Special Article

Guidance for Fluorescence *in Situ* Hybridization Testing in Hematologic Disorders

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Fluorescence *in situ* hybridization (FISH) provides an important adjunct to conventional cytogenetics and molecular studies in the evaluation of chromosome abnormalities associated with hematologic malignancies. FISH employs DNA probes and methods that are generally not Food and Drug Administration-approved, and therefore, their use as analyte-specific reagents involves unique pre- and postanalytical requirements. We provide an overview of the technical parameters influencing a reliable FISH result and encourage laboratories to adopt specific procedures and policies in implementing metaphase and interphase FISH testing. A rigorous technologist training program relative to specific types of probes is detailed, as well as guidance for consistent interpretation of findings, including typical and atypical abnormal results. Details are provided on commonly used dual-fusion, extra signal, and break-apart probes, correct FISH nomenclature in the reporting of results, and the use of FISH in relation to other laboratory testing in the

ongoing monitoring of disease. This article provides laboratory directors detailed guidance to be used in conjunction with existing regulations to successfully implement a FISH testing program or to assess current practices, allowing for optimal clinical testing for patient care. (J Mol Diagn 2007, 9:134–143; DOI: 10.2353/jmoldx.2007.060128)

The World Health Organization recent classification of tumors of hematopoietic and lymphoid tissues emphasizes the importance of chromosome abnormalities for accurate diagnosis, appropriate treatment, and monitoring response to therapy.¹ In certain scenarios, fluorescence *in situ* hybridization (FISH) analysis offers one of the most sensitive, specific, and reliable strategies for identifying acquired chromosomal changes associated with hematologic disorders. With the growth in the understanding of the importance of cytogenetic abnormalities associated with these diseases and the availability of commercial FISH probes, this area of clinical laboratory testing is rapidly expanding. Here, we offer guidance for initiating, validating, routinely performing, and reporting FISH studies for hematologic disorders. The recommendations in this article provide detailed assistance for implementing FISH testing and are meant to assist laboratories with complying with existing

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Table 1. Major Indications for Performing FISH at Diagnosis

1) To detect abnormalities usually* detected by CC	
AML	t(8;21)/CBFA2-ETO, t(15;17)/PML-RARA, inv(16)/CBFB-MYH11
CML	t(9;22)/BCR-ABL1
B-ALL	t(1;19)/E2A-PBX1, t(?;11)/MLL, t(9;22)/BCR-ABL1, hyperdiploidy
2) To detect abnormalities not usually† detected by CC	
CLL/SLL	del(13q14)/?miRNA15-16, del(11q22)/?ATM, del(17p13)/TP53, +12
PCM	del(13q14), hyperdiploidy, t(4;14)/FGFR3-IGH
B-ALL	t(12;21)/ETV6-RUNX1, t(?;11)/MLL
T-ALL	t(5;14)/HOX11L2-, del(1p)/SIL-SCL, episomal amplification 9q34/NUP214-ABL1
CEL	del(4q12)/FIP1L1-PDGFRB
3) No metaphases	
4) Clarification of complex CC	
5) Surrogate marker	
CEL	CHIC2 deletion as a marker of del(4q12)/FIP1L1-PDGFRB

CC, conventional cytogenetics; B-ALL, precursor B-cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; PCM, plasma cell myeloma; T-ALL, precursor T-cell acute lymphoblastic leukemia; CEL, chronic eosinophilic leukemia.

*Usually indicates false-negative CC in ~5 to 10% of cases.

†Not usually indicates that 50 to 100% of these abnormalities might be missed by CC.

regulations and guidelines from the Clinical Laboratory Improvement Amendments (CLIA), the American College of Medical Genetics (ACMG), and the College of American Pathologists.

