of the laboratory internal QA 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 1988 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.

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, 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.

USE OF ALTERNATIVE TECHNOLOGIES FOR MECHANISM DETERMINATION

Determination of the mechanism leading to the detected copy-number change may be considered on a case-by-case basis because this may lead to better determination of recurrence risk in constitutional studies and provide clinically useful information in neoplastic cases (e.g., confirmation of gene fusion). Some mechanisms can be identified through the combination of both the copy-number change(s) and recognition of the genomic location of the altered material, or the genomic structure surrounding the alteration. Examples include unbalanced translocations and insertions, iso- or isodicentric chromosomes, and ring or marker chromosomes. The appropriate alternative technology may depend on the size, type, and location of the identified copy-number change(s) 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 the published "Technical standards for the interpretation and reporting of constitutional copy-number variants"[21] and "Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders"[12] as well as "Standards and

guidelines for documenting suspected consanguinity as an incidental finding of genomic testing."[36]

METHODOLOGY AND DISCLAIMERS

All reports should include a brief description of the 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 regions. Therefore, a normal microarray result does not exclude single nucleotide variants (SNVs) or insertions/deletions (indels) not covered by the platform, 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, single nucleotide variants (SNVs) or insertions/deletions (indels) not covered by the platform, 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 the 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.

RETENTION OF FILES AND DOCUMENTATION

Laboratories should be explicit in their policies as to which file types and for 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 (section 493.1105) require storage of analytic systems records and test reports for at least two 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 reanalysis 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

CMA technologies provide a high-resolution copy-number view of the whole genome. The clinical application of this technology for constitutional and neoplastic disorders requires extensive clinical validation to ensure the results reported to the health-care provider are accurate and reliable for patient care decision making. The technical laboratory standards described here provide detailed guidance for performing this validation, including considerations for pre- and postnatal constitutional and neoplastic applications.

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. The interpretation of the data from microarray analysis into clinically relevant information is a difficult and complex undertaking. No algorithm for copy-number change interpretation can substitute for adequate training and knowledge in the fields of medical genetics, pathology, and oncology. We recommend that CMA analysis be performed in laboratories overseen by individuals with appropriate professional training (i.e., certified by the American Board of Medical Genetics and Genomics [ABMGG] in clinical cytogenetics, clinical molecular genetics or laboratory genetics and genomics or certified by the American Board of Pathology in molecular genetic pathology) and that the interpretation and reporting of clinical microarray findings be performed by these same certified individuals.

Received: 4 May 2021; Revised: 4 May 2021; Accepted: 4 May 2021;
Published online: 15 June 2021

REFERENCES

1. Manning, M. & Hudgins, L. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet. Med.* **12**, 742–745 (2010).
2. Miller, D. T. 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.* **86**, 749–764 (2010).
3. Committee on Genetics and the Society for Maternal–Fetal Medicine. Committee Opinion No. 682: Microarrays and next-generation sequencing technology: the use of advanced genetic diagnostic tools in obstetrics and gynecology. *Obstet. Gynecol.* **128**, e262–e268 (2016).
4. Cherry, A. M. et al. 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.* **19**, 845–850 (2017).
5. Arber, D. A. et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* **127**, 2391–2405 (2016).
6. Chun, K. et al. Assessing copy number aberrations and copy-neutral loss-of-heterozygosity across the genome as best practice: An evidence-based review from the Cancer Genomics Consortium (CGC) working group for chronic lymphocytic leukemia. *Cancer Genet.* **228–229**, 236–250 (2018).
7. Dougherty, M. J., Tooke, L. S., Sullivan, L. M., Hakonarson, H., Wainwright, L. M. & Biegel, J. A. Clinical utilization of high-resolution single nucleotide polymorphism based oligonucleotide arrays in diagnostic studies of pediatric patients with solid tumors. *Cancer Genet.* **205**, 42–54 (2012).
8. Kanagal-Shamanna, R. et al. Assessing copy number aberrations and copy neutral loss of heterozygosity across the genome as best practice: an evidence based review of clinical utility from the Cancer Genomics Consortium (CGC) working group for myelodysplastic syndrome, myelodysplastic/myeloproliferative and myeloproliferative neoplasms. *Cancer Genet.* **228–229**, 197–217 (2018).
9. Wang, Y., Miller, S., Roulston, D., Bixby, D. & Shao, L. Genome-wide single-nucleotide polymorphism array analysis improves prognostication of acute lymphoblastic leukemia/lymphoma. *J. Mol. Diagn.* **18**, 595–603 (2016).
10. Xu, X. et al. Assessing copy number abnormalities and copy-neutral loss-of-heterozygosity across the genome as best practice in diagnostic evaluation of acute myeloid leukemia: an evidence-based review from the Cancer Genomics Consortium (CGC) myeloid neoplasms working group. *Cancer Genet.* **228–229**, 218–235 (2018).
11. Roth, J. J. et al. Diagnostic application of high resolution single nucleotide polymorphism array analysis for children with brain tumors. *Cancer Genet.* **207**, 111–123 (2014).
12. Mikhail, F. M. et al. Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC). *Genet. Med.* **21**, 1903–1916 (2019).
13. South, S. T., Lee, C., Lamb, A. N., Higgins, A. W. & Kearney, H. M. ACMG standards and guidelines for constitutional cytogenomic microarray analysis, including

postnatal and prenatal applications: revision 2013. *Genet. Med.* **15**, 901–909 (2013).
14. Cooley, L. D., Lebo, M., Li, M. M., Slovak, M. L. & Wolff, D. J. American College of Medical Genetics and Genomics technical standards and guidelines: microarray analysis for chromosome abnormalities in neoplastic disorders. *Genet. Med.* **15**, 484–494 (2013).
15. Kearney, H. M., South, S. T., Wolff, D. J., Lamb, A., Hamosh, A. & Rao, K. W. 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.* **13**, 676–679 (2011).
16. Lee, C., Iafrate, A. J. & Brothman, A. R. Copy number variations and clinical cytogenetic diagnosis of constitutional disorders. *Nat. Genet.* **39**, S48–54 (2007).
17. Riggs, E. R. et al. Towards an evidence-based process for the clinical interpretation of copy number variation. *Clin. Genet.* **81**, 403–412 (2012).
18. Sayers, E. W. et al. Database resources of the National Center for Biotechnology Information. *Nucleic. Acids. Res.* **40**, D13–D25 (2012).
19. Firth, H. V. et al. DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources. *Am. J. Hum. Genet.* **84**, 524–533 (2009).
20. Zhang, J., Feuk, L., Duggan, G. E., Khaja, R. & Scherer, S. W. Development of bioinformatics resources for display and analysis of copy number and other structural variants in the human genome. *Cytogenet. Genome Res.* **115**, 205–214 (2006).
21. Riggs, E. R. et al. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). *Genet. Med.* **22**, 245–257 (2020).
22. van Heesch, S. et al. Systematic biases in DNA copy number originate from isolation procedures. *Genome Biol.* **14**, R33 (2013).
23. Mason-Suares, H. et al. Density matters: comparison of array platforms for detection of copy-number variation and copy-neutral abnormalities. *Genet. Med.* **15**, 706–712 (2013).
24. Conlin, L. K. et al. Mechanisms of mosaicism, chimerism and uniparental disomy identified by single nucleotide polymorphism array analysis. *Hum. Mol. Genet.* **19**, 1263–1275 (2010).
25. Valli, R., Maserati, E., Marletta, C., Pressato, B., Lo Curto, F. & Pasquali, F. Evaluating chromosomal mosaicism by array comparative genomic hybridization in hematological malignancies: the proposal of a formula. *Cancer Genet.* **204**, 216–218 (2011).
26. Markello, T. C. et al. Sensitive quantification of mosaicism using high density SNP arrays and the cumulative distribution function. *Mol. Genet. Metab.* **105**, 665–671 (2012).
27. Rowsey, R., Znoyko, I. & Wolff, D. J. Whole-genome single nucleotide polymorphism microarray for copy number and loss of heterozygosity analysis in tumors. *Methods Mol. Biol.* **1908**, 89–111 (2019).
28. Dugoff, L., Norton, M. E. & Kuller, J. A. The use of chromosomal microarray for prenatal diagnosis. *Am. J. Obstet. Gynecol.* **215**, B2–B9 (2016).
29. Vanakker, O. et al. Implementation of genomic arrays in prenatal diagnosis: the Belgian approach to meet the challenges. *Eur. J. Med. Genet.* **57**, 151–156 (2014).
30. Sahoo, T. et al. Prenatal diagnosis of chromosomal abnormalities using array-based comparative genomic hybridization. *Genet. Med.* **8**, 719–727 (2006).
31. Filges, I. et al. aCGH on chorionic villi mirrors the complexity of fetoplacental mosaicism in prenatal diagnosis. *Prenat. Diagn.* **31**, 473–478 (2011).
32. Gu, S., Jernegan, M., Van den Veyver, I. B., Peacock, S., Smith, J. & Breman, A. Chromosomal microarray analysis on uncultured chorionic villus sampling can be complicated by confined placental mosaicism for aneuploidy and microdeletions. *Prenat. Diagn.* **38**, 858–865 (2018).
33. Yong, P. J., McFadden, D. E. & Robinson, W. P. Developmental origin of chorionic villus cultures from spontaneous abortion and chorionic villus sampling. *J. Obstet. Gynaecol. Can.* **33**, 449–452 (2011).
34. Li, M. M. et al. Points to consider for reporting of germline variation in patients undergoing tumor testing: a statement of the American College of Medical Genetics and Genomics (ACMG). *Genet. Med.* **22**, 1142–1148 (2020).
35. Pinto, D. et al. Comprehensive assessment of array-based platforms and calling algorithms for detection of copy number variants. *Nat. Biotechnol.* **29**, 512–520 (2011).
36. Rehder, C. W., David, K. L., Hirsch, B., Toriello, H. V., Wilson, C. M. & Kearney, H. M. American College of Medical Genetics and Genomics: standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing. *Genet. Med.* **15**, 150–152 (2013).

ACKNOWLEDGEMENTS

B.A.S. was an ABMGG laboratory genetics and genomics postdoctoral fellow at Duke University Health System and a trainee member of the ACMG Laboratory Quality Assurance Committee during the preparation of this document.

COMPETING INTERESTS

All members of this workgroup are directors of clinical laboratories that use chromosomal microarray technologies.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to ACMG.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

APPENDIX 6

Technical laboratory standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen)

(See following page)

ACMG TECHNICAL STANDARDS | Genetics inMedicine

Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen)

Erin Rooney Riggs, MS, CGC[1], Erica F. Andersen, PhD[2,3], Athena M. Cherry, PhD[4], Sibel Kantarci, PhD[5], Hutton Kearney, PhD[6], Ankita Patel, PhD[7], Gordana Raca, MD, PhD[8], Deborah I. Ritter, PhD[9], Sarah T. South, PhD[10], Erik C. Thorland, PhD[6], Daniel Pineda-Alvarez, MD[11], Swaroop Aradhya, PhD[4,11] and Christa Lese Martin, PhD[1]

Purpose: Copy-number analysis to detect disease-causing losses and gains across the genome is recommended for the evaluation of individuals with neurodevelopmental disorders and/or multiple congenital anomalies, as well as for fetuses with ultrasound abnormalities. In the decade that this analysis has been in widespread clinical use, tremendous strides have been made in understanding the effects of copy-number variants (CNVs) in both affected individuals and the general population. However, continued broad implementation of array and next-generation sequencing–based technologies will expand the types of CNVs encountered in the clinical setting, as well as our understanding of their impact on human health.

Methods: To assist clinical laboratories in the classification and reporting of CNVs, irrespective of the technology used to identify them, the American College of Medical Genetics and Genomics has developed the following professional standards in collaboration with the National Institutes of Health (NIH)–funded Clinical Genome Resource (ClinGen) project.

Results: This update introduces a quantitative, evidence-based scoring framework; encourages the implementation of the five-tier classification system widely used in sequence variant classification; and recommends "uncoupling" the evidence-based classification of a variant from its potential implications for a particular individual.

Conclusion: These professional standards will guide the evaluation of constitutional CNVs and encourage consistency and transparency across clinical laboratories.

Genetics in Medicine (2020) 22:245–257; https://doi.org/10.1038/s41436-019-0686-8

Keywords: copy-number variant; interpretation; classification; CNV; scoring metric

INTRODUCTION

Genome-wide assessment of copy-number variants (CNVs), including losses (deletions) and gains (duplications and triplications), is recommended as a first-tier approach for the postnatal evaluation of individuals with intellectual disability, developmental delay, autism spectrum disorder, and/or multiple

[1]Autism & Developmental Medicine Institute, Geisinger, Danville, PA, USA; [2]ARUP Laboratories, Salt Lake City, UT, USA; [3]Department of Pathology, University of Utah, Salt Lake City, UT, USA; [4]Stanford University School of Medicine, Stanford, CA, USA; [5]Quest Diagnostics Nichols Institute, San Juan Capistrano, CA, USA; [6]Genomics Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA; [7]Lineagen, Salt Lake City, UT, USA; [8]Children's Hospital Los Angeles, Los Angeles, CA, USA; [9]Texas Children's Cancer Center, Baylor College of Medicine, Houston, TX, USA; [10]AncestryDNA, Lehi, UT, USA; [11]Invitae, San Francisco, CA, USA. Correspondence: ACMG (documents@acmg.net)

The Board of Directors of the American College of Medical Genetics and Genomics approved this technical standard on 23 September 2019.

Submitted 18 October 2019; accepted: 18 October 2019
Published online: 6 November 2019

congenital anomalies, as well as for prenatal evaluation of fetuses with structural anomalies observed by ultrasound.[1–3] For over a decade, CNV analysis by chromosomal microarray (CMA) has been broadly implemented in the clinical setting for detection of genomic imbalances at a much higher resolution than conventional cytogenetic methods (e.g., G-banded karyotype). In some cases, exon-focused array designs have also been used for detecting CNVs involving individual genes associated with monogenic disorders. More recently, next-generation sequencing (NGS)–based CNV analysis is increasingly used in clinical testing through genome, exome, or gene panel sequencing. Together, these methods have enabled genome-wide detection of CNVs, ranging in size from single exons to whole chromosomes in clinically affected individuals, as well as in the general population.

Though many recurrent CNVs (such as those flanked by segmental duplications) have been well characterized, most CNVs are unique, requiring further investigation to determine their potential clinical significance. This can be challenging for several reasons, including absent, limited, or conflicting associations with clinical phenotypes described in published literature and genomics databases. Accurate clinical interpretation of CNVs requires consistent methods of evaluating the genomic content of a CNV region and correlating clinical findings with those reported in the medical literature, with the ultimate goal of producing consistent, evidence-based clinical classification across laboratories.[4] Inconsistency among laboratories can create confusion for clinicians and their patients, leaving them unable to confidently use genetic information to manage health-care decisions.[5] Standards that are widely available, up-to-date, and flexible enough to incorporate lessons learned from the ever-evolving genomics knowledge base should help to reduce discordance in clinical classifications.

METHODS

To assist in the evaluation of CNVs and promote consistency and transparency in classification and reporting across clinical laboratories, the American College of Medical Genetics and Genomics (ACMG) and the National Institutes of Health (NIH)–funded Clinical Genome Resource (ClinGen) project[6] formed a collaborative working group with the goal of updating the existing ACMG professional clinical laboratory practice standards for evaluating CNVs.[7] The working group held an in-person meeting in the fall of 2015 to review the existing version of the interpretation standards[7] and discuss how laboratories had incorporated them (and any modifications) into their clinical practice, as well as new resources, tools, and technologies that became available in the intervening years. Through group consensus, evidence categories most relevant to CNV classification were determined (including genomic content, dosage sensitivity predictions and curations, predicted functional effect, clinical overlap with patients in the medical literature, evidence from case and control databases, and inheritance patterns for individual CNVs), and a relative weight was assigned to each. In this manner, a semiquantitative point-based scoring system was developed (described in detail in Supplemental Material 1).

Development of the new framework was an iterative process; working group members tested the analysis metrics using cases observed in their clinical laboratories and provided feedback for refinement that ensured objective and rigorous assessment of the available evidence. In 2017, after the framework had been developed and assessed by the working group, we identified a group of 11 additional board-certified clinical cytogeneticists to further evaluate both the performance of the analysis metrics and their usability in the clinical setting. Using both the outside reviewers and the committee members, we evaluated a total of 114 CNVs (58 deletions, 56 duplications); most CNVs ($n = 111$) were each evaluated by two independent reviewers. A full description of the validation process is provided in Supplemental Material 2. Feedback from this process led to the current version of the scoring metrics.

Proposed criteria for the evaluation of constitutional copy-number variants

These standards build upon the previous version[7] by introducing a semiquantitative point-based scoring metric for CNV classification. Owing to the distinct properties and inherent differences between copy-number losses and copy-number gains, separate scoring metrics were developed for each (Tables **1** and **2**, respectively); each scored evidence category is labeled (1A, 1B, etc.) for easy referencing. Full descriptions of each evidence category, including caveats to consider while scoring and illustrative examples, are provided in Supplemental Material 1. We strongly recommend the user to carefully review the explanatory material provided in the Supplement before utilizing these scoring metrics in clinical practice. Example cases scored using the metrics are provided in Supplemental Material 3.

