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

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**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.**

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## 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.

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### 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 $\mu$m 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 $\mu$m sections should not be used for testing specimens that are cut at a thickness of 3 $\mu$m.

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.