FISH Procedures and Probes

The FISH methods widely used in clinical laboratory studies involve hybridization of a fluorochrome-labeled DNA probe to an *in situ* chromosomal target. FISH can be applied to a variety of specimen types. Metaphase preparations from cultured cells that are routinely used for cytogenetic analysis are considered the “gold standard” because chromosome morphology and position of the signals can be visualized directly. However, a major advantage of FISH is that it can also be performed on nondividing interphase cells. Interphase nucleus assessment from uncultured preparations allows for a rapid screening for specific chromosome rearrangements or numerical abnormalities associated with hematologic malignancies. Interphase analysis may also be performed on bone marrow cell suspensions routinely used for conventional cytogenetics, paraffin-embedded tissue sections, or disaggregated cells from paraffin blocks, bone marrow, or blood smears, and touch-preparations of cells from lymph nodes or solid tumors.

Pre-Analytical Issues

Clinical Indications for FISH Analysis

The initial assessment of many hematologic malignancies typically includes morphological, histological, immunophenotypic (flow cytometric and/or immunohistochemical), and conventional cytogenetic analyses. Data gleaned from cytogenetic analysis can be pathognomonic for specific leukemias in the World Health Organization classification (for example, acute myeloid leukemias with recurrent cytogenetic abnormalities and chronic myelogenous leukemia) and are likely to assume an even greater role in defining specific entities in future

classifications.¹ Despite conventional karyotyping's current role as the standard for such definitions, it has become clear that FISH studies may also be an integral component of the diagnostic evaluation, particularly where the abnormality is “cryptic” (ie, not evident by conventional karyotyping).

Although somewhat overlapping, FISH can be considered to be of special utility in the following diagnostic scenarios (Table 1): 1) abnormalities with a low frequency, but well-documented percentage, of false-negative cytogenetic results, particularly in scenarios where the clinical, hematologic, and pathological parameters suggest a specific abnormality; 2) abnormalities with a high frequency of “false-negative” cytogenetics; 3) interphase analysis, when conventional cytogenetics fails or is not possible, for example, on fixed tissue; 4) to clarify abnormal or complex conventional karyotypic findings; and 5) as a surrogate marker for a primary genetic event.

For example, when FISH is the preferred laboratory method, it is recommended that cases of chronic myelogenous leukemia (CML) be studied at diagnosis by cytogenetic analysis and molecular cytogenetic methods to determine the initial clonal abnormalities and the FISH signal pattern both for prognostic information and for follow-up studies, respectively.² In acute lymphoblastic leukemia (ALL), where the genetics of the leukemic cells are frequently used for risk stratification and therapeutic decisions, FISH can be used to detect *BCR/ABL1*, *ETV6(TEL)/RUNX1(AML1)*, and *MLL* gene rearrangements and hyperdiploidy with extra copies of chromosomes 4, 10, and 17. In acute myeloid leukemia (AML), FISH to detect *CBFA2T1(ETO)/RUNX1*, *PML/RARA*, *CBFB/MYH11*, and *MLL* gene rearrangements may be performed, as indicated based on the morphological and immunophenotypic findings in the bone marrow.³ When acute promyelocytic leukemia is the suspected diagnosis, FISH, or another method with rapid turnaround time, should be performed as quickly as possible with same-day or next-day turnaround to allow for timely treatment with all-*trans*-retinoic acid.⁴

In some of these diagnostic situations, polymerase chain reaction (PCR)-based assays can be performed

instead of FISH analysis, with the decision of which assay to perform often dictated by factors such as local expertise/availability of the appropriate technology. However, one exception to this generalization is for the detection of numeric abnormalities (gains and losses of whole chromosomes or deletions/duplications), where FISH is clearly superior to PCR-based assays. Furthermore, there are also well-documented scenarios in which FISH is preferred to PCR-based assays, in particular, DNA-based assays, where breakpoint heterogeneity compromises the diagnostic utility of the PCR assay. Examples of such false-negative PCR assays include detection of the t(11;14)/CCND1-IGH translocation in mantle cell lymphoma and t(14;18)/IGH-BCL2 translocation in follicular lymphoma. In addition to these false negatives, there is the potential concern of false-positive PCR results as a result of contamination or the detection of disease-associated gene fusions in normal individuals. Whereas some studies have proposed a role for FISH in minimal residual disease analysis, in situations where PCR-based assays are available, the latter are clearly preferred given their greater analytic sensitivity.