As clinical laboratories incorporate more NGS-based techniques for CNV detection and integrate results from multiple technologies (some capable of identifying both copy-number and sequence variants), consistency across interpretation processes and reporting is critical. Thus, where possible, evidence categories and concepts presented in this CNV scoring system were developed to align with terminology and processes currently utilized for clinical sequence variant classification and interpretation.[8]

The point values assigned to each piece of evidence roughly correspond to the categorical strengths of evidence present in the sequence variant interpretation guidelines[8] as well as recommendations put forth by the ClinGen Sequence Variant Interpretation (SVI) Working Group to model the ACMG/Association for Molecular Pathology (AMP) sequence variant interpretation guidelines into a more quantitative Bayesian framework;[9] however, it is important to note that these numbers have not been statistically derived. In general, evidence receiving 0.90 points or higher is considered “very strong"; 0.45 points is considered “strong"; 0.30 points is considered “moderate"; and 0.15 points or lower is considered “supporting” evidence. Scores

Table 1 CNV interpretation scoring metric: copy-number loss

Section 1: Initial assessment of genomic content			
Evidence type	**Evidence**	**Suggested points/case**	**Max score**
Copy-number loss content	**1A**. Contains protein-coding or other known functionally important elements.	0 (Continue evaluation)	0
	1B. Does NOT contain protein-coding or any known functionally important elements.	−0.60	−0.60
Section 2: Overlap with established/predicted haploinsufficiency (HI) or established benign genes/genomic regions *(Skip to section 3 if your copy-number loss DOES NOT overlap these types of genes/regions)*			
Overlap with ESTABLISHED HI genes or genomic regions and consideration of reason for referral	**2A**. Complete overlap of an established HI gene/genomic region.	1.00	1.00
	2B. Partial overlap of an established HI genomic region • The observed CNV does NOT contain the known causative gene or critical region for this established HI genomic region OR • Unclear if known causative gene or critical region is affected OR • No specific causative gene or critical region has been established for this HI genomic region	0 (Continue evaluation)	0
	2C. Partial overlap with the 5′ end of an established HI gene (3′ end of the gene not involved)…	See categories below	
	2C-1. …and coding sequence is involved	0.90 *(range: 0.45 to 1.00)*	1.00
	2C-2. …and only the 5′ UTR is involved	0 *(range: 0 to 0.45)*	0.45
	2D. Partial overlap with the 3′ end of an established HI gene (5′ end of the gene not involved)…	See categories below	
	2D-1. …and only the 3′ untranslated region is involved.	0 (Continue evaluation)	0
	2D-2. …and only the last exon is involved. Other established pathogenic variants have been reported in this exon.	0.90 *(range: 0.45 to 0.90)*	0.90
	2D-3. …and only the last exon is involved. No other established pathogenic variants have been reported in this exon.	0.30 *(range: 0 to 0.45)*	0.45
	2D-4. …and it includes other exons in addition to the last exon. Nonsense-mediated decay is expected to occur.	0.90 *(range: 0.45 to 1.00)*	1.00
	2E. Both breakpoints are within the same gene (intragenic CNV; gene-level sequence variant).	See ClinGen SVI working group PVS1 specifications • PVS1 = 0.90 *(Range: 0.45 to 0.90)* • PVS1_Strong = 0.45 *(Range: 0.30 to 0.90)* • PVS1_Moderate *or* PM4 (in-frame indels) = 0.30 *(Range: 0.15 to 0.45)* • PVS1_Supporting = 0.15 *(Range: 0 to 0.30)*	See categories at left

Table 1 continued

		• N/A = No points, but continue evaluation	
Overlap with ESTABLISHED benign genes or genomic regions	**2F**. Completely contained within an established benign CNV region.	−1	−1
	2G. Overlaps an established benign CNV, but includes additional genomic material.	0 (Continue evaluation)	0
Haploinsufficiency predictors	**2H**. Two or more HI predictors suggest that AT LEAST ONE gene in the interval is HI.	0.15	0.15
Section 3: Evaluation of gene number			
Number of protein-coding RefSeq genes wholly or partially included in the copy-number loss	**3A**. 0–24 genes	0	0
	3B. 25–34 genes	0.45	0.45
	3C. 35+ genes	0.90	0.90
Section 4: Detailed evaluation of genomic content using cases from published literature, public databases, and/or internal lab data *(Skip to section 5 if either your CNV overlapped with an established HI gene/region in section 2, OR there have been no reports associating either the CNV or any genes within the CNV with human phenotypes caused by loss of function [LOF] or copy-number loss)*			
Individual case evidence—de novo occurrences	Reported proband (from literature, public databases, or internal lab data) has either: • A complete deletion of or a LOF variant within gene encompassed by the observed copy-number loss OR • An overlapping copy-number loss similar in genomic content to the observed copy-number loss AND…	See categories below	
	4A. …the reported phenotype is highly specific and relatively unique to the gene or genomic region,	Confirmed de novo: 0.45 points each Assumed de novo: 0.30 points each *(range: 0.15 to 0.45)*	0.90 (total)
	4B. …the reported phenotype is consistent with the gene/genomic region, is highly specific, but not necessarily unique to the gene/genomic region.	Confirmed de novo: 0.30 points each Assumed de novo: 0.15 point each *(range: 0 to 0.45)*	
	4C. …the reported phenotype is consistent with the gene/genomic region, but not highly specific and/or with high genetic heterogeneity.	Confirmed de novo: 0.15 point each Assumed de novo: 0.10 point each *(range: 0 to 0.30)*	
Individual case evidence—inconsistent phenotype	**4D**. …the reported phenotype is NOT consistent with what is expected for the gene/genomic region or not consistent in general.	0 points each *(range: 0 to −0.30)*	−0.30 (total)
Individual case evidence—unknown inheritance	**4E**. Reported proband has a highly specific phenotype consistent with the gene/genomic region, but the inheritance of the variant is unknown.	0.10 points each *(range: 0 to 0.15)*	0.30 (total)
Individual case evidence—segregation among similarly affected family members	**4F**. 3–4 observed segregations	0.15	0.45
	4G. 5–6 observed segregations	0.30	
	4H. 7 or more observed segregations	0.45	
Individual case evidence—nonsegregations	**4I**. Variant is NOT found in another individual in the proband's family AFFECTED with a consistent, specific, well-defined phenotype (no known phenocopies).	−0.45 points per family *(range: 0 to −0.45)*	−0.90 (total)
	4J. Variant IS found in another individual in the proband's family UNAFFECTED with the specific, well-defined phenotype observed in the proband.	−0.30 points per family *(range: 0 to −0.30)*	−0.90 (total)

Table 1 continued

	4K. Variant IS found in another individual in the proband's family UNAFFECTED with the nonspecific phenotype observed in the proband.	−0.15 points per family *(range: 0 to −0.15)*	−0.30 (total)
Case–control and population evidence	**4L**. Statistically significant increase amongst observations in cases (**with a consistent, specific, well-defined phenotype**) compared with controls.	0.45 per study *(range: 0 to 0.45 per study)*	0.45 (total)
	4M. Statistically significant increase amongst observations in cases (**without a consistent, nonspecific phenotype OR unknown phenotype**) compared with controls.	0.30 per study *(range: 0 to 0.30 per study)*	0.45 (total)
	4N. No statistically significant difference between observations in cases and controls.	−0.90 (per study) *(range: 0 to −0.90 per study)*	−0.90 (total)
	4O. Overlap with common population variation.	−1 *(range: 0 to −1)*	−1
Section 5: Evaluation of inheritance pattern/family history for patient being studied			
Observed copy-number loss is de novo	**5A**. *Use appropriate category from de novo scoring section in section 4.*	*Use de novo scoring categories from section 4 (4A–4D) to determine score*	0.45
Observed copy-number loss is inherited	**5B**. Patient with **specific, well-defined** phenotype and no family history. CNV is inherited from an apparently unaffected parent.	−0.30 *(range: 0 to −0.45)*	−0.45
	5C. Patient with **nonspecific** phenotype and no family history. CNV is inherited from an apparently unaffected parent.	−0.15 *(range: 0 to −0.30)*	−0.30
	5D. CNV segregates with a consistent phenotype observed in the patient's family.	*Use segregation scoring categories from section 4 (4F–4H) to determine score*	0.45
Observed copy-number loss—nonsegregations	**5E**. *Use appropriate category from nonsegregation section in section 4.*	*Use nonsegregation scoring categories from section 4 (4I–4K) to determine score*	−0.45
Other	**5F**. Inheritance information is unavailable or uninformative.	0	0
	5G. Inheritance information is unavailable or uninformative. The patient phenotype is nonspecific, but is consistent with what has been described in similar cases.	0.10 *(range: 0 to 0.15)*	0.15
	5H. Inheritance information is unavailable or uninformative. The patient phenotype is highly specific and consistent with what has been described in similar cases.	0.30 *(range: 0 to 0.30)*	0.30

Only those CNVs otherwise meeting the reporting thresholds determined by your laboratory should be evaluated using this metric. See Supplemental Material 1 for a detailed description of each evidence category. Scoring: *pathogenic* 0.99 or more points, *likely pathogenic* 0.90 to 0.98 points, *variant of uncertain significance* 0.89 to −0.89 points, *likely benign* −0.90 to −0.98 points, *benign* −0.99 or fewer points.
CNV copy-number variant, *SVI* sequence variant interpretation, *UTR* untranslated region.

Table 2 CNV interpretation scoring metric: copy-number gain

Evidence type	Evidence	Suggested points/case	Max score
Section 1: Initial assessment of genomic content			
Copy-number gain content	**1A**. Contains protein-coding or other known functionally important elements.	0 (Continue evaluation)	0
	1B. Does NOT contain protein-coding or any known functionally important elements.	−0.60	−0.60
Section 2: Overlap with established triplosensitive (TS), haploinsufficient (HI), or benign genes or genomic regions ***(Skip to section 3 if the copy-number gain DOES NOT overlap these types of genes/regions)***			
Overlap with ESTABLISHED TS genes or genomic regions	**2A**. Complete overlap; the TS gene or minimal critical region is fully contained within the observed copy-number gain.	1	1
	2B. Partial overlap of an established TS region • The observed CNV does NOT contain the known causative gene or critical region for this established TS genomic region OR • Unclear if the known causative gene or critical region is affected OR • No specific causative gene or critical region has been established for this TS genomic region.	0 (Continue evaluation)	0
Overlap with ESTABLISHED benign copy-number gain genes or genomic regions	**2C**. Identical in gene content to the established benign copy-number gain.	−1	−1
	2D. Smaller than established benign copy-number gain, breakpoint(s) does not interrupt protein-coding genes.	−1	−1
	2E. Smaller than established benign copy-number gain, breakpoint(s) potentially interrupts protein-coding gene.	0 (Continue evaluation)	0
	2F. Larger than known benign copy-number gain, does not include additional protein-coding genes.	−1 *(range: 0 to −1.00)*	−1
	2G. Overlaps a benign copy-number gain but includes additional genomic material.	0 (Continue evaluation)	0
Overlap with ESTABLISHED HI gene(s)	**2H**. HI gene fully contained within observed copy-number gain.	0 (Continue evaluation)	0
Breakpoint(s) within ESTABLISHED HI genes	**2I**. Both breakpoints are within the same gene (gene-level sequence variant, possibly resulting in loss of function [LOF]).	See ClinGen SVI working group PVS1 specifications • PVS1 = 0.90 *(Range: 0.45 to 0.90)* • PVS1_Strong = 0.45 *(Range: 0.30 to 0.90)* • N/A = 0 (Continue evaluation)	
	2J. One breakpoint is within an established HI gene, patient's phenotype is either inconsistent with what is expected for LOF of that gene OR unknown.	0 (Continue evaluation)	0
	2K. One breakpoint is within an established HI gene, patient's phenotype is highly specific and consistent with what is expected for LOF of that gene.	0.45	0.45
Breakpoints within other gene(s)	**2L**. One or both breakpoints are within gene(s) of no established clinical significance.	0 (Continue evaluation)	0
Section 3: Evaluation of gene number			
Number of protein-coding RefSeq genes wholly or partially included in the copy-number gain	**3A**. 0–34 genes	0	0
	3B. 35–49 genes	0.45	0.45
	3C. 50 or more genes	0.90	0.90
Section 4: Detailed evaluation of genomic content using cases from published literature, public databases, and/or internal lab data ***(Note: If there have been no reports associating either the copy-number gain or any of the genes therein with human phenotypes caused by triplosensitivity, skip to section 5)***			
Individual case evidence—de novo occurrences	Reported proband (from literature, public databases, or internal lab data) has either: • complete duplication of one or more genes within the observed copy-number gain OR • an overlapping copy-number gain similar in genomic content to the observed copy-number gain AND…	See categories below	
	4A. …the reported phenotype is highly specific and relatively unique to the gene or genomic region.	Confirmed de novo: 0.45 points each Assumed de novo: 0.30 points each *(range: 0.15 to 0.45)*	0.90 (total)
	4B. …the reported phenotype is consistent with the gene/genomic region, is highly specific, but is not necessarily unique to the gene/genomic region.	Confirmed de novo: 0.30 points each Assumed de novo: 0.15 point each *(range: 0 to 0.45)*	
	4C. …the reported phenotype is consistent with the gene/genomic region, but not highly specific and/or with high genetic heterogeneity.	Confirmed de novo: 0.15 point each Assumed de novo: 0.10 point each *(range: 0 to 0.30)*	
Individual case evidence—inconsistent phenotype	**4D**. …the reported phenotype is NOT consistent with the gene/genomic region or not consistent in general.	0 points each *(range: 0 to −0.30)*	−0.30 (total)
Individual case evidence—unknown inheritance	**4E**. Reported proband has a highly specific phenotype consistent with the gene/genomic region, but the inheritance of the variant is unknown.	0.10 points each *(range: 0 to 0.15)*	0.30 (total)

Table 2 continued

Individual case evidence—segregation among similarly affected family members	**4F**. 3–4 observed segregations	0.15	0.45
	4G. 5–6 observed segregations	0.30	
	4H. 7 or more observed segregations	0.45	
Individual case evidence—nonsegregations	**4I**. Variant is NOT found in another individual in the proband's family AFFECTED with a consistent, specific, well-defined phenotype (no known phenocopies).	−0.45 points per family *(range: 0 to −0.45)*	−0.90 (total)
	4J. Variant IS found in another individual in the proband's family UNAFFECTED with the specific, well-defined phenotype observed in the proband.	−0.30 points per family *(range: 0 to −0.30)*	−0.90 (total)
	4K. Variant IS found in another individual in the proband's family UNAFFECTED with the nonspecific phenotype observed in the proband.	−0.15 points per family *(range: 0 to −0.15)*	−0.30 (total)
Case–control and population evidence	**4L**. Statistically significant increase among observations in cases (with a consistent, **specific, well-defined phenotype**) compared with controls.	0.45 per study *(range: 0 to 0.45 per study)*	0.45 (total)
	4M. Statistically significant increase among observations in cases (with a consistent, **nonspecific phenotype or unknown phenotype**) compared with controls.	0.30 per study *(range: 0 to 0.30 per study)*	0.45 (total)
	4N. No statistically significant difference between observations in cases and controls.	−0.90 per study *(range: 0 to −0.90 per study)*	−0.90 (total)
	4O. Overlap with common population variation.	−1 *(range: 0 to −1)*	−1
Section 5: Evaluation of inheritance patterns/family history for patient being studied			
Observed copy-number gain is de novo	**5A**. *Use appropriate category from de novo scoring section in section 4.*	*Use de novo scoring categories from section 4 (4A–4D) to determine score*	0.45
Observed copy-number gain is inherited	**5B**. Patient with a **specific, well-defined phenotype** and no family history. Copy-number gain is inherited from an apparently unaffected parent.	−0.30 *(range: 0 to −0.45)*	−0.45
	5C. Patient with **nonspecific phenotype** and no family history. Copy-number gain is inherited from an apparently unaffected parent.	−0.15 *(range: 0 to −0.30)*	−0.30
	5D. CNV segregates with consistent phenotype observed in the patient's family.	*Use segregation scoring categories from in section 4 (4F–4H) to determine score*	0.45
Observed copy-number gain—nonsegregations	**5E**. *Use appropriate category from nonsegregation section in section 4.*	*Use nonsegregation scoring categories from section 4 (4I–4K) to determine score*	−0.45
	5F. Inheritance information is unavailable or uninformative.	0	0
	5G. Inheritance information is unavailable or uninformative. The patient phenotype is nonspecific, but is consistent with what has been described in similar cases.	0.10 *(range: 0 to 0.15)*	0.15
	5H. Inheritance information is unavailable or uninformative. The patient phenotype is highly specific and consistent with what has been described in similar cases.	0.15 *(range: 0 to 0.30)*	0.30

Only those CNVs otherwise meeting the reporting thresholds determined by your laboratory should be evaluated using this metric. See Supplemental Material 1 for full description of each evidence category. Scoring: *pathogenic* 0.99 or more points, *likely pathogenic* 0.90 to 0.98 points, *variant of uncertain significance* 0.89 to −0.89 points, *likely benign* −0.90 to −0.98 points, *benign* −0.99 or fewer points.
CNV copy-number variant, *SVI* sequence variant interpretation.

for each observed piece of evidence, both in support of (positive values) and refuting (negative values) pathogenicity, are summed to arrive at a CNV classification. CNVs with a final point value ≥0.99 are considered pathogenic, while point values between 0.90 and 0.98 are considered likely pathogenic; this approach aligns with the sequence variant interpretation guidelines[8] (i.e., variants interpreted as pathogenic should have a 99% level of confidence and variants interpreted as likely pathogenic should have a 90% level of confidence). The variant of uncertain significance (VUS) category is the broadest, corresponding to points between −0.89 and 0.89, while refuting evidence arriving at scores between −0.90 and −0.98, or ≤−0.99 are considered likely benign and benign, respectively.

To facilitate use of this semiquantitative system, a web-based CNV classification calculator based on these scoring metrics is publicly available (http://cnvcalc.clinicalgenome.org/cnvcalc/). This tool allows users to apply points for individual evidence categories for a given CNV and will automatically calculate the final point value and corresponding CNV classification. This tool will be continually supported and updated, allowing timely integration of new information as it emerges.

These standards were developed for evaluating evidence in the context of constitutional CNVs, including those detected during postnatal or prenatal testing. Laboratories may choose to use specific reporting practices based on factors such as CNV classification and clinical context, and these may vary across different test types and clinical settings (e.g., choosing to only report likely pathogenic or pathogenic variants associated with dominantly inherited conditions in a prenatal setting). These specific reporting practices should be documented in the laboratory's interpretation and reporting protocol.

These standards do not apply to acquired CNVs in neoplasia. In addition, this document does not address analytical validation of CNV detection methods, which have been addressed elsewhere, and assumes that any laboratory using the provided standards is confident that a reported CNV represents a true biological event.[10] These standards serve as a reference for clinicians to enable them to understand the complexity of CNV interpretation and to appropriately communicate test results to patients and families. Although these standards attempt to comprehensively incorporate commonly available resources and processes used in CNV classification and interpretation, it is important to recognize that no singular algorithm will be applicable in all potential scenarios. The semiquantitative scoring framework is meant to serve as a guide. Professional judgment should always be used when evaluating the evidence surrounding a particular genomic variant and assigning a classification.

Recommended variant classification categories

Using the scoring metrics described in Supplemental Material 1, a laboratory geneticist should assign any CNV reported in a patient to one of five main classification categories. It is strongly recommended that consistent terminology for these categories be used in clinical reporting to facilitate unambiguous communication of clinical significance throughout the medical community.

The classification categories represent a significant update from the previous version of these guidelines.[7] To align closely with recommendations in the ACMG/AMP sequence variant interpretation guidelines[8] and with the manner in which these terms are now commonly used, we have updated the existing three-tiered system of clinical significance (in which the term "variant of uncertain significance" had the optional qualifiers of "likely pathogenic" or "likely benign") to the five-tiered system described below.