For mature B-cell disorders, genetic studies provide important independent prognostic information. Routine cytogenetic analyses often yield normal results because of the poor *in vitro* growth of mature B-cell populations; therefore, FISH is a useful tool for detecting chromosomal aberrations. For chronic lymphocytic leukemia/small lymphocytic lymphoma, FISH allows for patient stratification into good (deletion 13q14), intermediate (trisomy 12), and poor (deletion of *ATM* and deletion of *TP53*) prognosis categories, in addition to providing a means to monitor disease progression.^{5,6} For plasma cell myeloma, FISH for deletion 13q14 or monosomy 13, translocations involving chromosome 14, especially t(4;14), trisomy of chromosomes 7, 9, and 15, and deletion of the *TP53* gene provide prognostic information.⁷

FISH can be a useful tool to monitor remission status when clonal chromosome abnormalities have been identified at diagnosis and appropriate probes are available. For CML, sequential FISH studies can be useful to determine changes in clinical status in response to therapy and to assess for residual disease in concert with reverse transcription-PCR analysis for *BCR/ABL1*. In situations where PCR-based assays are available, these are the preferred methodology for minimal residual disease assessment given their greater analytic sensitivity. In patients with sex-mismatched bone marrow transplants for whom graft rejection, marrow suppression, or disease relapse is a clinical consideration, monitoring with a FISH assay that combines sex chromosome probes with or without probes to detect the patient's clonal abnormality can be valuable for graft assessment and to detect residual or recurrent disease.

FISH Test Validation

There are only a few commercially manufactured probe kits that have been approved by the Food and Drug

Administration for *in vitro* diagnostic testing. These FISH kits must meet the sensitivity and specificity parameters stated in package inserts provided by the manufacturer.

The majority of probes used for clinical FISH testing are considered analyte-specific reagents, ie, reagents that are produced under good manufacturing practice guidelines set forth by the Food and Drug Administration, but their safety and efficacy must be established by the user. When a new analyte-specific reagent probe is introduced in the laboratory, extensive validation is needed, including specific validation of the probe itself (*probe validation*) and validation of the procedures using the probe (*analytical validation*) (ACMG. Standards and Guidelines for Clinical Genetic Laboratories, Section E: Clinical Cytogenetics. <http://www.acmg.net>, accessed December 12, 2005).⁸ Initially, it is important to become familiar with a probe's parameters, including signal intensity and pattern and any cross-hybridization that is likely to confound test results. This can be accomplished by assessing probe characteristics on several known positive and/or negative specimens. Probe validation consists of localizing the probe to the correct chromosomal band on normal metaphase cells and determining its sensitivity and specificity (ACMG Standards and Guidelines for Clinical Genetic Laboratories, Section E: Clinical Cytogenetics). Probes may be localized by hybridization to metaphase cells with identification of the appropriate target chromosome region using reverse 4,6-diamino-2-phenol-indole chromosomal staining, sequential G-, R-, or Q-chromosome banding to FISH, or other methods that allow for specific chromosome identification. Localization should ensure that the tested probe is the intended probe and that there is no probe contamination or significant cross-hybridization. Probe sensitivity, defined as the percentage of metaphases with the expected signal pattern at the correct chromosomal location, should be established by analysis of the hybridization of the probe to chromosomes representing at least 200 distinct genomic targets derived from each of at least five control male individuals (includes all 24 haploid chromosomes). The genomic targets may represent distinct chromosomes or distinct chromatids depending on the location of the probe. Likewise, probe specificity, or the percentage of signals that hybridize to the correct locus and no other location, may be assessed by studying at least 200 cells from a minimum of five male individuals. An adequate number of cells and loci should be scored to ensure that the probe has the sensitivity and specificity required for the clinical testing being performed.⁹ Probes used for hematologic malignancy studies should have a high analytic sensitivity and specificity (>95%), particularly if they are to be used for minimal residual disease assessment.