Pathogenic

Pathogenic (P) CNVs are those that score 0.99 points or higher using the evidence scoring metric (Supplemental Material 1). Although the full clinical effect of a CNV on a patient's phenotype may not be known (due to zygosity or other reasons), the pathogenic nature of the CNV should not be in question.

Examples of P CNVs may include (1) CNVs reported in association with consistent clinical phenotypes across multiple peer-reviewed publications, with well-documented penetrance and expressivity, even if reduced and/or variable; (2) unique CNVs that overlap completely with an established dosage-sensitive region; and (3) multigenic CNVs in which at least one gene is known to be dosage sensitive,[11] even if the other genes are of uncertain significance.

Except for well-established cytogenetic heteromorphisms, this category will include most cytogenetically visible alterations (generally >5 Mb). In the absence of loci clearly associated with defined genetic syndromes within the interval, cytogenetically visible alterations should still be cautiously evaluated, taking the gene content into consideration.

Likely pathogenic

Likely pathogenic (LP) CNVs are those that score between 0.90 and 0.98 points using the evidence scoring metric. In general, these variants have strong evidence to suggest that they will ultimately be determined to be disease-causing, but not enough yet to definitively assert pathogenicity. Several evidence types outlined within the scoring metrics could be combined to reach the LP point threshold. However, some particularly strong pieces of evidence may result in the CNV being classified as LP without the need for additional evidence (although additional information could be added to bring the classification to P). Examples of this type of evidence may include (1) deletions involving the 5' end (plus additional coding sequence) of established haploinsufficient (HI) genes (in scenarios where there are no known alternative start sites) (category 2C-1, deletion metric); (2) deletions involving multiple exons (through the 3' end of the gene) in an established HI gene (category 2D-4); and (3) deletions or duplications involving genes with multiple case reports reported in consistent, highly specific phenotypes.

Uncertain significance

Variants of uncertain significance (VUS) are those that score between −0.89 and 0.89 points using the evidence scoring metric. This represents a broad category and may include findings that are later demonstrated with additional evidence to be either pathogenic or benign. Some CNVs in this category may have more evidence than others to indicate involvement in disease and the likelihood of additional evidence surfacing through published literature may be higher. However, at the time of reporting, if insufficient evidence is available for confident determination of definitive clinical significance and the CNV meets the reporting criteria established by the laboratory, the CNV should be described as a variant of uncertain significance.

Examples of VUS may include (1) a CNV that exceeds a laboratory's size threshold for reporting but has no genes in the affected genomic interval (category 1B); (2) a CNV described in a small number of cases in the general population, but not at a high enough frequency to be considered a polymorphism (>1%) (category 4O, with a downgraded score due to frequency); (3) a CNV that contains a small number of genes, but it is not known whether the genes in the interval are dosage sensitive (category 3A); (4) a CNV described in multiple contradictory publications and/or databases, without firm conclusions regarding clinical significance (multiple categories); (5) a CNV within an individual gene (category 2E, deletion metric, and 2I, duplication metric) with an unclear effect on the transcript reading frame.

Likely benign

Likely benign (LB) CNVs are those that score between −0.90 and −0.98 points using the evidence scoring metric. In general, these variants have strong evidence to suggest that they are likely not involved in Mendelian disease, but do not yet have enough evidence to state this definitively.

Examples of LB CNVs may include (1) variants with no statistically significant difference between observations in cases and controls (category 4N), and (2) variants observed frequently in the general population (although at a lower frequency than 1%, a conventionally accepted threshold for a common polymorphism [category 4O]).

Benign

Benign CNVs are those that score −0.99 or fewer points using the evidence scoring metric. These CNVs have typically been reported in multiple peer-reviewed publications or annotated in curated databases as benign variants, particularly if the nature of the copy-number variation has been well characterized (e.g., copy-number variation of the salivary amylase gene[12]) and/or the CNV represents a common polymorphism. To qualify as a benign 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 copy-number variants in the constitutional setting

In recent years, innovations in microarray and NGS technologies have expanded the diagnostic application of clinical CNV analysis and interpretation from chromosomal microarrays to single- and multigene sequencing panels, and exome or genome sequencing. Each of these tests may have distinct clinical reporting specifications. The following recommendations describe elements of a clinical laboratory report that are necessary to precisely describe the nature of a CNV and clearly communicate the evidence related to its classification and clinical significance. Other required elements of a clinical report (e.g., methodology and relevant disclaimers) are outlined in detail in the ACMG Technical Standards and Guidelines.

Reporting criteria

The laboratory report should include a description of the criteria used for both inclusion of a CNV in the report (e.g., classification type, CNV size) and classification of the CNV (e.g., the scoring metrics included in this document). Laboratories may or may not choose to disclose benign or likely benign CNVs, and this should be indicated in the report and their laboratory reporting protocol.

Description of each CNV detected

Each CNV should be described with the elements below. Appropriate nomenclature from the International System for Human Cytogenomic Nomenclature (ISCN) or the Human Genome Variation Society (HGVS) should be included in the report, but should not serve as a substitute for a clear description of the genomic imbalance for clinical professionals unfamiliar with these conventions.

- Cytogenetic location (chromosome number and cytogenetic band designation).
- CNV size and linear coordinates with the genome build specified. Genomic coordinates for the minimum predicted interval should be specified. When applicable, particularly when gene content of the CNV is unclear, the maximal genomic coordinates may also be provided.
- Copy-number state (e.g., single-copy gain or loss) with CNV mechanism specified when understood (e.g., tandem duplication). Assessment of mechanism may require additional testing methods.
- For intragenic CNVs: Appropriate naming conventions in this scenario may be dependent on the platform used to detect these variants. If the variant is identified using NGS-based technologies, HGVS nomenclature may be preferable, including gene name (using valid Human Genome Organisation Gene Nomenclature Committee [HGNC] nomenclature), transcript, and exons involved. If the variant is identified using CMA, ISCN nomenclature

is generally recommended. The naming convention selected should recognize a location, genomic content, and certainty or uncertainty of precise breakpoints.

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 validated/curated (i.e., not predicted or hypothetical) genes in the interval be included, when possible, to facilitate periodic reviews of relevant medical literature. The incorporation of links to websites that list the genes in an interval is not recommended because the links may not faithfully direct the clinician to the appropriate gene content in the future. If all genes in the interval are not listed on the report, it is suggested that at least the total number of genes in the CNV interval be provided to highlight the extent of genomic imbalance; other potentially clinically relevant elements may also be noted.

Clear statement of variant classification and clinical significance

Regardless of the type of variant being assessed (CNV, sequence variant, etc.), determining a variant's classification should be performed independently from determining how it contributes to the diagnosis of the individual in whom it is discovered. Uncoupling variant classification (P, LP, etc.) from clinical significance in the context of an individual patient's diagnosis is key to objective and consistent interpretation of genomic variants. While the phenotype of the proband should be taken into account when assessing evidence supporting the pathogenicity of a CNV, classification should not be solely driven by the presentation of the patient under investigation (without consideration of other available evidence). For example, there is compelling evidence in the literature that deletion of a particular gene results in disease X; a laboratory evaluating a deletion of this gene is able to reach 0.99 points using the scoring metric, suggesting a classification of pathogenic. The laboratory should not then disregard all previously collected evidence and classify the variant as "uncertain significance" solely because their patient did not display features of disease X.

The classification of a particular variant should be based upon the evidence available to support or refute its pathogenicity at a given point in time; that body of evidence is ostensibly the same for every patient found to have that variant at that same point in time. As such, the variant should receive the same variant classification (P, LP, VUS, etc.), regardless of the clinical significance it has for each patient (which may differ). For example, there is substantial evidence demonstrating that a particular gene on the X chromosome causes disease via a loss-of-function mechanism. Given the body of evidence, deletions involving this gene should receive the classification of pathogenic each time they are observed, regardless of whether they are observed in hemizygous males or heterozygous females. Within the report, the laboratory should explain the potential consequences of such a deletion for the patient under study—in a male this variant could represent a diagnostic finding; in a female this variant could represent carrier status. Therefore, each description of a CNV should include a clear statement of its classification and the evidence supporting it, as outlined in these recommendations, as well as the clinical significance of that variant for the patient being tested. See Supplemental Material 4 for examples of how these concepts may be conveyed during reporting.

Special considerations regarding reporting: clinically significant findings unrelated to the reason for referral

Occasionally, a CNV may be identified that, although unrelated to the patient's reason for referral, may indicate presymptomatic status for a late-onset disorder or may reveal an ongoing clinically unrecognized condition (i.e., an incidental finding[13]). Some examples of these include deletions involving known tumor suppressor genes,[14] male infertility due to deletions involving the AZF region on the Y chromosome,[15] a deletion disrupting a gene for hereditary spastic paraplegia in a child referred for autism,[16] etc. It is often not possible to specifically avoid interrogation of the types of loci mentioned in the aforementioned cases, because such findings may occur as part of a large CNV involving multiple genes. It is impractical to provide a predefined list of all possible diagnoses to allow a patient to consent specifically to the interrogation of and reporting for each disorder. Therefore, referring clinicians must have a clear understanding of the potential for these discoveries, and patients/families should be duly informed before test ordering. An informed consent process is strongly recommended.

It is recommended that P or LP CNVs indicative of presymptomatic status be reported to facilitate appropriate and timely access to medical care. Individual laboratories may adopt nondisclosure policies for specific conditions and state them as such in their clinical reports.

The ACMG Secondary Findings Working Group has been established to identify genes "associated with highly penetrant genetic disorders and established interventions aimed at preventing or significantly reducing morbidity and mortality."[13] When evaluating CNVs involving these genes, it is important to remember the mechanism of disease associated with each. If haploinsufficiency or triplosensitivity is not an established mutational mechanism for a specific gene, a deletion or duplication is not likely to be clinically relevant. If the mechanism of disease is consistent with haploinsufficiency or triplosensitivity, these CNVs should be reported. Dosage sensitivity evaluations of the genes currently on the ACMG secondary findings list are available at the following link: https://www.ncbi.nlm.nih.gov/projects/dbvar/clingen/acmg.shtml.

Clinically significant findings seemingly unrelated to the reason for referral represent another situation where it is important to separate the variant classification from clinical correlation. Historically, the reason for referral has influenced the CNV interpretation process; anecdotally, variants with clear evidence for pathogenicity have been classified as VUS because they did not "explain" the patient's stated reason for referral. The reasons for referral provided to laboratories may not always represent a complete picture of the patient's phenotypic features, and assumptions that a patient does or does not have a particular feature are not prudent without appropriate consultation with the referring clinician. Open channels of communication between the laboratory and the ordering physician are critical to guide clinical correlation.[17]

It is certainly appropriate to consider available phenotype information about a given patient as evidence in variant evaluation; if the patient undergoing testing has a phenotype that is consistent with the described phenotype for an observed CNV, this may be considered evidence supporting pathogenicity. It is not appropriate, however, to provide a different classification for the same CNV simply because it was identified in an individual with a different reason for referral. For example, there is substantial evidence demonstrating that loss-of-function variants in gene X result in hearing loss. If a laboratory observes a deletion of this gene in an individual referred for hearing loss, and the exact same deletion in another individual referred for speech delay, they should not interpret that variant as P in the former case and VUS in the latter. The variant should be classified as P in both instances. The variant is directly relevant to the reason for referral in the individual with hearing loss, but may represent an incidental finding or an explanation for an unobserved/unreported phenotype in the second. The pathogenicity of the variant, however, should not be in question given the depth of the supporting evidence. The reason for referral alone should not be used to justify varying classifications for the same CNV in different individuals.

Special considerations regarding reporting: carrier status

Detection of some CNVs, particularly deletions, will indicate carrier status for autosomal recessive or X-linked disorders mapping within the CNV interval. Although exhaustive reporting of carrier status may be considered difficult to standardize and beyond the intended scope of genome-wide microarrays (particularly for very large multigenic events), improvements in informatics could support reporting of such information in the future. Individual laboratories may choose to adopt specific disclosure policies for recessive conditions. If a laboratory chooses to include a list of carrier alleles, its reports should clearly separate the primary CNV results related to the reason for referral from a secondary list of carrier status alleles. If carrier status is not routinely assessed, reports should clearly state that carrier status may not be disclosed, and that any clinical concern for recessive disorders should be communicated to the reporting laboratory for appropriate consideration. There are some situations when disclosure of carrier status is recommended:

1. Well-characterized disorders where loss of function is the established disease mechanism. In such cases, there may be justification for reporting carrier status to provide opportunity for reproductive counseling and additional testing in the proband or relevant family members, particularly when the carrier frequency is reasonably high, and/or screening is commonly available (e.g., cystic fibrosis). 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 routinely detect carrier status for any condition.
2. Disorders with clinical features consistent with the patient's reason for referral. In such cases, a laboratory may have identified a CNV that represents one allele of an expected pair consistent with the referral diagnosis. The laboratory may then recommend ancillary molecular testing for this disorder in an effort to identify the other disease-causing allele. This should be restricted to well-described 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 pathogenic variant.
3. CNVs involving dosage-sensitive genes on the X chromosome in females. Given the significant reproductive risk to female carriers of X-linked conditions, we recommend reporting these variants because it provides the opportunity for the patient and relevant family members to pursue additional testing/counseling as needed. Additionally, females may manifest symptoms in many X-linked disorders; these variants may ultimately have an impact on their medical management.

To make these nuances clearer to users of the laboratory report, we recommend dividing the report into sections describing primary variants considered relevant to the stated reason for referral separately from any variants that represent secondary or incidental findings or carrier status. Laboratories may decide at their discretion if additional subcategories are necessary.

Recommendation for appropriate clinical follow-up

The laboratory report should include recommendations for any necessary further cytogenetic characterization of the CNV, genetic counseling, and evaluation of relevant family members as appropriate. In addition, when a CNV is of uncertain significance, the report may include a recommendation for continued surveillance of the medical literature for new information that may alter the classification of the CNV and provide clarification on its clinical significance. The responsibility for monitoring the medical literature for a specific patient lies primarily with the physician with an ongoing patient relationship,[18] but laboratories may choose to offer amended reports when reclassifications occur.

CONCLUDING REMARKS

Understanding the clinical relevance of CNVs is a complex, continually evolving process that constitutes the practice of medicine. As evident from the numerous considerations outlined in this document, no one formula or algorithm for CNV interpretation will substitute for adequate training in genetics and sound clinical judgment. We recommend that clinical reporting of constitutional CNVs be performed by individuals with appropriate professional training and certification (those individuals certified by the American Board of Medical Genetics and Genomics [ABMGG] in clinical cytogenetics, molecular genetics, and/or laboratory genetics and genomics). In addition, given the complexity of CNV interpretation, the different laboratory methodologies utilized for CNV characterization, and the evaluation of additional family members, an ideal laboratory setting for CNV analysis should include both cytogenetic and molecular genetic expertise.

This document for the first time lays out explicit guidance for interpreting CNVs that occur within individual genes. As detecting CNVs from sequencing-based platforms becomes more commonplace, it is important that CNV and single-nucleotide variant (SNV) analyses are appropriately aligned in their approaches to variant classification. Ideally, a CNV should receive the same classification whether it was detected on a CMA or an NGS platform, and whether or not it was interpreted by someone board-certified in cytogenetics or molecular genetics. The recommendations presented here (and in Supplemental Material 1) represent an initial effort to move toward more consistent CNV interpretation between laboratories and across technologies.

Systematic approaches to variant interpretation (such as this one) will evolve over time, particularly as knowledge regarding the relationships between genomic variation and human health improve. Groups are encouraged to use this framework as a guide, always using professional judgment when opting to incorporate emerging knowledge, methods, and resources, and documenting the process by which this evidence is used to arrive at a variant classification.

To summarize, major updates from the previous document[7] include:

- CNV classification categories will change to the five-tier classification system recommended in the ACMG/AMP sequence variant interpretation guidelines.[8]
- Variants should be classified consistently between patients; while patient presentation and/or reason for referral may be used as evidence to support a particular classification, this information should not be used to justify disparate classifications of the same variant. Variant classifications should be based on evidence; at a given point in time, evidence supporting/refuting a given variant's pathogenicity should be the same. Therefore, the classification of that variant should be the same regardless of patient-specific factors such as reason for referral, sex, age, etc.
- Laboratories should consider utilizing headers or subsections in the clinical report to clearly communicate primary versus incidental or secondary findings, such as carrier status for autosomal recessive conditions, pathogenic variants unrelated to the stated reason for referral, etc. (examples may be found in Supplemental Material 4).
- Explicit new guidance for interpreting CNVs occurring within individual genes (intragenic deletions and duplications) (described in detail in Supplemental Material 1).
- Points-based scoring rubrics (Tables **1** and **2**) to guide laboratories toward more consistent CNV interpretations. We anticipate that updates to these metrics will be required as laboratories gain experience using them, and as evidence and technologies change.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-019-0686-8) contains supplementary material, which is available to authorized users.

ACKNOWLEDGEMENTS

We acknowledge the contributions of the volunteers who piloted early versions of the scoring metrics and provided invaluable feedback: Rachel Burnside, Alka Chaubey, Laura Conlin, James Harraway, Vanessa Horner, Dominic McMullan, Jeanne Meck, Sian Morgan, Karen Tsuchiya, Yiping Shen, and Karen Swisshelm. ClinGen is primarily funded by the National Human Genome Research Institute (NHGRI) through the following three grants: U41HG006834, U41HG009649, and U41HG009650.

DISCLOSURE

E.F.A., A.M.C., S.K., H.K., G.R., S.T.S., E.C.T., D.P.-A., and S.A. are directors of fee-for-service testing laboratories performing tests mentioned in this technical standard. The other authors declare no conflicts 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. American College of Obstetricians and Gynecologists Committee on Genetics. Committee opinion no. 581: the use of chromosomal microarray analysis in prenatal diagnosis. Obstet Gynecol. 2013;122: 1374–1377.
4. Brothman AR, Dolan MM, Goodman BK, et al. College of American Pathologists/American College of Medical Genetics proficiency testing for constitutional cytogenomic microarray analysis. Genet Med. 2011; 13:765–769.
5. 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.

6. Rehm HL, Berg JS, Brooks LD, et al. ClinGen—the Clinical Genome Resource. N Engl J Med. 2015;372:2235–2242.
7. 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.
8. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405–424.
9. Tavtigian SV, Greenblatt MS, Harrison SM, et al. Modeling the ACMG/AMP variant classification guidelines as a Bayesian classification framework. Genet Med. 2018;20:1054–1060.
10. Kearney HM, South ST, Wolff DJ, et al. 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.
11. 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.
12. 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.
13. Kalia SS, Adelman K, Bale SJ, et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. Genet Med. 2017;19:249–255.
14. Adam MP, Justice AN, Schelley S, Kwan A, Hudgins L, Martin CL. Clinical utility of array comparative genomic hybridization: uncovering tumor susceptibility in individuals with developmental delay. J Pediatr. 2009;154:143–146.
15. Vogt PH. Genomic heterogeneity and instability of the AZF locus on the human Y chromosome. Mol Cell Endocrinol. 2004;224:1–9.
16. Boone PM, Soens ZT, Campbell IM, et al. Incidental copy-number variants identified by routine genome testing in a clinical population. Genet Med. 2013;15:45–54.
17. Bush LW, Beck AE, Biesecker LG, et al. Professional responsibilities regarding the provision, publication, and dissemination of patient phenotypes in the context of clinical genetic and genomic testing: points to consider—a statement of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2018;20:169–171.
18. Hirschhorn K, Fleisher LD, Godmilow L, et al. Duty to re-contact. Genet Med. 1999;1:171–172.

The supplementary materials for the "Technical laboratory standards for the interpretation and reporting of constitutional CNVs" are listed below (with hyperlinks to the individual supplements):

1. **Supplemental Figures**
2. **Supplemental Material 1: Using the copy number variation (CNV) scoring metrics**
3. **Supplemental Material 2: Validation of the CNV scoring metrics**
4. **Supplemental Material 3: Case examples**
5. **Supplemental Material 4: Example reports**

APPENDIX 7

Interpretation and reporting of large regions of homozygosity and suspected consanguinity/ uniparental disomy, 2021 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

(See following page)

Genetics in Medicine (2022) 24, 255–261

ELSEVIER

Genetics in Medicine

www.journals.elsevier.com/genetics-in-medicine

ACMG TECHNICAL STANDARD

Interpretation and reporting of large regions of homozygosity and suspected consanguinity/uniparental disomy, 2021 revision: A technical standard of the American College of Medical Genetics and Genomics (ACMG)

Patrick R. Gonzales[1], Erica F. Andersen[2,3], Teneille R. Brown[3], Vanessa L. Horner[4], Juli Horwitz[5], Catherine W. Rehder[6], Natasha L. Rudy[7], Nathaniel H. Robin[7], Erik C. Thorland[8]; on behalf of the ACMG Laboratory Quality Assurance Committee[9,*]

Disclaimer: This technical standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this technical standard is voluntary and does not necessarily assure a successful medical outcome. This technical standard 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, clinical laboratory geneticists should apply their 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 this technical standard. They also are advised to take notice of the date any particular technical standard 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.

ARTICLE INFO

Article history:
Received 5 October 2021
Accepted 6 October 2021
Available online 3 December 2021

Keywords:
Chromosomal microarray
Consanguinity
Homozygosity
Sequencing
Uniparental disomy

ABSTRACT

Genomic testing, including single-nucleotide variation (formerly single-nucleotide polymorphism)–based chromosomal microarray and exome and genome sequencing, can detect long regions of homozygosity (ROH) within the genome. Genomic testing can also detect possible uniparental disomy (UPD). Platforms that can detect ROH and possible UPD have matured since the initial American College of Medical Genetics and Genomics (ACMG) standard was published in 2013, and the detection of ROH and UPD by these platforms has shown utility in diagnosis of patients with genetic/genomic disorders. The presence of these segments, when distributed across multiple chromosomes, may indicate a familial relationship between the proband's parents. This technical standard describes the detection of possible consanguinity and UPD by genomic testing, as well as the factors confounding the inference of a specific parental relationship or UPD. Current bioethical and legal issues regarding detection and reporting of consanguinity are also discussed.

The Board of Directors of the American College of Medical Genetics and Genomics approved this technical standard on 27 September 2021.
Author Juli Horwitz has retired from LabCorp.
*Correspondence: ACMG. *E-mail address:* documents@acmg.net
Affiliations are at the end of the document.

doi: https://doi.org/10.1016/j.gim.2021.10.004

Introduction

Previous American College of Medical Genetics and Genomics (ACMG) standards addressed the documentation of suspected consanguinity as an incidental finding of genomic testing, when using single-nucleotide variation (formerly single-nucleotide polymorphism)–based chromosomal microarray (CMA) and exome or genome sequencing (ES/GS).[1] Those standards were developed to harmonize practices within the clinical genetics laboratory community when reporting regions of homozygosity (ROH) detected by CMA.[2] At present, the use of CMA and ES/GS for constitutional analysis of children and adults with developmental delay, intellectual disability, congenital anomalies, and neurobehavioral disorders, along with prenatal specimens, is a routine practice.[3-7] Because the detection of ROH is no longer considered an incidental finding, there is an increased need to address the bioethical, social, and legal ramifications of these findings. Standards for the field when reporting ROH consistent with uniparental disomy (UPD) are also addressed.[8] The updated standards presented here are designed to assist clinical laboratories in the management of CMA and ES/GS findings that suggest parental consanguinity or UPD, with an emphasis on detection and reporting results back to the ordering clinician. The standards are not intended to address CMA and ES/GS findings in neoplastic testing.

Methods

The workgroup tasked with this update comprised laboratory geneticists, clinical geneticists, genetic counselors, and a law professor trained in bioethics. This technical laboratory standard was informed by a targeted review of the literature and current guidelines. Resources consulted included PubMed and relevant ACMG guidelines. The workgroup members also used consensus expert opinion and empirical data to inform their recommendations. Conflicts of interest for workgroup members were reviewed per ACMG policy and are listed at the end of the paper. The ACMG Laboratory Quality Assurance Committee reviewed the document, providing further input on the content, and a final draft was presented to the ACMG Board of Directors for review and approval to post on the ACMG website for member comment. Upon posting to the ACMG website, an email and a link were sent to all ACMG members inviting participation in the 30-day open comment process. All members' comments and additional evidence received were assessed by the authors, and these recommendations were incorporated into the document as deemed appropriate. Member comments and author responses were reviewed by representatives of the ACMG Laboratory Quality Assurance Committee and the ACMG Board of Directors. The final document was approved for publication by the ACMG Board of Directors.

Significance of ROH

Most CMA platforms use a combination of probes designed to assess copy number and probes to genotype single-nucleotide variations. In addition to copy number changes (ie, deletions, duplications, amplifications), these array platforms can identify ROH, often in the form of 1 or more long contiguous stretches of homozygosity. Detection of ROH is also possible using next-generation sequencing methods, including ES/GS.[9-12] These autozygous segments, synonymous with identity by descent (IBD), originate from a common ancestor and may indicate a consanguineous relationship between the proband's parents.[13] Consanguinity confers increased homozygosity, which leads to an increased risk of autosomal recessive (AR) disorders.[14] The detection of ROH can lead to the identification of AR candidate loci.[13,15,16] When observed on a single chromosome, large ROH can be indicative of UPD.[16] More commonly, when unique ROH are observed to be distributed throughout the genome, they represent segments of autozygosity/IBD. Detection of ROH and UPD by these platforms has become a useful clinical tool in the diagnosis of patients with genetic/genomic disorders.[17] In a study with a large population of patients tested for a broad range of clinical conditions, including intellectual disability and congenital anomalies, approximately 4.4% of tested samples (651/14,574 consecutive cases) showed multiple ROH of >10 Mb in length suggesting IBD, and ~1.2% showed ROH of >10 Mb on a single chromosome suggestive of UPD.[18] However, the frequency of UPD in newborns is estimated to be quite rare, ~1 in 3500 births (0.029%).[19] Effects of UPD can vary based on whether the chromosome in question is imprinted (eg, chromosome 15 and Prader-Willi or Angelman syndrome)[8] or if the presence of 2 identical copies of a parental chromosome can unmask deleterious recessive alleles and lead to AR disorders (eg, neonatal diabetes mellitus and congenital hypothyroidism; *GLIS3* at 9p24.2).[6] Detection of these homozygous regions by CMA may lead to a recommendation of additional diagnostic confirmation by ES/GS[18,20] or molecular confirmation of putative UPD.[21] Results obtained may reveal a familial relationship or consanguinity between parents. The findings may provide evidence of abuse, especially if 1 parent is a minor at the time of conception, vulnerable, or intellectually disabled.[15,22] However, laboratories should consider the possibility of marriage between first cousins, which is legal in many states within the United States and practiced in many cultures.[23] It is estimated that approximately one-fifth of the global population resides in communities in which consanguineous matches are traditional and a cultural norm. Such populations include, but are not limited to, Middle Eastern, East Indian, and North African ancestry.[23-26]

Detection of Consanguinity

The clinical suspicion for an AR etiology should be high when evaluating a child referred to genetics with clinical signs and symptoms of an illness, born to healthy consanguineous parents.[27] Genomic regions that are IBD originate from a common ancestor, and the proportion of the genome that is autozygous correlates with parental relatedness. The average proportion of the autosomal genome that is IBD in offspring of related parents is given by the coefficient of inbreeding (F).[28] For example, on average, 6.25% (1/16th) of the genome in an offspring of first cousins (F = 1/16) is IBD. Given these percentages of IBD, the offspring of first-cousin (F = 1/16) and double-first-cousin (F = 1/8) mating will be identified by ROH in CMA and ES/GS testing. While the coefficient of inbreeding provides a theoretical value,[28] significant deviations from the expected values do occur.

Because smaller ROH (<3 Mb) spread throughout the genome are common even in outbred populations, laboratories typically set a size threshold of >3 to 5 Mb under which segments are not considered significant.[13,29,30] The size threshold may be platform-dependent; for example, it has been demonstrated that lower density microarrays may overestimate ROH,[18,31] so for lower density arrays a larger size threshold may be needed. Hypothetically, in the offspring of a second-cousin mating, an average of four 12.5 Mb ROH per genome will be present, although both the number and the size of homozygous segments can be highly variable.[28] When ROH involving multiple chromosomes is 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 (approximately 2881 Mb for GRCh37/hg19). The sex chromosomes are typically excluded from the calculation as males have 1 X and 1 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 size threshold set by the laboratory may be flagged for inclusion in the calculation.[13] This percentage can then be compared to the theoretical value derived from the coefficient of inbreeding for any given parental relationship.[28]

Because recombination during meiosis is a somewhat random process, the variation from the theoretical value increases with each meiosis,[25] such that in some cases, 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 standard deviation is 2.43%.[28] These expected percentages are based on a single common ancestor in an outbred population; 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 and may be caused by stochastic events, multiple loops of consanguinity, small gene pools, and unknown family structures (adoptions, misattributed paternity, etc).[28] Certain populations that have gone through a population bottleneck, eg, Native American populations, typically have at least 1 large ROH due to this.[32] 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. CMA 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.[1]

Concerns for abuse arise when IBD proportions suggest that the parents of the proband are first- or second-degree relatives, particularly when 1 parent is a minor at the time of conception, vulnerable, or intellectually disabled. Among the progeny of first-degree (F = 1/4; 0.25) and second-degree (F = 1/8; 0.125) relatives, the number of meioses separating the parents is sufficiently low, such that the standard deviation is relatively low. Therefore, when high percentages of the genome (≥10%) are IBD and several large segments of homozygosity are present, it is reasonable to suspect a close parental relationship.[1]

Detection of UPD

UPD occurs when both homologs of a chromosome are inherited from 1 parent, typically through defects in segregation of homologous chromosomes in meiosis via nondisjunction.[33] The inheritance of 2 homologous/nonidentical copies of a parental chromosome via nondisjunction in meiosis I leads to heterodisomy, whereas both nondisjunction in meiosis II and monosomy rescue can result in isodisomy.[8] The most common mechanism for UPD is trisomy rescue or reduction to disomy in a conceptus derived from a fertilization resulting in 3 copies of a given chromosome.[21] Although rare, monosomy rescue can occur in a conceptus with a monosomic chromosome after fertilization, which is increased to disomy by duplication.[16] UPD of chromosomes with clinical relevance include chromosomes 6, 7, 11, 14, 15, and 20, with imprinting or parent-of-origin effects leading to aberrant expression/repression of certain genes or genomic regions.[8]

UPD is suspected based on ROH detectable by various genomic technologies, including CMA and ES/GS.[8-12,21] Isodisomy is detected as a large ROH, typically on a single chromosome, including the pericentromeric region, and in some cases the entire chromosome.[16,34] In contrast, heterodisomy may be detected by 1 or more large ROH on a single chromosome that does not include the pericentromeric region.[16,34] However, because UPD is not always accompanied by large ROH, up to one-third of all UPD cases may be undetectable using CMA.[8,21] Recent literature has proposed reporting criteria for different genomic testing

platforms, including CMA[8,21,35] and ES/GS.[8] For postnatal CMA detection of UPD, Hoppman et al[21] proposed the following: telomeric ROH cutoffs of ≥5 Mb for any chromosome, with increased scrutiny for any possible telomeric ROH on imprinted chromosomes; ≥10 Mb for interstitial ROH on imprinted chromosomes; ≥15 Mb for interstitial ROH on nonimprinted chromosomes. Hoppman et al[21] did not propose cutoffs for multiple interstitial ROH on a single chromosome, which suggest UPD, but referred to Papenhausen et al[16] who proposed using an additive cutoff of >15 Mb for multiple interstitial ROH on 1 chromosome. For prenatal CMA testing, Wang et al[35] proposed the following: presence of ROH on a single, entire chromosome (isodisomy), and a single large (≥20 Mb) or multiple segments of ROH on a single chromosome (uniparental isodisomy and heterodisomy [iso/hetero UPD]). Del Gaudio et al[8] discussed UPD results via ES/GS in excess of 10 Mb and recommended that they be reported as nondiagnostic findings when such findings are consented to, with recommendations for confirmation by a clinically validated orthogonal genomic assay. In any instance of possible UPD result, follow-up testing is indicated to rule out false positives.[8]

Recommendations for Pretest Counseling

It is recommended that each patient/family undergoing CMA and ES/GS testing receive pretest counseling. The consenting process for CMA and other genomic testing should include the possibility of revealing ROH/consanguinity and/or UPD.

Recommendations for Reporting Findings of Consanguinity to the Ordering Clinician

It is important to recognize that detection of 1 or more ROH, in and of itself, is not diagnostic for a particular genetic disorder. However, the detection of segments that are homozygous does increase the likelihood that the proband has inherited 2 copies of a deleterious allele for an AR disorder. Clinicians may find utility in this knowledge if the patient's phenotype matches that of an AR disorder for which 1 or more candidate genes are located within 1 of these segments.[22,36] 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 in ROH designated by the clinician that may be relevant to the proband's phenotype.[1] Laboratories should set a cutoff for the percentage of homozygosity that is reported as excess homozygosity detected. A cutoff of 2% to 3% of the autosomal genome for reporting ROH is recommended based on the progeny of second cousins, in whom the average percentage of the genome that is IBD is 1.56%, using segmental ROH cutoffs of >3 to 5 Mb to account for possible ethnicity-specific or isolated population loops of ancestral consanguinity.[30] Given that consanguineous matches occur frequently in many cultures,[30,32] the presence of excess homozygosity should not be the final diagnosis for the proband. Instead, the information may be used to help determine the most likely regions within the genome that harbor AR variants consistent with the proband's phenotype. 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 ROH, regardless of size or mechanism, which can include deletions. This automated calculation is also typically inflated by small ROH that are more likely representative of regions of suppressed recombination or linkage disequilibrium (identity by state). Limiting this calculation to segments >3 to 5 Mb is more likely to result in the inclusion of segments that are truly IBD.[30] However, at the discretion of the laboratory director, regions below the cutoff may be reported for certain cases. In general, larger ROH may harbor diagnostic recessive variants.[6] Because there is typically little phenotypic information available to correlate between genes in putative homozygous regions and possible homozygous variants in fetal testing via prenatal CMA, a cutoff of ROH >5% of the autosomal genome in fetal testing is recommended. A >5% reporting threshold will be sufficient to cover most first-cousin (6.25% ± 2.43%) and closer matings[28] where the known risk of AR disorders starts to rise significantly.[13]

Special Considerations

The observation of a possible first- or second-degree parental relationship, particularly when 1 parent of the proband is known to be a minor at the time of conception, vulnerable, or intellectually disabled, raises a suspicion for abuse involving that parent. For pediatric specimens, laboratories do not typically have information regarding the parents' ages, intellectual status, or family structure; therefore, they do not have adequate information to communicate a suspicion for abuse to any authoritative agency. Thus, when the percentage of homozygosity reaches a level that could be consistent with a first- or second-degree parental relationship (>10% ROH with multiple ROH of >3–5 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[1]:

"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 parental relationship is suspected based on the results of the analysis. The clinician is the most appropriate person to correlate laboratory results with family history and to investigate any concern for abuse. 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.[1]

Given that the analysis of ROH can reveal possible incidents of incest, ethical and legal issues must be taken into consideration. Grote et al[37] addressed variable approaches to genetic counseling when addressing CMA findings of ROH associated with putative parental relatedness. Because the detection of ROH and possible UPD has clinical utility, the possibility of identifying ROH should be addressed as part of the standard of care within the informed consent process. Through this process, the proband's parents or guardians should be counseled on the possibility of findings such as ROH that suggest parental consanguinity. Although this may have medical implications (eg, raising the likelihood of an AR disease), it may also suggest an incestuous relationship. If the parent of the proband being tested was a minor at the time of conception, had diminished mental capacity themselves, or was otherwise considered vulnerable, then this may indicate criminal abuse. In such circumstances, there may be a legal obligation to report these findings to welfare agencies. There is no uniform law that dictates what must be reported, although the federal Child Abuse Prevention and Treatment Act does require each state to develop a system for mandatory reporting.[38] Because the relevant statutes are state-based, they vary as to who must report, when reporting must occur, and what exactly must be reported. However, if neither parent was a minor, intellectually disabled, or considered vulnerable, a finding of consanguinity in the proband is not to be considered reasonable evidence of abuse and not subject to mandatory disclosure. In addition, marriage between first cousins is legal in some states within the United States, and some ethnic groups have cultural norms of consanguinity, thus these possibilities must also be considered.[23]

Violations for failing to report also vary but can include criminal penalties.[39] Notably, a lack of certainty regarding the occurrence of incest will typically not excuse reporting obligations. In most states, a duty to report is triggered when there is a reasonable suspicion of incest.[40] In some states, mandatory reporters include anyone who has a reasonable suspicion that child abuse has occurred,[41] and other states list specific mandatory reporters, such as those engaged in the healing arts[42] or employees at universities or the hospitals themselves.[43]

Further, the mandatory disclosure is not expected to violate physician–patient confidentiality or the privacy rule of the Health Insurance Portability and Accountability Act because several courts have found that confidentiality must give way to the need to report.[44] Most state laws provide broad immunity from a civil suit for those who report in good faith.[45] Even so, mandatory reporters should reveal only the minimum amount of information to comply with the statute, so as to maximally protect patient confidentiality. All individuals with access to genetic information indicating suspected incest should consult their state's reporting requirements.