Analytical validation requires an appreciation of probe and test parameters that allows for interpretation of the FISH result. Analytical validation for metaphase cell analysis is inherent in the probe validation process. For probes that will be used for interphase cell analysis, analytical validation requires normal reference ranges be calculated from the evaluation of cytogenetically characterized cases. The data are used to establish cutoffs for

designating a result as normal or abnormal (ie, distinguishing true positives from false positives). One way to establish a normative database for a dual-color, dual-fusion *BCR/ABL1* probe for bone marrow samples would be to gather data from at least 500 interphase cells from bone marrow samples from each of 20 *BCR/ABL1* fusion-negative individuals. For paraffin-embedded tissues, a normative database for a dual-color, dual-fusion probe could be generated by assessing the signal patterns for all of the available disease tissue (if there are less than 500 cells available) from 20 individuals without the rearrangement being validated. The normal cutoff point would be determined by statistical analysis using a binominal distribution assessment.¹⁰ Because the normative data are not distributed in a typical bell-shaped curve, cutoff points cannot be based on SD calculations.¹⁰

The normal cutoff for an analysis of 200 cells can be calculated for FISH results using the Microsoft Excel β inverse function, = BETAINV(Confidence level, false-positive cells plus 1, number of cells analyzed).¹⁰ This formula calculates a one-sided upper confidence limit for a specified percentage proportion based on an exact computation for the binomial distribution. This can be done by examining results for 20 normal specimens for the particular sample type being validated and identifying the specimen with the greatest number of false-positive nuclei for any given signal pattern. This number of false-positive cells is inserted into the β inverse function to determine the normal cutoff for detection of a true abnormal clone. To illustrate how to calculate the normal cutoff, consider the following example for a 95% confidence level in which four false-positive cells for any given signal pattern were identified among 200 nuclei. In the formula bar in Microsoft Excel, enter: = BETAINV(0.95,5200); the result is 4.43% cutoff or 8.86 cells. In other words, the formula would read = BETAINV(0.95 upper bound percentile, four false-positive cells plus 1, analysis of 200 cells). Based on this calculation, 9.0 cells is the abnormal cutoff because fractions of cells cannot be analyzed. The observation of 9.0 or more cells with the aberrant pattern in a study of a total of 200 cells analyzed would be an abnormal result. Note that a normal cutoff needs to be established for different levels of cells counted. Hence, the initial assessment for the database generation of 500 nuclei for 20 control individuals can be used to establish cutoffs for 200, 250, or 300 cells, or whatever the laboratory determines to be a clinically relevant number of cells to score.

To validate a FISH test, known normal and abnormal cases should be assessed to establish clearly defined scoring criteria for determining that the assay is acceptable. Laboratories may choose to have a series of cases split between the home institution and a reference laboratory. Results should be compared, including images of the hybridization patterns. The clinical test should not be offered until the Laboratory Director is confident of the laboratory's ability to produce accurate results following a review of the data from the parallel series and investigation of the reason for any discrepant results. CLIA regulations require that there also be biannual or continuous review of the performance characteristics of each FISH test to assess for trends that might suggest problems with the assay (ACMG

Standards and Guidelines for Clinical Genetic Laboratories, Section E: Clinical Cytogenetics). In addition to continuous quality monitoring of individual probes, biannual proficiency testing must be performed on each FISH category.

Analytical Issues

Staff Training

Ideally, at least one individual in the laboratory should have considerable experience and expertise in FISH. Experience may be obtained from attending course offerings or sponsored workshops or from visiting another laboratory with the expertise.

Laboratories should maintain a FISH training manual in which each probe or probe set used is described. Photographic images or drawings of abnormal patterns (both simple abnormal patterns and more complex variant patterns) and normal patterns should be included, along with helpful comments regarding the use of particular probes. Probe manufacturer material and data sheets and pertinent references should also be included in this manual. As technologists demonstrate competency, it may also be useful to include a log sheet documenting the completion of training on each probe set.