Although relaying sensitive information of this nature to patients and families is commonplace for medical geneticists and genetic counselors, they must be aware of the legal and ethical implications as well. Even in the absence of a legal duty to report potential abuse, there may be ethical obligations based on nonmaleficence or autonomy. The potential for criminal prosecution will complicate what is already a difficult conversation. It is often wise to include the institution's social worker to assist with the provision of follow-up social services as well as legal counsel to be sure that all legal requirements are accurately and completely followed. Laboratories with findings suggestive of incest should also consider how to best report this sensitive information to clinicians. In some cases, it might be necessary to use the word incest itself rather than potentially obfuscating this information in terms such as ROH or even consanguinity.

Conclusion

The ability to detect ROH is an important clinical tool, with clear utility in the context of the detection of AR conditions and UPD. A secondary consequence of this observation is the possible discovery of a consanguineous relationship between the proband's parents. This possibility should be a point of emphasis in pretest counseling. Although a specific relationship cannot be determined using 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.[1]

Conflict of Interest

The authors declare no conflicts of interest.

Affiliations

[1]University of Kansas Medical Center, Kansas City, KS; [2]ARUP Laboratories, Salt Lake City, UT; [3]The University

of Utah, Salt Lake City, UT; [4]University of Wisconsin-Madison, Madison, WI; [5]Labcorp, Santa Fe, NM; [6]Duke University Health System, Durham, NC; [7]The University of Alabama at Birmingham, Birmingham, AL; [8]Mayo Clinic, Rochester, MN; [9]American College of Medical Genetics and Genomics, Bethesda, MD

References

1. 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):150–152. http://doi.org/10.1038/gim.2012.169.
2. Grote L, Myers M, Lovell A, Saal H, Lipscomb Sund KL. Variability in laboratory reporting practices for regions of homozygosity indicating parental relatedness as identified by SNP microarray testing. *Genet Med*. 2012;14(12):971–976. http://doi.org/10.1038/gim.2012.83.
3. South ST, Lee C, Lamb AN, Higgins AW, Kearney HM. ACMG Standards and Guidelines for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013. *Genet Med*. 2013;15(11):901–909. http://doi.org/10.1038/gim.2013.129.
4. South ST, Brothman AR. Clinical laboratory implementation of cytogenomic microarrays. *Cytogenet Genome Res*. 2011;135(3-4):203–211. http://doi.org/10.1159/000331425.
5. Marchuk DS, Crooks K, Strande N, et al. Increasing the diagnostic yield of exome sequencing by copy number variant analysis. *PLoS One*. 2018;13(12):e0209185. http://doi.org/10.1371/journal.pone.0209185.
6. Wakeling MN, Laver TW, Wright CF, et al. Homozygosity mapping provides supporting evidence of pathogenicity in recessive Mendelian disease. *Genet Med*. 2019;21(4):982–986. https://doi.org/10.1038/s41436-018-0281-4.
7. Malinowski J, Miller DT, Demmer L, et al. Systematic evidence-based review: outcomes from exome and genome sequencing for pediatric patients with congenital anomalies or intellectual disability. *Genet Med*. 2020;22(6):986–1004. http://doi.org/10.1038/s41436-020-0771-z.
8. Del Gaudio D, Shinawi M, Astbury C, et al. Diagnostic testing for uniparental disomy: a points to consider statement from the American College of Medical Genetics and Genomics (ACMG). *Genet Med*. 2020;22(7):1133–1141. http://doi.org/10.1038/s41436-020-0782-9.
9. Carr IM, Bhaskar S, O'Sullivan J, et al. Autozygosity mapping with exome sequence data. *Hum Mutat*. 2013;34(1):50–56. http://doi.org/10.1002/humu.22220.
10. King DA, Fitzgerald TW, Miller R, et al. A novel method for detecting uniparental disomy from trio genotypes identifies a significant excess in children with developmental disorders. *Genome Res*. 2014;24(4):673–687. http://doi.org/10.1101/gr.160465.113.
11. Bis DM, Schüle R, Reichbauer J, et al. Uniparental disomy determined by whole-exome sequencing in a spectrum of rare motoneuron diseases and ataxias. *Mol Genet Genomic Med*. 2017;5(3):280–286. http://doi.org/10.1002/mgg3.285.
12. Retterer K, Juusola J, Cho MT, et al. Clinical application of whole-exome sequencing across clinical indications. *Genet Med*. 2016;18(7):696–704. http://doi.org/10.1038/gim.2015.148.
13. 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(4):595–613. https://doi.org/10.1016/j.cll.2011.08.003.
14. Abbas HA, Yunis K. The effect of consanguinity on neonatal outcomes and health. *Hum Hered*. 2014;77(1-4):87–92. http://doi.org/10.1159/000362125.
15. Schaaf CP, Scott DA, Wiszniewska J, Beaudet AL. Identification of incestuous parental relationships by SNP-based DNA microarrays. *Lancet*. 2011;377(9765):555–556. https://doi.org/10.1016/S0140-6736(11)60201-8.
16. Papenhausen P, Schwartz S, Risheg H, et al. UPD detection using homozygosity profiling with a SNP genotyping microarray. *Am J Med Genet A*. 2011;155A(4):757–768. http://doi.org/10.1002/ajmg.a.33939.
17. Sund KL, Rehder CW. Detection and reporting of homozygosity associated with consanguinity in the clinical laboratory. *Hum Hered*. 2014;77(1-4):217–224. http://doi.org/10.1159/000362448.
18. Wang JC, Ross L, Mahon LW, et al. Regions of homozygosity identified by oligonucleotide SNP arrays: evaluating the incidence and clinical utility. *Eur J Hum Genet*. 2015;23(5):663–671. http://doi.org/10.1038/ejhg.2014.153.
19. Liehr T. *Uniparental Disomy (UPD) in Clinical Genetics: A Guide for Clinicians and Patients*. Springer-Verlag; 2014.
20. Alabdullatif MA, Al Dhaibani MA, Khassawneh MY, El-Hattab AW. Chromosomal microarray in a highly consanguineous population: diagnostic yield, utility of regions of homozygosity, and novel mutations. *Clin Genet*. 2017;91(4):616–622. http://doi.org/10.1111/cge.12872.
21. Hoppman N, Rumilla K, Lauer E, Kearney H, Thorland E. Patterns of homozygosity in patients with uniparental disomy: detection rate and suggested reporting thresholds for SNP microarrays. *Genet Med*. 2018;20(12):1522–1527. http://doi.org/10.1038/gim.2018.24.
22. Sund KL, Zimmerman SL, Thomas C, et al. Regions of homozygosity identified by SNP microarray analysis aid in the diagnosis of autosomal recessive disease and incidentally detect parental blood relationships. *Genet Med*. 2013;15(1):70–78. http://doi.org/10.1038/gim.2012.94.
23. Oniya O, Neves K, Ahmed B, Konje JC. A review of the reproductive consequences of consanguinity. *Eur J Obstet Gynecol Reprod Biol*. 2019;232:87–96. http://doi.org/10.1016/j.ejogrb.2018.10.042.
24. Hamamy H. Consanguineous marriages: preconception consultation in primary health care settings. *J Commun Genet*. 2012;3(3):185–192. http://doi.org/10.1007/s12687-011-0072-y.
25. McQuillan R, Leutenegger AL, Abdel-Rahman R, et al. Runs of homozygosity in European populations. *Am J Hum Genet*. 2008;83(3):359–372. https://doi.org/10.1016/j.ajhg.2008.08.007.
26. Fan YS, Ouyang X, Peng J, et al. Frequent detection of parental consanguinity in children with developmental disorders by a combined CGH and SNP microarray. *Mol Cytogenet*. 2013;6(1):38. http://doi.org/10.1186/1755-8166-6-38.
27. Alkuraya FS. Discovery of rare homozygous mutations from studies of consanguineous pedigrees. *Curr Protoc Hum Genet*; 2012 Chapter 6: Unit6.12. https://doi.org/10.1002/0471142905.hg0612s75.
28. 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. http://doi.org/10.1111/j.1469-1809.2006.00263.x.
29. Hildebrandt F, Heeringa SF, Rüschendorf F, et al. A systematic approach to mapping recessive disease genes in individuals from outbred populations. *PLoS Genet*. 2009;5(1):e1000353. http://doi.org/10.1371/journal.pgen.1000353.
30. Pemberton TJ, Absher D, Feldman MW, Myers RM, Rosenberg NA, Li JZ. Genomic patterns of homozygosity in worldwide human populations. *Am J Hum Genet*. 2012;91(2):275–292. http://doi.org/10.1016/j.ajhg.2012.06.014.
31. 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(9):706–712. http://doi.org/10.1038/gim.2013.36.
32. Ceballos FC, Joshi PK, Clark DW, Ramsay M, Wilson JF. Runs of homozygosity: windows into population history and trait architecture. *Nat Rev Genet*. 2018;19(4):220–234. http://doi.org/10.1038/nrg.2017.109.
33. Yamazawa K, Ogata T, Ferguson-Smith AC. Uniparental disomy and human disease: an overview. *Am J Med Genet C Semin Med Genet*. 2010;154C(3):329–334. http://doi.org/10.1002/ajmg.c.30270.

34. Conlin LK, Thiel BD, Bonnemann CG, et al. Mechanisms of mosaicism, chimerism and uniparental disomy identified by single nucleotide polymorphism array analysis. *Hum Mol Genet*. 2010;19(7):1263–1275. http://doi.org/10.1093/hmg/ddq003.
35. Wang JC, Radcliff J, Coe SJ, Mahon LW. Effects of platforms, size filter cutoffs, and targeted regions of cytogenomic microarray on detection of copy number variants and uniparental disomy in prenatal diagnosis: results from 5026 pregnancies. *Prenat Diagn*. 2019;39(3):137–156. http://doi.org/10.1002/pd.5375.
36. Wierenga KJ, Jiang Z, Yang AC, Mulvihill JJ, Tsinoremas NF. A clinical evaluation tool for SNP arrays, especially for autosomal recessive conditions in offspring of consanguineous parents. *Genet Med*. 2013;15(5):354–360. http://doi.org/10.1038/gim.2012.136.
37. Grote L, Myers M, Lovell A, Saal H, Sund KL. Variable approaches to genetic counseling for microarray regions of homozygosity associated with parental relatedness. *Am J Med Genet A*. 2014;164A(1):87–98. http://doi.org/10.1002/ajmg.a.36206.
38. 42 USC 5106a: Grants to States for child abuse or neglect prevention and treatment programs. The Child Abuse Prevention and Treatment Act 1974. https://uscode.house.gov/view.xhtml?req=(title:42%20section:5106a%20edition:prelim. Accessed November 18, 2021.
39. Tinsley JE. Failure to report suspected case of child abuse. *Am Jur 2d*. 1975;6:345 (updated 2019).
40. Shotton AC. Making reasonable efforts in child abuse and neglect cases: ten years later. *Calif West Law Rev*. 1990;26(2):223–256.
41. Utah Code Annotated § 62A-4a-403 (2021). https://le.utah.gov/xcode/Title62A/Chapter4a/62A-4a-S403.html. Accessed November 18, 2021.
42. California Penal Code § 11165.7 (2021). https://leginfo.legislature.ca.gov/faces/codes_displaySection.xhtml?sectionNum=11165.7.&lawCode=PEN. Accessed November 18, 2021.
43. Alabama code title 26. Infants and incompetents § 26-14-3 (2021). FindLaw. Updated January 1, 2019. https://codes.findlaw.com/al/title-26-infants-and-incompetents/al-code-sect-26-14-3.html. Accessed November 18, 2021.
44. Malia TR. Validity, construction, and application of statute limiting physician–patient privilege in judicial proceedings relating to child abuse or neglect. *ALR (4th)*. 1986;44:649 (updated 2019).
45. Veilleux DR. Annotation, validity, construction, and application of state statute requiring doctor or other person to report child abuse. *ALR (4th)*. 1989;73:782 (updated 2019).

APPENDIX 8

Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC)

(See following page)

ACMG TECHNICAL STANDARD | Genetics inMedicine

Technical laboratory standards for interpretation and reporting of acquired copy-number abnormalities and copy-neutral loss of heterozygosity in neoplastic disorders: a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC)

Fady M. Mikhail, MD, PhD[1], Jaclyn A. Biegel, PhD[2], Linda D. Cooley, MD, MBA[3], Adrian M. Dubuc, PhD[4], Betsy Hirsch, PhD[5], Vanessa L. Horner, PhD[6], Scott Newman, PhD[7], Lina Shao, MD, PhD[8], Daynna J. Wolff, PhD[9] and Gordana Raca, MD, PhD[2]

The detection of acquired copy-number abnormalities (CNAs) and copy-neutral loss of heterozygosity (CN-LOH) in neoplastic disorders by chromosomal microarray analysis (CMA) has significantly increased over the past few years with respect to both the number of laboratories utilizing this technology and the broader number of tumor types being assayed. This highlights the importance of standardizing the interpretation and reporting of acquired variants among laboratories. To address this need, a clinical laboratory-focused workgroup was established to draft recommendations for the interpretation and reporting of acquired CNAs and CN-LOH in neoplastic disorders. This project is a collaboration between the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC). The recommendations put forth by the workgroup are based on literature review, empirical data, and expert consensus of the workgroup members. A four-tier evidence-based categorization system for acquired CNAs and CN-LOH was developed, which is based on the level of available evidence regarding their diagnostic, prognostic, and therapeutic relevance: tier 1, variants with strong clinical significance; tier 2, variants with some clinical significance; tier 3, clonal variants with no documented neoplastic disease association; and tier 4, benign or likely benign variants. These recommendations also provide a list of standardized definitions of terms used in the reporting of CMA findings, as well as a framework for the clinical reporting of acquired CNAs and CN-LOH, and recommendations for how to deal with suspected clinically significant germline variants.

Genetics in Medicine (2019) 21:1903–1915; https://doi.org/10.1038/s41436-019-0545-7

Keywords: copy-number abnormalities; acquired variants; cancer; chromosomal microarray; interpretation

INTRODUCTION

Genomic testing of hematologic malignancies and solid tumors at the time of disease presentation provides information that is crucial for diagnosis and management. This evaluation may include G-banded chromosome analysis, fluorescence in situ hybridization (FISH) analysis, chromosomal microarray analysis (CMA), gene expression and fusion studies, targeted gene sequencing, as well as gene sequencing panels.

The somatic genomic variants detected in the tumor tissue play a critical role in the patient's clinical management by aiding in the diagnosis, providing prognostic information, and helping in the choice of appropriate therapy. The types of somatic variants observed include numerical and structural chromosomal abnormalities, single-nucleotide variants (SNVs), nucleotide-level deletions, duplications and insertions (i.e., indels), and gene-level deletions and duplications. One type of somatic structural chromosomal rearrangements

Correspondence: Fady M. Mikhail (fmikhail@uab.edu). #Affiliations are listed at the end of the paper.

The Board of Directors of the American College of Medical Genetics and Genomics approved this technical standard on 25 March 2019. The Board of Directors of the Cancer Genomics Consortium approved this technical standard on 27 March 2019.

Submitted 3 May 2019; accepted: 5 May 2019
Published online: 29 May 2019

common in neoplastic disorders are copy-number abnormalities (CNAs), which are changes that represent acquired gains and losses of chromosomal material. High-resolution genome-wide CMA is being widely used in clinical laboratories to detect acquired CNAs and copy-neutral loss of heterozygosity (CN-LOH) in neoplastic disorders, and is providing important insights into the unique genomic copy-number profile of different tumor tissues. In recent years, the clinical utility of CMA has been well established in the diagnosis of several neoplastic disorders.[1–7]

The American College of Medical Genetics and Genomics (ACMG) technical standards and guidelines for CMA in neoplastic disorders includes a section on CMA results evaluation and interpretation.[8] This document provided the initial framework for interpreting CMA results in neoplastic disorders; however, it describes broad principles. The use of CMA in neoplastic disorders has significantly increased over the past few years with respect to both the number of laboratories utilizing this technology and the broader number of tumor types being assayed. In practice, the interpretation of CMA results remains relatively subjective and lacks standardization, resulting in inconsistent practices between clinical laboratories. The CNAs and CN-LOH detected by CMA in neoplastic disorders are in many cases critical for optimal patient care. This necessitates standardized interpretation and reporting of acquired variants using an evidence-based system to accurately establish their clinical significance.

To address this need, a clinical laboratory-focused workgroup was established to draft recommendations for the interpretations and reporting of acquired CNAs and CN-LOH in neoplastic disorders. This project is a collaboration between the ACMG Laboratory Quality Assurance (QA) Committee and the Cancer Genomics Consortium (CGC). The workgroup developed recommendations for categorizing CNAs and CN-LOH detected by CMA in neoplastic disorders into specific standard categories (i.e., tiers) of clinical significance based on objective criteria using an evidence-based weighting system. The term "acquired variants" is used in this document to refer to both acquired CNAs and CN-LOH.

These newly developed recommendations, which are described in detail below, include:

1. Standardized definitions of terms used to describe single variants or patterns of variants detected by CMA
2. A four-tier evidence-based categorization system for acquired CNAs and CN-LOH, which is based on the level of available evidence regarding their diagnostic, prognostic, and therapeutic relevance
3. CNA and CN-LOH examples in tiers 1 and 2 in various hematologic malignancies and solid tumors
4. Considerations regarding the interpretation and reporting of unanticipated clinically significant germline variants
5. A framework to standardize the clinical reporting of acquired CNAs and CN-LOH

Even though these proposed technical laboratory standards are intended for interpretation and reporting of acquired variants detected by CMA in neoplastic disorders, the newly developed recommendations should be applicable to acquired structural variants (including CNAs) detected by sequencing-based approaches, as the clinical testing practices move increasingly toward these technologies.