One scheme for FISH training is to treat all like-designed probes as a unit, with group 1 including enumeration probes (eg, one color chromosome 8 α -satellite centromere probe, two color X/Y probes); group 2 including dual-color, dual-fusion probes (eg, *BCR/ABL1*, *IGH/BCL2*, *PML/RARA*); group 3 including single-fusion, extra-signal (ES) probes (eg, *ETV6/RUNX1*, *BCR/ABL1* ES); and group 4, including break-apart probes (eg, *CBFB*, *MLL*).

Each of the four subgroups has unique features that should be focused on during training. For enumeration, the trainee learns to count signals accurately. Particular emphasis should be placed on distinguishing a "split" signal (two signals in very close proximity, representing two chromatids from a single chromosome) from two true separate signals. For the dual-color, dual-fusion translocation probes, for which the abnormal pattern is typically represented by the presence of two fusion signals, the trainee's goals are to learn the criteria necessary for labeling two signals as overlapping or fused and to recognize other clinically significant variant patterns [eg, a t(9;22;15) that would not yield a typical dual-fusion pattern but would be detected at initial diagnosis by the high level of single-fusion cells present]. The ES translocation probes are probe sets for which the abnormal pattern is represented by a single fusion plus a small extra signal representing a residual portion of one of the involved loci. The goals for the trainee are to learn to distinguish two overlapping signals from technical artifact and to learn to differentiate a small true residual signal from nonspecific hybridization or debris. For break-apart probe analyses, probes for the region 5' of a designated breakpoint, labeled with one color, and probes for the region 3' of the breakpoint, labeled in another color, are assessed. For these probes, an overlapping red/green or fused yellow

signal represents the normal pattern, and separate red and green signals indicate the presence of a rearrangement. Here, the emphasis should be on judging the distance between a red and a green signal that is necessary to call them "separate," to meet criteria for designating the cell abnormal.

The following training scheme is used to illustrate how to ensure comparability of scoring between different technologists and to ensure achievement and maintenance of competency in evaluating FISH results for the *BCR/ABL1* dual-color, dual-fusion probe set.

1. The trainee should read through the laboratory FISH training manual, including probe manufacturer's material and data sheet, and review how the signal patterns in the interphase cell relates to the metaphase cell chromosomal aberration under study.

2. Training should begin with assessment of a positive diagnostic specimen that has been shown to have a straight-forward dual-fusion positive pattern in the majority of cells, eg, two fusion signals, one separate *BCR*, and one separate *ABL1* signal. An experienced technologist should sit with the trainee and describe in detail how the slides and microscope fields are to be scanned, how to judge whether the hybridization reaction is adequate, how to designate an individual cell's pattern, and how to tabulate results.

3. The trainee should then score a series of known positive and negative cases. Previously scored FISH cases, if stored in the freezer, can be rescored months after their initial preparation. The positive cases should include both diagnostic cases (with high percentages of *BCR/ABL1* fusion-positive cells), as well as follow-up cases (with low percentages of *BCR/ABL1* fusion-positive cells). In addition, cases with variant FISH patterns, such as those with a three-way translocation [eg, t(9;22;15)], which would be expected to have a single fusion rather than a double fusion pattern, could also be included.

4. After the trainer has confidence in the trainee's scoring, the trainee should score a series of cases independently, and the results should be correlated between the scorers. There should be complete agreement between scorers as to the final diagnosis of the case, normal or abnormal (eg, *BCR/ABL1* fusion present or not), and there should be close agreement between the percentages of abnormal cells scored. If, on the series of positive cases, the trainee consistently gets a lower or higher rate of abnormal cells than the trainer, side-by-side scoring should ensue to identify the basis for the differences, and a new series of abnormal and normal cases should be evaluated.

Scoring

A routine FISH evaluation should be scored by two technologists. The technologists should be familiar with any unique scoring criteria that were established as part of the test validation process. The technologists must also have an understanding of general technical problems that may influence a test result, such as poor slide preparation, chromosomes or interphase nuclei of poor mor-

phology with reduced signal intensity, or over- or underdenaturation.

Technologists reading FISH must be knowledgeable about the reason the test is being performed. They should also be familiar with any previous G-band chromosome findings and any previous FISH study results that were obtained for that patient. Laboratory personnel should know manufacturer's specifications and limitations with respect to the behavior of any probe or probe set, such as the size of the genomic target, the expected signal patterns, and the matrix being studied. For example, paraffin-embedded tissues commonly require unique evaluation parameters.

Control Probes

CLIA requires the use of standard controls in laboratory testing, including FISH studies. For metaphase FISH, it is recommended that clinical FISH tests include control probes to tag the chromosome(s) of interest. Such probes afford a limited level of quality control by providing an internal control of hybridization efficiency. The target sequence on a normal chromosome serves as the best control of technical variables. If a probe is used that does not have an inherent chromosome control signal (ie, an X or Y chromosome probe analysis in a male with clonal loss of the Y chromosome), another sample that is known to have the probe target (a normal 46, XY male for this example) should be run in parallel with the patient sample.¹¹

When interphase analysis is performed, a useful control measure is assessment of several metaphase cells, when available. Visualizing the probes on metaphase cells allows for validation that the correct probe was used for the study and may be used to confirm an abnormal result. In addition, metaphase assessment may be needed if an atypical abnormal FISH pattern is observed. Understandably, some samples, such as hematologic blood and direct preparations, may not have available metaphase cells, and use of another internal control (for another locus on the same chromosome, for example) may be necessary.

New lots of reagents, including probes, must be tested before being put into use. Probe lot validation may be done by comparing the new lot and the old lot of probe side by side on the same slide preparation and ensuring that the results are equivalent. To monitor FISH testing over time to assess adverse technical trends, the laboratory should periodically check assay performance (including control probes) as part of biannual quality monitoring.¹¹

Postanalytical Issues

Interpretation of Results

FISH results should be interpreted within the broader context of probe and analytical validation (ACMG Standards and Guidelines for Clinical Genetic Laboratories, Section E: Clinical Cytogenetics), including the laborato-

ry's own normative database and confidence interval established in the ongoing use of any probes. The director and/or clinical consultant must have an understanding of factors intrinsic to cell culture and slide preparation before FISH evaluation that may influence a result's interpretation. The interpretation of FISH results should include consideration of the reason for referral for testing and, when available, additional laboratory findings including conventional cytogenetic analysis, histology, and immunophenotype.¹²

Result Reporting

A system for FISH nomenclature, the International System for Human Cytogenetic Nomenclature (2005), including both metaphase and interphase analysis, has been developed.¹³ Although the system may seem confusing to those not working directly with chromosomes, correct nomenclature designations are important to convey the precise nature of a result. Metaphase FISH International System for Human Cytogenetic Nomenclature (ISCN) nomenclature for a male patient with a 9;22 translocation resulting in fusion of the *BCR* and *ABL1* genes studied with conventional banding and with a dual-color, single-fusion *BCR/ABL1* probe set would be written as 46,XY,t(9;22)(q34;q11.2).ish (9;22)(ABL1-;BCR+,ABL1+), indicating that the probe sequence from the *ABL1* locus is missing from the derivative chromosome 9 and is present on the derivative chromosome 22 distal to the *BCR* locus.

The same rearrangement expressed in interphase FISH nomenclature but using a dual-color, dual-fusion probe set would be written as follows: nuc ish (ABL1x3),(BCRx3),(ABL1 con BCRx2), indicating that each of the probes has been split apart and juxtaposed by the translocation.

The use of such precise ISCN nomenclature is valued by laboratories in the initial diagnostic workup and continued monitoring of patients with a specific chromosome abnormality. However, the report should also contain a statement as to the normalcy/abnormalcy of a FISH result. The report should also clearly indicate the percentage of abnormal and normal cells and whether FISH results are from metaphase or interphase cells or from both. Specific naming of the probes used to obtain results, including the name of the manufacturer, must also be included on the written report. The report must indicate any specific limitations of the assay, some of which may be described in the probe manufacturer's package insert.