METHODS

These technical laboratory standards were informed by a review of the literature and current guidelines. Resources consulted included PubMed; current World Health Organization (WHO) and National Comprehensive Cancer Network (NCCN) guidelines; and relevant ACMG, Association for Molecular Pathology (AMP), and College of American Pathologists (CAP) guidelines. The workgroup members also used their expert opinion and empirical data to inform their recommendations. The proposed four-tier evidence-based categorization system for CNAs and CN-LOH was refined and extensively tested among the workgroup members using clinical cases from the members' diagnostic laboratories. In addition, input from the greater cancer genomics community was solicited during the annual CGC meeting when this tier system was first presented. Any conflicts of interests for workgroup members are listed at the end of the paper. The ACMG Laboratory QA Committee reviewed the document providing further input on the content, and a final draft was presented to the ACMG Board of Directors for review and approval to post on the ACMG website for member comment. Upon posting to the ACMG website, an email and link were sent to all ACMG members inviting participation in the 30-day open comment process. All members' comments and additional evidence received were assessed by the authors, and these recommendations were incorporated into the document as deemed appropriate. Member comments and author responses were reviewed by representatives of the ACMG Laboratory QA Committee and the ACMG Board of Directors. The final document was approved for publication by the ACMG and the CGC Board of Directors.

DEFINITIONS OF SPECIFIC TERMS USED TO DESCRIBE SINGLE VARIANTS OR PATTERNS OF VARIANTS DETECTED BY CMA

In an attempt to standardize the terminology used to communicate results of clinical CMA, the workgroup reached consensus on the definitions of specific terms shown in Box **1**, which describe genomic variants commonly detected by CMA in neoplastic disorders.[9–11]

PROPOSED FOUR-TIER EVIDENCE-BASED CATEGORIZATION SYSTEM FOR ACQUIRED CNAS AND CN-LOH

The interpretation of clinical significance of acquired genomic variants is based on their impact on clinical care, including diagnostic, prognostic, and therapeutic significance. The weight of clinical impact of a genomic variant is gauged by the level of available evidence regarding its association with a specific diagnosis, disease outcome, and/or response to a

Box 1: Specific terms used to describe single variants or patterns of variants detected by CMA

- **Size/location of variant:**

  Focal: Relatively small change (typically less than 5 Mb) that usually contains a known or suspected driver cancer gene
  Whole arm: Change that involves the entire chromosome short (p) or long (q) arm
  Whole chromosome: Change that involves the entire chromosome
  Interstitial: Change mediated by at least two breaks within a chromosome p or q arm
  Terminal: Change that includes the end of the p or q arm of the chromosome
  Intragenic: Change that occurs within a single gene
  Proximal/distal: Describes a position relative to the centromere and moving outward on the chromosome p or q arm

- **Type of variant:**

  Gain/loss: Type of copy-number change observed. It is recommended that the term "gain" be used rather than "duplication." Attempts should be made to determine the relative gain/loss in polyploid samples.
  Copy-number abnormalities (CNAs): Neoplastic disease-associated changes that represent acquired gains or losses of chromosome material.
  Copy-neutral loss of heterozygosity (CN-LOH): Allelic imbalance without an associated copy-number change. This is a somatic process occurring in tumors, and terms such as absence of heterozygosity (AOH), identity by descent (IBD), and uniparental disomy (UPD) should be used when the change is germline.
  Amplification: High copy-number gain of sequences, typically containing oncogene(s) that are important for the cancer being studied. Note that the term should not be used to describe a single copy gain of chromosomal material or to describe gain due to polysomy. Standard thresholds used to represent amplification typically range from 3–5 fold increases over baseline ploidy (e.g., intrachromosomal amplification of chromosome 21 [iAMP21] in B-ALL) to >100 copies per genome (e.g., *MYCN* amplified neuroblastoma) and will vary depending on the type of tumor. The laboratory should establish specific copy-number threshold cutoffs that will be used to identify clonally amplified regions by correlating CMA results to established methodologies for different tumor types.
  Chromothripsis: A copy-number profile that has alternating copy states in a single region—typically a single chromosome or chromosome arm—that contains at least ten distinct alternating copy-number segments.[9–11]
  Intrachromosomal complexity: Summary of chromosomal regions that include more than two copy-number states, are largely confined to a single chromosome or chromosome arm, and contain at least five distinct copy-number segments. If clinically significant abnormalities (tiers 1 or 2) fall within a complex region, they may be reported individually.
  Genomic complexity: Pattern of chromosome instability predominantly due to structural alterations resulting in widespread gains and losses of chromosomes or chromosomal regions in the majority of chromosomes.

particular treatment. The evidence used for variant categorization is weighted differently based on its likely impact on clinical decision-making. Additionally, the type and size of study providing this evidence is considered in the interpretation of the variants, with professional practice guidelines, large collaborative studies, and replicated studies carrying more weight than individual case reports. Based on literature review and workgroup consensus, the workgroup proposes categorizing genomic variants detected by CMA in neoplastic disorders into four tiers according to the level of evidence for their clinical significance as described below (Fig. 1). The workgroup adapted the levels of evidence published by the Oxford Centre for Evidence-Based Medicine (CEBM).[12] An attempt was made to keep these newly developed recommendations aligned, to the extent possible, with the recently published standards and guidelines for the interpretation and reporting of sequence variants in cancer.[13] The sequence variant guidelines introduce the concept that the interpretation of somatic variants should focus on their impact on clinical care. In addition to their oncogenic role, they may be associated with a favorable or adverse prognosis, with sensitivity, resistance, or toxicity to a specific therapy, with eligibility for clinical trials, and/or with better diagnostic accuracy. The principles put forward for interpretation of somatic sequence variants are applicable for interpretation of somatic CNAs and CN-LOH, which can also serve as biomarkers of prognosis, sensitivity, or resistance to targeted therapies, and/or can support a diagnosis of a particular tumor type. In addition, there is an increasing trend in genomic oncology testing to use consolidated sequencing-based assays to detect somatic SNVs, indels, CNAs, and abnormal gene fusions in selected cancer-related genes. Reporting results of such integrated assays would not be practical if disparate sets of rules had to be applied for interpretation of SNVs and indels versus CNAs and CN-LOH. With the prediction that unbiased genome-wide evaluation

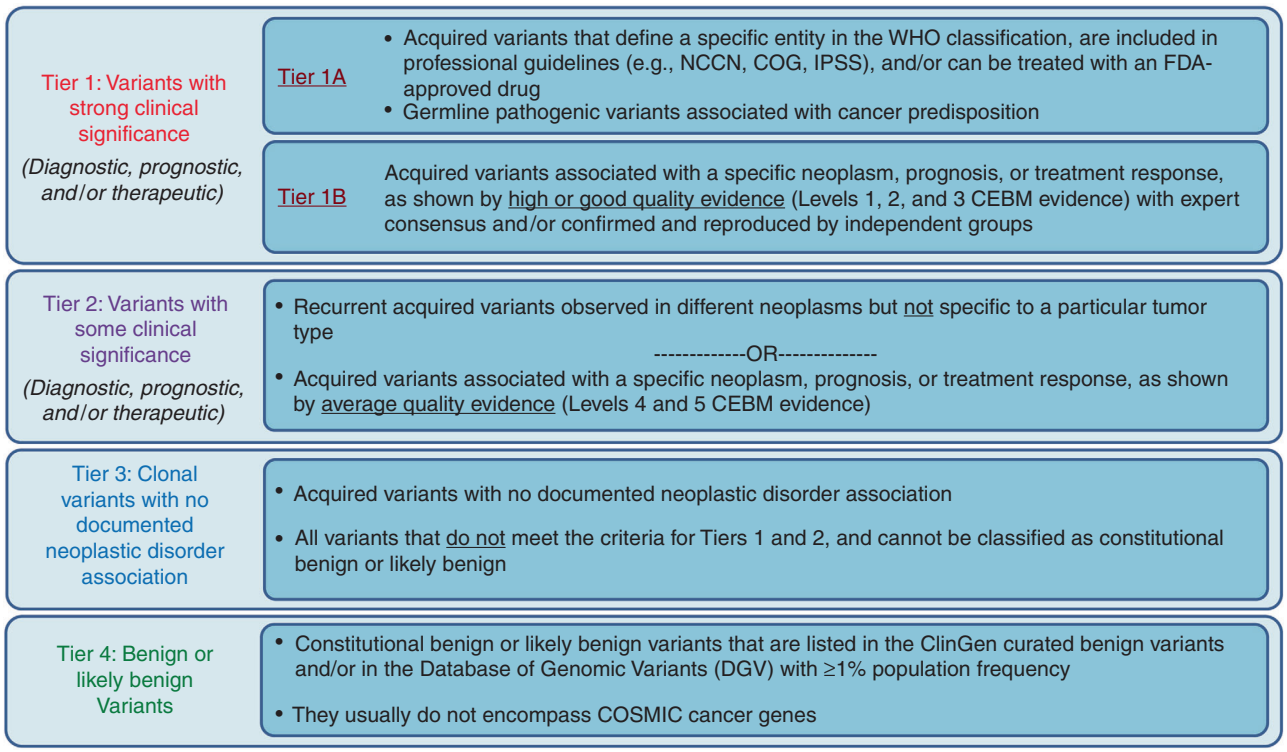

Fig. 1 Four-tier evidence-based categorization system for acquired copy-number abnormalities (CNAs) and copy-neutral loss of heterozygosity (CN-LOH) detected by chromosomal microarray analysis (CMA). *CEBM* Oxford Centre for Evidence-Based Medicine, *COG* Children's Oncology Group, *COSMIC* Catalogue of Somatic Mutations in Cancer, *IPSS* International Prognostic Scoring System for myelodysplastic syndromes, *NCCN* National Comprehensive Cancer Network, *WHO* World Health Organization.

for different types of genetic and genomic variants (including both sequence variants and numerical and structural chromosome rearrangements) may become feasible for cancer samples in the near future, a unified approach for the clinical interpretation, classification, and reporting of all somatic variants will become a necessity.

Tables 1 and 2 provide examples of CNAs and CN-LOH in tiers 1 and 2 in various hematologic malignancies and solid tumors.

I. **Tier 1 (variants with strong clinical significance):** Variants with strong diagnostic, prognostic, and/or therapeutic clinical significance. They have been demonstrated to play a critical role in the oncogenic process under investigation. Based on the level of evidence available, tier 1 variants are further subdivided into:
   a. **Tier 1A:** Acquired variants or a specific pattern of acquired variants that fulfill one or more of the following criteria:
      – Define a specific entity in the WHO classification.
      – Are included in professional clinical practice guidelines as clinically significant variants (e.g., NCCN, Children's Oncology Group (COG), Myelodysplastic Syndromes (MDS) International Prognostic Scoring System, International Myeloma Working Group Criteria).
      – Can be treated by a targeted FDA approved drug. Tier 1A also includes germline pathogenic variants associated with cancer predisposition.

   b. **Tier 1B:** Acquired variants or a specific pattern of acquired variants with either:
      – High quality evidence (levels 1 and 2 CEBM evidence) in the literature that shows association with a specific neoplasm, prognosis, or treatment response. This includes well-powered studies in the form of randomized controlled clinical trials, systematic review and meta-analysis of these studies, and cohort studies with consensus from experts in the field.
      – Good quality evidence (level 3 CEBM evidence) in the literature that shows association with a specific neoplasm, prognosis, or treatment response. This includes multiple (at least two) smaller clinical studies in the form of cohort or case–control studies that have been confirmed and reproduced by different independent groups.

II. **Tier 2 (variants with some clinical significance):** Acquired variants or a specific pattern of acquired

Table 1 Tier examples in hematologic malignancies

Disease	Tier 1A	Reference (PMID)	Tier 1B	Reference (PMID)	Tier 2	Reference (PMID)
Myeloid						
Acute myeloid leukemia (AML)	−5/5q del[P] −7[P]	NCCN 27895058	*KMT2A* (*MLL*) partial tandem duplication[P] 13q CN-LOH[P]	12149299 22417203 26033747 11585760 25270908	9q del[R]	8207990
Myelodysplastic syndromes (MDS)	−5/5q del[D,P,T] −7/7q del[D,P] Trisomy 8[P] 11q del[D,P] 12p del[D,P] −13/13q del[D,P] 17p del/*i*(17q)[D,P]	WHO 2016 NCCN MDS IPSS-R	7q CN-LOH[P] 11q CN-LOH[P]	17954704 21285439	1p CN-LOH[R] 1q gain[R] Trisomy 21[R]	21285439 24123380
Myeloid/lymphoid neoplasms with eosinophilia	4q12 del resulting in *FIP1L1-PDGFRA* fusion[D,P,T]	WHO 2016				
Lymphoid						
B-lymphoblastic leukemia/lymphoma	Xp22.33/Yp11.32 del resulting in *P2RY8-CRLF2* fusion[D (Ph-like),P,T] 5q32q33 del resulting in *EBF1-PDGFRB* fusion[D (Ph-like),P,T] 9q34.1 dup resulting in *NUP214-ABL1* fusion[D (Ph-like),P,T] iAMP21 CNAs pattern[D,P] Hyperdiploid B-ALL with typical pattern of gains[D,P] Hypodiploid B-ALL with typical pattern of losses and doubled near-haploid/hypodiploid B-ALL[D,P]	WHO 2016 25207766	*IKZF1* del (7p12.2)[P] *ERG* del (21q22.2)[D,P]	27815723 26202931 24064621 27776115	*CDKN2A/2B* del (9p21.3)[R] *ETV6* del (12p13.2)[R] *PAX5* del (9p13.2)[R] *RB1* del (13q14.2)[R]	17344859 23508010
T-lymphoblastic leukemia/lymphoma			*TCR* rearrangements with CNAs at the breakpoints or in the unbalanced form[D] 9q34.1 amp resulting in *NUP214-ABL1* fusion[D,P,T] 1p33 del resulting in *STIL-TAL1* fusion[D]	28671688 18835836 17609427 15361874 18923437 19562638 25304610	6q del[R] *CDKN2A/2B* biallelic del (9p21.3)[R]	9552025 2207332 18838613
Chronic lymphocytic leukemia (CLL)	11q22.3 del (*ATM* and/or *BIRC3*)[P,T] Trisomy 12[P] 13q14.2 del (*MIR15A/16-1*)[P] 17p13.1 del (*TP53*)[P,T]	WHO 2016 NCCN	2p12p25.3 gain (*MYCN*)[P] 9p21.3 del (*CDKN2A*) in CLL transformed to RS[P]	19406473 21749360 23044996 24004666 9531609	Trisomy 19[R] 6q del[R] 14q24.1q32.3 del[R]	17593029 21788947 14712287 21281237 10482982 24729385 20649559
Plasma cell neoplasms	Hyperdiploidy with trisomies of odd-numbered chromosomes[P] 1q21 gain[P] −17/17p13.1 del (*TP53*)[P]	IMWG 21292777 21292778 25212883 27249749	1p del[P]	19448682 20929319 23892719 24460694	14q del[R,P] 16q del[R,P]	20616218 27157252 22565645 27157252 23716545
Burkitt-like lymphoma with 11q aberration	11q CNAs pattern[D,P]	WHO 2016 26980727				

This table lists examples of tiers 1 and 2 genomic variants and is not intended to provide a comprehensive list of variants in each disease. It reflects the evidence available at the time the current technical standards were written.

amp amplification, *CNA* copy-number abnormality, *CN-LOH* copy-neutral loss of heterozygosity, *D* diagnostic, *del* deletion, *dup* duplication, *iAMP21* intrachromosomal amplification of chromosome 21, *IMWG* International Myeloma Working Group, *IPSS-R* Revised International Prognostic Scoring System for myelodysplastic syndromes, *NCCN* National Comprehensive Cancer Network, *P* prognostic, *R* recurrent, *RS* Richter syndrome, *T* therapeutic, *WHO* World Health Organization.

Table 2 Tier examples in solid tumors

Disease	Tier 1A	Reference (PMID)	Tier 1B	Reference (PMID)	Tier 2	Reference (PMID)
Central nervous system (CNS)						
Pilocytic astrocytoma	7q34 dup/del resulting in *KIAA1549-BRAF* fusion[D] 17q11.2 del (*NF1*)[GL]	WHO 2016				
Supratentorial ependymoma	11q13.1 del resulting in *C11orf95-RELA* fusion[D]	WHO 2016 25965575	Chromothripsis 11q[D]	24553141 25965575		
Ependymoma	−22/22q12.2 del (*NF2*)[D]	WHO 2016	1q gain[P] 9p21.3 del (*CDKN2A*)[P]	28371821 22338015 20516456		
ETMR, *C19MC*-altered	19q13.42 gain/amp[D] +2 with 19q13.42 gain/amp[D]	WHO 2016				
MB WNT pathway	Monosomy 6[D]	WHO 2016				
MB SHH pathway	9q22.32 del/LOH (*PTCH1*)[D] 10q23.31 del/LOH (*PTEN*)[D] *GLI2* amp[D] *MYCN* amp[D] 10q24.32 del (*SUFU*)[GL] 17p13.1 del/LOH (*TP53*)[D,P,GL]	WHO 2016 25403219	Chromothripsis 17p[D,P]	22265402 24651015 29753700		
MB non-WNT/non-SHH	17p del and/or 17q gain idic(17p11.2)[D] *MYC* amp[D,P] *MYCN* amp[D]	WHO 2016				
Glioblastoma IDH wild type—adult	+7, −10 (*PTEN*)[D,P] 9p21.3 del/LOH (*CDKN2A*)[D,P] −13/13q14.2 del (*RB1*)[D,P] *PDGFRA* amp[D] *EGFR* amp[D]	WHO 2016				
Glioblastoma—pediatric	+7, 17p13.1 del/LOH (*TP53*)[D,P] *PDGFRA* amp[D,P]	WHO 2016 27582545	*MET* amp[D,T]	28966033 27748748		
Oligodendroglioma	1p and 19q co-del[D,T]	WHO 2016				
Meningioma, acoustic neuroma	22q12.2 del (*NF2*)[GL] −22/22q del[D]	WHO 2016	9p del (*CDKN2A*)[P]	11485924 11958372		
Atypical teratoid/rhabdoid tumor	−22/22q del[D] 22q11.23 del/LOH (*SMARCB1*)[D,GL] 19p13.2 del/LOH (*SMARCA4*)[D,GL]	WHO 2016				
Choroid plexus carcinoma	17p13.1 del (*TP53*)[GL]	WHO 2016				
Chordoma	22q11.23 del (*SMARCB1*)[D]	29119645	10q23.31 del (*PTEN*)[D] 9p21.3 del (*CDKN2A*)[D]	24983247 21602918		
Hemangioblastoma	3p25.3 del (*VHL*)[GL]	20301636 (Gene Reviews)				
Pineoblastoma	14q32.13 del (*DICER1*)[D,GL] 13q14.2 del (*RB1*)[GL]	WHO 2016				
Pediatric embryonal tumors						
Neuroblastoma	*MYCN* amp[D,P] 1p del[P] 11q del and 17q gain[D,P] *ALK* amp[T] Near-triploid[P]	26389190 (NCI guidelines)			3p del[P] 14q del[R]	15800319 12538451 11729208
Wilms tumor	11p del/LOH[D,P,GL] 17p13.1 del (*TP53*)[P] 1q gain, 16q del[D,P]	26389282 (NCI guidelines) 20301471 (Gene Reviews)				
Alveolar rhabdomyosarcoma	*PAX-FOXO1* gene fusion amp[P]	22447499				
Renal cell carcinoma (RCC)						
Clear cell RCC	3p25.3 del/LOH (*VHL*)[D] *VHL, FLCN* del[GL]	WHO 2016 26448938 24550497 23797736	14q loss[P] 9p loss[P]	26448938 21725288 26790128 25315157		
Papillary RCC-type I	Gain 7 and 17[D]	WHO 2016 26448938 25790038 28780132			Gain 12, 16, 20, -Y[R]	26448938
Chromophobe RCC	Hypodiploidy with loss 1, 2, 6, 10, 13, 17, 21[D,R] 17p11.2 del (*FLCN*)[GL]	WHO 2016 19562744 26448938				
Breast	*ERBB2* amp[P]	29523670 (NCCN guidelines)	6q25.1 tandem dup resulting in *ESR1-CCDC170* fusion[P]	25099679	*CCND1* amp[T]	26059247
Lung	*EGFR* amp[T]	23552377	6q22.1 del resulting in *GOPC-ROS1* fusion[T] *FGFR1* amp[T] *MET* amp[T,P]	25870798 25535693 21160078 27664533		
Soft tissue						
Liposarcoma, atypical lipomatous tumors	*MDM2, CDK4* amp[D]	WHO 2013				
Desmoid-type fibromatosis	5q22.2 del (*APC*)[GL]	24554300				
Infantile fibrosarcoma			+8, +11, +17, +20[D,R]	11801301		
Lipoblastoma					Gain 8[R]	11549588
Bone						
Osteochondroma	8q24.11 del (*EXT1*)[GL] 11p11.2 del (*EXT2*)[GL]	20301413 (Gene Reviews)				
Osteosarcoma	17p13.1 del (*TP53*)[D]	WHO 2013	*MDM2, CDK4* amp[D]	20196171 21336260		

Table 2 continued

Disease	Tier 1A	Reference (PMID)	Tier 1B	Reference (PMID)	Tier 2	Reference (PMID)
Ewing sarcoma					1q gain, 16q loss^{D} Gain 8^{R}	11672775
Gastrointestinal stromal tumor (GIST)			−1p, −14, −22^{D}	10919666 16982739 23942094		
Mesothelioma	3p21.1 del (*BAP1*)^{GL}	28713672			3p del (*BAP1*)^{R} 9p del (*CDKN2A*)^{R} −22 (*NF2*)^{R}	21642991 26928227 28713672

This table lists examples of tiers 1 and 2 genomic variants and is not intended to provide a comprehensive list of variants in each disease. It reflects the evidence available at the time the current technical standards were written.

amp amplification, *D* diagnostic, *del* deletion, *dup* duplication, *ETMR* embryonal tumor with multilayered rosettes, *GL* germline, *LOH* loss of heterozygosity, *MB* medulloblastoma, *NCCN* National Comprehensive Cancer Network, *NCI* National Cancer Institute, *P* prognostic, *R* recurrent, *T* therapeutic, *WHO* World Health Organization.

variants with some diagnostic, prognostic, and/or therapeutic clinical significance. They include:

- Recurrent variants observed in different neoplasms but *not* specific to a particular tumor type, and usually encompassing Catalogue of Somatic Mutations in Cancer (COSMIC) census cancer genes(s).
- Acquired variants or a specific pattern of acquired variants with average quality evidence (levels 4 and 5 CEBM evidence) in the literature that shows association with a specific neoplasm, prognosis, or treatment response. This includes a small case series or multiple (at least two) case reports that describe the association.

III. **Tier 3 (clonal variants with no documented neoplastic disorder association):** Acquired clonal variants with no documented neoplastic disorder association. All variants that *do not* meet the criteria for tiers 1 and 2 and cannot be classified as constitutional benign or likely benign, can be classified as tier 3 variants. Tier 3 variants are defined as "acquired clonal variants with no documented neoplastic disorder association" rather than "acquired clonal variants with uncertain clinical significance." This is because an "acquired clonal variant" is by default significant for this particular patient because it can be used as a marker for the neoplastic clone to monitor residual disease and/or relapse.

IV. **Tier 4 (benign or likely benign variants):** Constitutional benign or likely benign variants that are listed in the ClinGen curated benign variants and/or in the Database of Genomic Variants (DGV) with ≥1% population frequency, and usually do not encompass COSMIC cancer gene(s). It is not recommended to report tier 4 variants.

GENERAL AND SPECIAL CONSIDERATIONS

1. The interpretation of clinical significance of CNAs and CN-LOH using this tier system should be performed in the context of the clinical/pathologic diagnosis, as well as other laboratory tests including G-banded karyotype, FISH, and other relevant tests. This is crucial because some acquired variants will have different clinical significance in different neoplastic disorders. For example, 1q gain is associated with adverse prognosis in multiple myeloma (MM) (tier 1A),[14,15] while it does not have major prognostic significance in MDS (tier 2).[16] CNAs may also have different clinical significance depending on other cytogenetic or molecular diagnostic abnormalities present in the tumor. For example, loss of chromosome 7 or 7q deletion are typically associated with an inferior outcome in myeloid malignancies (tier 1A), but in acute myeloid leukemia (AML) with a *CBFB* gene rearrangement, they do not appear to significantly change the prognosis (tier 2).[17]
2. This tier system can be used to classify a specific pattern of CNAs and/or CN-LOH that is diagnostic of a specific neoplastic disease entity. This includes a characteristic pattern of whole chromosome gains/losses (e.g., hyperdiploid and hypodiploid B-ALL) and whole chromosome CN-LOH (e.g., doubled hypodiploid/near-haploid B-ALL).[18] It also includes a characteristic signature of gains and losses along one chromosome (e.g., intrachromosomal amplification of chromosome 21 [iAMP21] in B-ALL).[19] The pattern of acquired gains/losses can be classified collectively using the tier system.
3. Diagnostic balanced chromosomal abnormalities (e.g., translocations, inversions, and insertions) detected by G-banded karyotype and/or FISH testing but not by CMA should be discussed in the CMA report but should not be included in the classification using the tier system or listed in the results table/nomenclature string. When present in the unbalanced form and detected by CMA with breakpoints mapping within genes known to be associated with a specific gene fusion, these abnormalities can be classified using the tier system and listed in the results table/nomenclature string (e.g., the presence of an extra copy of the Philadelphia chromosome der(22)t(9;22)(q34;q11.2) in CML or ALL,[18] or an extra copy of the der(21)t(12;21)(p13;q22) in B-ALL, and the unbalanced der(19)t(1;19)(q23;p13) in B-ALL).[20]
4. An interstitial loss or gain involving one chromosome arm with recurring breakpoints in genes known to be involved in a specific gene fusion can be classified using

this tier system (e.g., 4q12 deletion that results in *FIP1L1-PDGFRA* fusion, PAR1 deletion at Xp22.33/Yp11.32 that results in *P2RY8-CRLF2* fusion, and 9q34.1 gain that results in *NUP214-ABL1* fusion).[18,21]

5. Interstitial or terminal losses or gains involving two chromosome arms with breakpoints within genes known to be associated with a specific gene fusion as a result of an interchromosomal rearrangement (e.g., translocation or insertion) or intrachromosomal rearrangement (e.g., inversion) should be interpreted according to the level of supporting evidence. They can be classified using this tier system with later confirmation of the gene fusion by other molecular techniques if there is enough supporting evidence, including the clinical/pathologic diagnosis, visible recurrent rearrangement by G-banded karyotype, and/or other acquired variant known to be associated with the gene fusion in question. In the absence of such supporting evidence, the report should describe the possibility of a gene fusion but without classifying the variants using the tier system until the fusion is confirmed by other molecular techniques.
6. Correlation of the CMA results with the G-banded karyotype and FISH results is strongly recommended because some professional clinical practice guidelines used to classify tier 1A variants are technique specific. For example, some chromosomal abnormalities can only be considered diagnostic/prognostic if detected by G-banded karyotype (e.g., MDS and MM prognostic criteria).
7. CMA has the potential to identify acquired variants associated with comorbid neoplastic disorders. For example, comorbid MDS-related variants may be identified in patients treated for chronic lymphocytic leukemia (CLL) or MM either because of prior therapy or age-related disease. These variants should be interpreted in the context of the clinical/pathologic diagnosis and correlated with G-banded karyotypes from both stimulated and unstimulated CLL or MM cultures. CMA performed in MM on CD138+ enriched cells is helpful in identifying MM-specific acquired variants.[7]
8. The term "CN-LOH" is used in this document to refer to a region with acquired allelic imbalance (i.e., homozygosity) without an associated copy-number change (i.e., copy-neutral), which is a common finding in cancer. The term "copy-neutral" is used to allow distinction from loss of heterozygous single-nucleotide polymorphism (SNP) calls due to a one copy-number loss (i.e., heterozygous deletion). However, in some cases LOH can also be observed with a copy-number gain. Examples include high-level amplification involving only one allele, and the copresence of a clone with trisomy of a particular chromosome and a subclone that lost one copy of that chromosome resulting in whole chromosome LOH.
9. Regions of CN-LOH may have a higher level of clinical significance if they span a gain-of-function variant in an oncogene and/or loss-of-function variant in a tumor suppressor gene documented in this patient. This is especially relevant in laboratories that do integrated reporting of CNAs, regions of CN-LOH, and sequence variants results.

DATABASES AND RESOURCES FOR INTERPRETATION OF CNAS AND CN-LOH IN NEOPLASTIC DISORDERS

A wealth of genomic information has been generated for different tumor types through chromosome analysis and large-scale genome sequencing projects, and the data have been consolidated into many public databases. However, the majority of such databases house information at a gene and variant level, and resources focused on incidence and significance of acquired CNAs and CN-LOH in neoplastic disorders are limited. In the absence of CNA-specific information, gene and variant-centered databases can be used to support interpretation of CNAs involving specific genes.

To allow utilization of gene and variant-focused data for informing interpretation of CNAs and CN-LOH in oncology samples, it is important to annotate the mechanism of action for genes and variants related to cancer. Such mechanisms typically include loss of function of tumor suppressors, gain of function of oncogenes, abnormal gene fusions, and translocations involving regulatory regions. If variants affecting a gene are proven to be loss-of-function variants, it can be extrapolated that a deletion of the same gene or a larger region containing that gene would also confer a loss of function.

A brief overview of resources that are useful in interpretation of CMA results in oncology is provided in Table 3. Such resources include:

1. Databases and data portals focusing directly on acquired CNAs and CN-LOH
2. Databases and data portals focusing on acquired sequence variants, which allow the evaluation of whether specific genes within the region affected by a CNA have been associated with the tumor type of interest
3. Knowledge bases that contain curated information on the significance of individual genes and acquired sequence variants in different tumor types
4. Chromosome-level databases and knowledge bases that compile data from conventional cytogenetic analysis and curations regarding the significance of chromosome aberrations detected by karyotyping
5. Databases of benign and pathogenic germline variants that allow exclusion of benign germline variants and interpretation of germline secondary findings

To facilitate review and interpretation of acquired CNAs data, laboratories are advised to curate and maintain lists of genes and regions of clinical relevance in a variety of tumor types. These lists support comprehensive and efficient recognition of disease-relevant loci, and allow consistency in interpretation. A laboratory can also opt to develop lists of predefined pertinent positives and negatives per tumor

Table 3 Selected databases relevant for interpretation of acquired CNAs

Resource type and utility	Resource name and description	Location (web address)
General/summary	**Video tutorial:** 'Introduction to Publicly Available Knowledge Bases to Aid Interpretations of Genomic Findings in Oncology' Provides overview of types and utility of online resources	Cancer Genomics Consortium YouTube channel: (https://www.youtube.com/watch?v=4dBh1Qkp8os)
Databases and knowledge bases of acquired CNAs in neoplastic disorders (Can be used to search for recurrent CNAs in the tumor of interest)	**The Cancer Genome Atlas (TCGA) Copy Number Portal:** Allows one to search and review high-resolution copy-number data from cancer samples in The Cancer Genome Atlas project	http://portals.broadinstitute.org/tcga/gistic/browseGisticAnalyses
	The Compendium of Cancer Genome Aberrations (CCGA): A knowledge base developed by the Cancer Genomics Consortium that compiles information about clinical significance of CNAs, CN-LOH, and balanced structural abnormalities in different tumors	http://www.ccga.io
Pan-cancer gene list	**Catalog of Somatic Mutations in Cancer (COSMIC) database Cancer Gene Census**	https://cancer.sanger.ac.uk/census
Cancer gene and variant databases and data portals (Can be used to evaluate the role of a particular gene (or genes) within a CNA or CN-LOH region in pathogenesis of the tumor type being tested; these resources may have overlapping data sets (from the same large-scale studies) but offer different solutions for data visualization and searches)	**Catalog of Somatic Mutations in Cancer (COSMIC):** A large source of manually curated somatic variant information hosted by the Sanger Institute; contains data from >35,000 cancer genomes from large-scale genome screening studies including TCGA and the International Cancer Genomics Consortium (ICGC)	http://cancer.sanger.ac.uk/cosmic
	ICGC Data Portal: An international consortium established to launch and coordinate worldwide large-scale genome sequencing projects for various tumor types; data from specific projects is available through the ICGC portal	https://dcc.icgc.org/
	cBioPortal: A source for visualization, analysis, and download of large-scale cancer genomics data sets, initially developed at Memorial Sloan Kettering Cancer Center and now maintained by a multi-institution team	http://www.cbioportal.org/
	National Cancer Institute (NCI) Genomic Data Commons (GDC): An information system that contains genomic and clinical data from NCI-funded projects as the Cancer Genome Atlas (TCGA) and the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program, as well as other cancer studies	https://portal.gdc.cancer.gov/
	PeCan Data Portal (Pediatric Cancer focused): A data portal developed and hosted by St. Jude Children's Research Hospital, which provides interactive visualizations of pediatric cancer variant data from large-scale childhood cancer genomic studies	https://pecan.stjude.org/home
Chromosome-level data sources (Contain data and knowledge about conventional cytogenetic studies in cancer)	**Mitelman Database:** A database that contains karyotype information for >69,000 tumor cases and allows searches based on abnormality, tumor type, and other criteria	https://cgap.nci.nih.gov/Chromosomes/Mitelman
	Atlas of Genetics and Cytogenetics in Oncology and Haematology: An expert curated knowledge base devoted to cytogenetics findings in cancer	http://atlasgeneticsoncology.org/
Knowledge bases with cancer gene and variant curations (Contain expert curated information and summaries about the clinical significance of genes and variants in cancer)	**Information about commonly used knowledge-bases compiled by the Variant Interpretation for Cancer Consortium (VICC):** A driver project of the Global Alliance for Genomics and Health (GA4GH)	http://cancervariants.org/resources/
	Clinical Interpretation of Variants in Cancer (CIViC): An open access, open source, community-driven knowledge base developed by researchers at the Genome Institute at Washington University School of Medicine	http://www.civicdb.org
	My Cancer Genome: A knowledge base developed and hosted by the Vanderbilt University Cancer Center	https://www.mycancergenome.org/
	OncoKB: A knowledge base developed and hosted by the Memorial Sloan Kettering Cancer Center	http://oncokb.org/#/
	Precision Medicine Knowledgebase (PMKB): A knowledge base developed and hosted by the Institute of Precision Medicine at Weill Cornell Medicine	https://pmkb.weill.cornell.edu/
Population database of benign CNVs (Allows to exclude CNVs that are common in the general population)	**Database of Genomic Variants (DGV):** A comprehensive catalog of normal structural variation in the human genome; the database contains copy-number variants and other structural variations identified in healthy control samples	http://dgv.tcag.ca/dgv/app/home
Databases and data portals of genes, variants, and CNVs associated with constitutional genetic disorders (May assist in interpretation of findings that are suspected to be germline)	**dbVar Human Structural Variant Data Hub:** Catalogs CNVs identified through the course of routine clinical cytogenomic testing in postnatal populations, with clinical assertions as classified by the original submitter	https://www.ncbi.nlm.nih.gov/dbvar/content/human_hub/
	DECIPHER (DatabasE of genomiC varIation and Phenotype in Humans using Ensembl Resources): A database of sequence variants or copy-number variants and main clinical findings from patients with genetic disorders	https://decipher.sanger.ac.uk/
	Online Mendelian Inheritance in Man: A catalog of genes implicated in single-gene (Mendelian) disorders	http://www.ncbi.nlm.nih.gov/omim/
	ClinVar: A National Center for Biotechnology Information (NCBI) maintained catalog of variants found in patient samples, with assertions made regarding their clinical significance, information about the submitter, and other supporting data; focused mostly on constitutional variants, may have utility in the interpretation of suspected germline findings	http://www.ncbi.nlm.nih.gov/clinvar/
	ClinGen: A National Institutes of Health (NIH)-funded central resource that defines the clinical relevance of genes and variants for use in precision medicine and research	https://www.clinicalgenome.org/
Sequence repositories (collect, store, and disseminate the nucleotide and amino acid sequence data) and **genome browsers** (provide context and visualization for genome features, such as genes or disease loci)	**NCBI Genome:** A NIH-sponsored sequence repository	https://www.ncbi.nlm.nih.gov/genome
	Ensembl: A genome browser developed and maintained by the European Molecular Biology Laboratory (EMBL)	https://www.ensembl.org/index.html?redirect=no
	UCSC Genome Browser: A genome browser developed and maintained by the University of California–Santa Cruz	https://genome.ucsc.edu/

CNA copy-number abnormalities, *CN-LOH* copy-neutral loss of heterozygosity, *CNV* copy-number variant.

type, and perform systematic careful evaluation for their presence.

The curated clinical-grade disease-specific gene lists can be maintained in both a spreadsheet and .bed file format. It is useful for such lists to be converted into a format compatible with the CMA software, so they can be uploaded and used as custom annotation tracks during case review. This allows quickly recognizing acquired CNAs that contain genes implicated in the tumor of interest. A comprehensive list of genes shown to contain variants causally implicated in cancer (so-called Cancer Gene Census) is maintained in the COSMIC database, and can be downloaded from its website (https://cancer.sanger.ac.uk/census).

Because the databases and data portals for search and visualization of acquired CNAs in neoplastic disorders are rare, clinical interpretation typically requires a review of primary literature. Such interpretation remains a complex and time-consuming task that requires appropriate professional training and certification in clinical cytogenetics and/or molecular diagnostics. It also necessitates familiarity with the CMA assay and an understanding of the specific tumor biology.