The report should clearly indicate both the diagnostic and prognostic significance of the FISH findings, including their relevance to the reason for referral for testing, the patient's age, and any pertinent clinicopathologic findings that may become available. Longitudinal studies of patients should be compared with their previous genetic test findings, and the report should make clear recommendations concerning future testing of such patients, eg, metaphase chromosome analysis, interphase FISH, and molecular reverse transcription-PCR testing.¹⁴

Because of the analyte-specific reagent nature of the majority of FISH probes, the Food and Drug Administration has mandated that a disclaimer regarding the use of analyte-specific reagents must also be included on the report. Sample wording for the disclaimer was suggested by the ACMG Standards and Guidelines for Clinical Genetic Laboratories, Section E: Clinical Cytogenetics as follows: "This test was developed and its performance characteristics determined by (Name) Laboratory as required by CLIA '88 regulations. It has not been cleared or approved for specific uses by the U.S. Food and Drug Administration."

Typical and Atypical Results

The high degree of specificity of probes generally makes their application and interpretation straightforward. However, it is not uncommon to have an atypical abnormal interphase FISH result. For example, findings for a dual-color, dual-fusion probe may show more than the expected number of fusion signals in a single cell or small population of cells or an atypical signal pattern indicating a probable variant of a disease-associated rearrangement. Although interphase FISH analysis provides information only on specific probes used and generally does not substitute for complete karyotype analysis, it may, under some disease circumstances, be the preferred means of identifying an abnormal clone, eg, FISH with the *ATM*, *CEP12*, *D13S319*, and *TP53* probe panel in B-cell chronic lymphocytic leukemia,⁶ discrimination of the inversion 16 in AML (M4-Eo),¹⁵ or for the diagnostic abnormality in post-therapy patients who have hypocellular marrows.

Specific Probe Examples

Dual-Color, Dual-Fusion Probes: *BCR/ABL1*

All patients with CML have an abnormal clone with fusion of *BCR* and *ABL1* loci; at least 90% of patients have a t(9;22)(q34;q11.2), and the rest have a complex or cryptic variant of this translocation. A similar *BCR/ABL1* fusion occurs in 6% of children with ALL and 17% of adults with ALL.¹⁶

The goal for treatment of CML is generally to achieve a complete cytogenetic metaphase and interphase FISH remission [ie, no metaphase cells with t(9;22) and interphase FISH results within normal limits] and a molecular PCR remission. The dual-color, dual-fusion *BCR*(green)/*ABL1*(red) probe (D-FISH) set allows for detection of all forms of the *BCR/ABL1* fusion (yellow), ie, t(9;22), variant translocations, and cryptic translocations or insertions.² The probe set is highly sensitive and consistent, and its technical application and interpretation can be mastered by most laboratories. Initial FISH studies of CML patients yield 85 to 99% of *BCR/ABL1*-positive nuclei in bone marrow before treatment. When treatment is successful, the post-treatment percentage of neoplastic nuclei progressively decreases to less than 1%. Studies indicate that analysis of 500 nuclei with a D-FISH on bone marrow

Figure 1. Representative interphase nuclei showing D-FISH signal patterns in cells with various Ph chromosomes. **A:** Nuclei with two red (*ABL1*) and two green (*BCR*) signals are normal. **B:** Nuclei that have one red, one green, and two fusion signals have a balanced t(9;22). **C:** Nuclei that have one red, one green, and three yellow fusion signals have an extra Ph chromosome. **D:** Nuclei that have amplification of the yellow *BCR/ABL1* fusion signal will show multiple copies of the *BCR/ABL1* fusion signal. **E:** Nuclei that have two red, two green, and one yellow fusion signal have a complex Ph chromosome. **F:** Nuclei that have one red, one green, and one fusion signal either have a masked Ph chromosome or have loss of the *ABL1* and *BCR* hybridization sites that are normally observed on the abnormal chromosome 9. **G:** Nuclei that have two red, one green, and one yellow fusion signal have loss of the *BCR* hybridization site that is translocated to the abnormal chromosome 9. **H:** Nuclei that have one red, two green, and one fusion signal have loss of the *ABL1* hybridization site that normally remains on the abnormal chromosome 9.