CONSIDERATIONS REGARDING INTERPRETATION AND REPORTING OF UNANTICIPATED CLINICALLY SIGNIFICANT GERMLINE VARIANTS

In addition to acquired clonal abnormalities, genome-wide analysis of tumor samples also detects constitutional germline copy-number variants (CNVs) and regions of absence of heterozygosity (AOH). These may include benign population variants, germline CNVs directly related to the neoplasm under investigation (e.g., germline deletions of tumor suppressor genes), and pathogenic CNVs that are diagnostic or predictive of a presymptomatic or unrecognized genetic condition unrelated to the patient's tumor. With the exception of CNVs that are associated with an increased risk of neoplasia, other germline variants are unanticipated and unrelated to the reason for CMA. Referring clinicians must have a clear understanding of the potential for these discoveries; the best practice would also include informing the patients and their families about the possibility of secondary findings before the test is ordered, and implementing a formal informed consent process. Before offering clinical CMA testing for oncology samples, laboratories should develop a process for appropriate follow-up if an unanticipated, likely germline abnormality is observed.

Indications that a detected CNV or AOH may be germline

Distinguishing between acquired CNAs/CN-LOH and constitutional CNVs/AOH in CMA may be challenging. The possibility that a variant may be germline should be considered in the following scenarios:

1. Involvement of 100% of the cells in a sample. Often, acquired variants involve only a subset of cells corresponding to the tumor clone. Review of the log2 ratio and SNP data allows determining if a variant is present in all or only a subset of cells in a sample. However, it is important to be aware of the following caveats:
   a. Some specimens may consist of pure tumor tissue and have acquired variants that involve close to 100% of the cells (e.g., a bone marrow specimen packed with leukemic blasts or a dissected tumor section).
   b. Copy-number losses encompassing cancer predisposition genes are particularly difficult to interpret by CMA alone. For small abnormalities with insufficient SNP data, CMA may not reliably differentiate a heterozygous loss in 100% of the cells from a homozygous loss in 50% of the cells. If CMA shows copy-number losses encompassing cancer predisposition genes, it might not be possible to distinguish between a germline heterozygous deletion of the gene in question in 100% of the cells versus acquired biallelic loss of the gene in 50% of the cells. Frequently encountered examples include the Fanconi anemia/DNA repair pathway genes (including *BRCA1* and *BRCA2*), *NF1*, *RB1*, and *PAX5*. Follow-up interphase FISH analysis using gene-specific probes can be helpful in distinguishing between these two possibilities.
2. Higher proportion of cells involved by a variant than expected by pathologic findings. For hematologic malignancies, a finding may be germline if it appears to involve a significantly greater proportion of cells than that expected based on the blast cell count or degree of involvement determined by morphology or flow cytometry. Correlation with hematopathology and flow cytometry/immunophenotyping data is valuable, and efforts to obtain this information are recommended. For solid tumors, a finding may be germline if the estimate of involvement by CMA is significantly greater than the estimation of tumor cell fraction provided by the submitting pathologist. However, estimating tumor fraction in solid tumors is often challenging and involves subjective judgment; this estimate may not always be perfectly correlated with CMA results.
3. Supporting clinical information may suggest that a CMA variant is germline:
   a. Some tumor types are frequently associated with the presence of predisposing germline variants. Examples include Wilms tumor, tuberous sclerosis complex (TSC1/TSC2) tumors, neurofibromas, adrenocortical carcinoma, and rhabdoid tumor (Supplementary Table 1). Laboratories should have an increased level of suspicion for germline variants when performing CMA for these tumor types.
   b. CNVs/AOH including known cancer predisposition genes (Supplementary Table 1) may be suspected as germline in patients with features of hereditary cancer syndromes, including diagnosis at unusually

young age, development of bilateral or multifocal tumors, or family/personal history of cancer.

c. CNVs involving genes and regions associated with known pathogenic microdeletion/microduplication syndromes may be suspected as germline in patients who have reported features consistent with the disorder in question. If the provided clinical information is limited, the laboratory may request additional details to allow accurate interpretation of the findings.

Interpretation and reporting of suspected germline variants

When reporting variants that are suspected to be germline, CNVs predisposing to cancer should be distinguished from variants unrelated to the patient's cancer diagnosis.

Germline CNVs directly related to the neoplasm under investigation (e.g., germline deletion of a tumor suppressor gene) should be reported as being of strong clinical significance (tier 1A) and discussed in the interpretation section of the report. This includes germline CNVs involving cancer predisposition genes listed in the 2016 ACMG secondary findings document.[22]

For likely germline CNVs not related to the neoplasm under investigation:

a. Laboratories should have an established policy for reporting CNVs that are likely germline and have been curated as pathogenic by ClinGen (including pathogenic CNVs associated with disorders that show incomplete penetrance) and/or span known haploinsufficient or triplosensitive genes.[23] These findings can influence clinical care for the patient and the family; as such, they should be included in the report and discussed as potentially constitutional clinically significant variants (see below).
b. Possibly constitutional CNVs unrelated to the patient's cancer diagnosis should not be classified into the tier system. For unambiguous reporting, the laboratory may have a separate section of the report for describing these variants.

Follow-up recommendations for suspected germline variants

If CMA of a tumor sample detects suspected germline CNVs/AOH, the report should contain recommendations for appropriate follow-up, including the following:

a. Referral to a genetic specialist for evaluation and counseling.
b. Confirmation of germline status by testing noninvolved tissue. For patients with solid tumors, a peripheral blood sample may be tested. For patients with hematologic malignancies, the optimal samples for germline testing are cultured skin fibroblasts, although a buccal swab or a peripheral blood sample at the time of complete remission may be acceptable.

Suggested language for reporting suspected constitutional findings:

Suspected germline variant

Based on (percent of cells involved, supporting clinical information, etc.), this finding may represent a germline variant. Genetic testing of a tissue that is not involved in the neoplastic process is recommended when the patient is in clinical remission to determine whether this is a germline or an acquired variant and to aid in determination of its clinical significance. If the variant is germline, genetic counseling is recommended for additional information about this variant and its clinical significance.

REPORTING RECOMMENDATIONS FOR ACQUIRED CNAS AND CN-LOH

The laboratory must ensure that the clinical report accurately describes the findings and clearly communicates their clinical significance. The report should include the preanalytic, analytic, and postanalytic factors that are relevant to the clinical interpretation of the findings, as well as elements that represent regulatory requirements (which are outlined in the ACMG Laboratory Standards and Guidelines; Section E8). Despite the large amount of information that must be included, the report should be as simple and concise as possible, formatted in a way that allows the results to be easily seen and understood, and the clinically critical information should appear at the beginning. Displaying the results in tables may be helpful to increase the overall clarity of the report, provided that the tables can be integrated into the medical record.

In contrast to reporting results of CMA testing for constitutional variants, reports for oncology specimens should not be limited to positive findings. In some cases, what the test does not detect may be of the same or even greater significance than the positive findings. It is strongly recommended that pertinent negatives relevant for clinical management are included in a disease-specific manner. This will typically include tier 1A variants that are used for clinical decision-making (as key prognostic markers or predictors of response or resistance to targeted treatments).

Detected CNAs and CN-LOH should be classified into the four-tier system described above. In complex cases, laboratories may opt not to specify tier classification for every variant individually, but should accurately point out and discuss in the interpretation section all the variants with strong or some clinical significance (tiers 1 and 2). It is not recommended that tier 4 variants (benign/likely benign) be included in the report.

If there is doubt about a variant being "acquired/clonal" versus "germline/constitutional," this should be discussed in the report, and such variants should not be tiered using the

classification system for acquired variants until this uncertainty is clarified.

For clear communication of the relevant and required information, it is recommended that the CMA clinical report be organized into the following sections: results, interpretation, recommendations (if applicable), references, and method description and disclaimers.

Results section

CMA results should be reported according to the current version of the International System for Human Cytogenomic Nomenclature (ISCN).[24] According to ISCN 2016, results can be reported using a table, as a nomenclature string, or both at the discretion of the laboratory director. If the results are displayed in a table, the following information should be included:

- Required
  - Chromosomes and corresponding bands involved in the variant
  - Type of variant (loss, gain, amplification, CN-LOH)
  - Genomic coordinates with designated genome build
- Recommended
  - Copy-number state and percentage of cells involved, estimated based on the log2 ratio and SNP data
  - Tier classification
- Optional
  - Variant size in kb or Mb
  - COSMIC cancer census genes within the affected region

Variants that constitute a diagnostic pattern may be classified collectively in the table using the tier system. The results table can be included either at the beginning or at the end of the report. In complex cases, it may be helpful to clinicians to emphasize clinically significant findings at the beginning of the report, and to place the complete results table at the end. In such cases, laboratories should consider including an additional abridged summary table with clinically significant variants at the beginning of the report.

While the clone structure cannot be ascertained with certainty by CMA, it is recommended to report the approximate percentage of cells (levels of mosaicism) for acquired variants to give an estimate of possible clones and subclones.

Full interpretation of clinically significant variants and a text summary integrating results

The full interpretation should include comments on the following variants:

- Clinically significant CNAs and/or CN-LOH (tier 1 and 2 variants).
- Clinically significant pattern of CNAs and/or CN-LOH (tier 1 and 2 variants).
- CNAs and/or CN-LOH of potential clinical significance (cannot be tiered at the time of reporting). This category addresses point 5 in "General and special considerations" when there is uncertainty about an acquired variant being indicative of a specific gene fusion in the absence of supporting evidence at the time of reporting.
- Optional: other clonal variants (tier 3 variants).

The comments may contain information about the prevalence and functional, prognostic, or predictive significance of the detected CNAs or CN-LOH in a particular tumor type. The laboratory may want to specifically point out the presence of abnormalities that are associated with response to a targeted treatment, in particular if they predict sensitivity to an FDA approved drug. However, specific treatment recommendations are not encouraged. A text summary should integrate CMA results and correlate them with the results of G-banded karyotype and FISH studies. This summary can be included at the beginning or at the end of the interpretation section. Key abnormalities detected by karyotyping and FISH should not be classified into tiers, but should be discussed in the summary with correlation to the CMA findings.

Recommendations

A recommendation section may be included when necessary based on the findings. For example, appropriate follow-up should be recommended in cases in which CMA findings may be germline (see "Follow-up recommendations for suspected germline variants"). Recommendations should also include molecular confirmation of clinically significant abnormalities that are predicted but cannot be established based solely on CMA results (this includes breakpoints suggestive of a particular abnormal gene fusion, CN-LOH suggestive of a variant in a particular oncogene or a tumor suppressor gene, etc.). Treatment recommendations (for the use of specific targeted therapies or enrollment into specific clinical trials) typically should not be included, considering that a treatment choice depends on many factors (other than the diagnosis provided on a test requisition and the CMA findings) that are unknown to the laboratory.

References

Key publications that were used as evidence to classify detected variants into tiers should be listed in the final report.

Methodology and disclaimers

Methodologic details should be presented at the bottom of the report and should include a brief description of the array platform and assay performance characteristics; this may include size resolution and limitations of the assay (e.g., lack of sensitivity for detecting abnormalities present in a low proportion of cells in the sample, inaccuracy in ploidy determination, inability to detect balanced rearrangements,

etc.). Criteria for inclusion of findings in the report and criteria for tier classification should be briefly stated.

The order of different report sections is at the discretion of the laboratory director. Laboratories should have the freedom to choose their own reporting format as long as the report includes the required information outlined above and clearly communicates clinically relevant findings. Laboratory report formats may be limited by a specific reporting system used by the associated hospital, medical center, or commercial entity. Several report examples for different tumor types, including cases with both simple and complex findings, are provided in the supplementary materials of this document.

SUMMARY

The technical standards for interpretation and reporting of acquired CNAs and CN-LOH in neoplastic disorders described were developed in response to an urgent need to standardize the interpretation and reporting of these acquired variants using an evidence-based system with objective criteria. These recommendations represent an expert consensus of the workgroup members based on literature review, empirical data, and their professional judgment. These recommendations describe a four-tier evidence-based categorization system for acquired CNAs and CN-LOH. They outline the variant classification criteria for each tier based on the level of evidence available, and provide examples in tiers 1 and 2 in various hematologic malignancies and solid tumors. This document also provides a list of standardized definitions of terms used in the reporting of these variants, and recommendations for handling suspected clinically significant germline variants. Finally, this document outlines a framework for the clinical reporting of acquired CNAs and CN-LOH. The workgroup believes that the technical standards presented here will help clinical laboratories in achieving better standardized interpretation of CMA results. The workgroup will be constantly reviewing and revising these recommendations based on feedback from the cancer genomic community through a follow-up evaluation mechanism established in collaboration with the ACMG and CGC.

SUPPLEMENTARY MATERIALS

- Supplementary Table 1 illustrating selected tumor suppressor genes associated with germline predisposition to cancer.
- CMA report examples in hematologic malignancies and solid tumors.
- Supplementary figures illustrating examples of amplification, chromothripsis, intrachromosomal complexity, and genomic complexity. The same pattern of acquired CNAs suggestive of a specific disease entity is demonstrated using different CMA platforms. To illustrate the clinical utility of this tier classification system in the interpretation of acquired CNAs derived from whole genome sequencing (WGS), examples of such abnormalities derived from WGS data are also included.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-019-0545-7) contains supplementary material, which is available to authorized users.

ACKNOWLEDGEMENTS

The authors would like to thank Beth Pitel and other members of the Mayo Clinic Genomics of Oncology Annotation Team (GOAT) for their valuable input regarding resources for interpretation of CNAs and CN-LOH in neoplastic disorders.

DISCLOSURE

All members of this workgroup are directors of clinical laboratories that use chromosomal microarray technologies.

REFERENCES

1. Dougherty MJ, Tooke LS, Sullivan LM, Hakonarson H, Wainwright LM, Biegel JA. Clinical utilization of high-resolution single nucleotide polymorphism based oligonucleotide arrays in diagnostic studies of pediatric patients with solid tumors. Cancer Genet. 2012;205:42–54.
2. Roth JJ, Santi M, Rorke-Adams LB, et al. Diagnostic application of high resolution single nucleotide polymorphism array analysis for children with brain tumors. Cancer Genet. 2014;207:111–123.
3. Wang Y, Miller S, Roulston D, Bixby D, Shao L. Genome-wide single-nucleotide polymorphism array analysis improves prognostication of acute lymphoblastic leukemia/lymphoma. J Mol Diagn. 2016;18:595–603.
4. Xu X, Bryke C, Sukhanova M, et al. Assessing copy number abnormalities and copy-neutral loss-of-heterozygosity across the genome as best practice in diagnostic evaluation of acute myeloid leukemia: an evidence-based review from the Cancer Genomics Consortium (CGC) myeloid neoplasms working group. Cancer Genet. 2018;228-229:218–235.
5. Kanagal-Shamanna R, Hodge JC, Tucker T, et al. Assessing copy number aberrations and copy neutral loss of heterozygosity across the genome as best practice: an evidence based review of clinical utility from the Cancer Genomics Consortium (CGC) working group for myelodysplastic syndrome, myelodysplastic/myeloproliferative and myeloproliferative neoplasms. Cancer Genet. 2018;228-229:197–217.
6. Chun K, Wenger GD, Chaubey A, et al. Assessing copy number aberrations and copy-neutral loss-of-heterozygosity across the genome as best practice: an evidence-based review from the Cancer Genomics Consortium (CGC) working group for chronic lymphocytic leukemia. Cancer Genet. 2018;228-229:236–250.
7. Pugh TJ, Fink JM, Lu X, et al. Assessing genome-wide copy number aberrations and copy-neutral loss-of-heterozygosity as best practice: an evidence-based review from the Cancer Genomics Consortium working group for plasma cell disorders. Cancer Genet. 2018;228-229:184–196.
8. 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.
9. Rausch T, Jones DT, Zapatka M, et al. Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. Cell. 2012;148:59–71.

10. Hirsch D, Kemmerling R, Davis S, et al. Chromothripsis and focal copy number alterations determine poor outcome in malignant melanoma. Cancer Res. 2013;73:1454–1460.
11. Korbel JO, Campbell PJ. Criteria for inference of chromothripsis in cancer genomes. Cell. 2013;152:1226–1236.
12. Howick J, Chalmers I, Glasziou P, et al. Oxford Centre for Evidence-Based Medicine (OCEBM) Levels of Evidence Working Group. The 2011 Oxford Levels of Evidence. May 1st 2016. https://www.cebm.net/index.aspx?o=5653.
13. Li MM, Datto M, Duncavage EJ, et al. Standards and guidelines for the interpretation and reporting of sequence variants in cancer: a joint consensus recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. J Mol Diagn. 2017;19:4–23.
14. Rajkumar SV. Updated diagnostic criteria and staging system for multiple myeloma. Am Soc Clin Oncol Educ Book. 2016;35:e418–23.
15. Shah GL, Landau H, Londono D, et al. Gain of chromosome 1q portends worse prognosis in multiple myeloma despite novel agent-based induction regimens and autologous transplantation. Leuk Lymphoma. 2017;58:1823–1831.
16. Greenberg PL, Tuechler H, Schanz J, et al. Revised International Prognostic Scoring System (IPSS-R) for myelodysplastic syndromes. Blood. 2012;120:2454–2465.
17. Grimwade D, Hills RK, Moorman AV, et al. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities amongst 5,876 younger adult patients treated in the UK Medical Research Council trials. Blood. 2010;116:354–365.
18. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127:2391–2405.
19. Harrison CJ. Blood spotlight on iAMP21 acute lymphoblastic leukemia (ALL), a high-risk pediatric disease. Blood. 2015;125:1383–1386.
20. 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.
21. KG Roberts, 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.
22. Kalia SS, Adelman K, Bale SJ, et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SFv2.0): a policy statement of the American College of Medical Genetics and Genomics. Genet Med. 2017;19:249–255.
23. 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.
24. McGowan-Jordan J, Simons A, Schmid M. An international system for human cytogenomic nomenclature. Basel, Switzerland: S Karger; 2016.

[1]Department of Genetics, University of Alabama at Birmingham, Birmingham, AL, USA. [2]Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA. [3]Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, University of Missouri Kansas City Medical School, Kansas City, MO, USA. [4]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. [5]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA. [6]Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI, USA. [7]Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN, USA. [8]Department of Pathology, University of Michigan, Ann Arbor, MI, USA. [9]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, USA

The supplementary materials for the "Technical laboratory standards for interpretation and reporting of acquired CNAs and CN-LOH in neoplastic disorders" are listed below (with hyperlinks to the individual supplements):

1. **Supplementary table 1**
2. **CMA report templates**
3. **CMA report examples**
4. **Supplementary figures**