or peripheral blood can detect less than 1% neoplastic cells.^{2,17}

Strict scoring criteria have been developed for D-FISH to reliably classify individual cells as either normal or abnormal.^{2,16,17} With D-FISH, normal cells have two *BCR* and two *ABL1* signals (Figure 1A). Patients with a conventional t(9;22)(q34;q11.2) have one *BCR*, one *ABL1*, and two fused *BCR* and *ABL1* signals (Figure 1B). Cells with multiple copies of the derivative chromosome 22 (Ph chromosome) have three or more *BCR/ABL1* fusion sig-

nals (Figure 1C). Some patients with CML on imatinib mesylate become “drug-resistant” due to amplification of the *BCR/ABL1* fusion gene; this is observed in individual neoplastic cells as a cluster of *BCR/ABL1* fusion signals (Figure 1D).

Patients with complex translocations have a unique signal pattern by D-FISH. For example, consider a patient with a t(5;9;22)(q31;q34;q11.2). In an abnormal metaphase from this patient, a *BCR/ABL1* fusion signal occurs on the derivative 22 (Ph chromosome), a small *ABL1* signal occurs on the abnormal chromosome 9, and a small *BCR* signal occurs on the abnormal chromosome 5 (Figure 1E). The *ABL1/BCR* fusion that is normally observed on the abnormal chromosome 9 with D-FISH does not occur in complex translocations.

Unusual *BCR/ABL1* signals are also observed among patients with cryptic rearrangements that originate from small insertions involving the *BCR* and *ABL1* loci. These insertions and other chromosomal rearrangements typically are not visible by conventional metaphase cytogenetic studies but are readily detectable by FISH (Figure 1, F–H). It is important to use a FISH technique to monitor response to therapy for patients with a cryptic rearrangement.

Nearly 20% of patients with a t(9;22)(q34;q11.2) have any one of three different atypical D-FISH patterns¹⁶ (Figure 1, F–H). Among these patients, there is loss of a portion of *BCR* or *ABL1* or both of these hybridization sites normally associated with the break and fusion point on the abnormal chromosome 9. Laboratory personnel need to be aware of these variant signal patterns to adjust their scoring criteria and to use different cutoff values for normal. For example, the normal cutoff may be 1.2 to 1.8% for two of these atypical signal patterns (Figure 1, F and G) and less than 1% for other atypical signal patterns (Figure 1H). It is useful to examine a few metaphase cells to establish the exact *BCR/ABL1* signal pattern for each patient.

Dual-Color, Single-Fusion, Extra Signal Probes: *ETV6/RUNX1*

The FISH assay for the cryptic 12;21 translocation (*ETV6/RUNX1* or, historically, *TEL/AML1*) has many applications in the diagnosis and monitoring of acute lymphoblastic leukemia. The 12;21 translocation is associated with a good prognosis and occurs in approximately 25% of childhood B-lineage-ALL, with a peak incidence at ages 2 to 5 years.^{18,19} The translocation is usually not seen in infants less than 1 year of age, and in adults with ALL the frequency is estimated between 1 and 3%.²⁰ In addition to the detection of the 12;21 translocation, the commercially available *TEL/AML1* ES probe (Vysis, Downer's Grove, IL) can also ascertain numeric and structural abnormalities of chromosomes 12 and 21, which are recurring abnormalities in ALL.

The 12;21 translocation cannot be seen on G-banded metaphases, and FISH and other molecular methods are needed to detect this rearrangement. Typically with an extra-signal, dual-color translocation probe set, one of

Figure 2. Probe patterns observed at diagnosis in the bone marrow of six patients with childhood B-lineage ALL. In each case, marrow slides were prepared by standard cytogenetic techniques for chromosome analysis and hybridized with Vysis' *TEL/AML1* ES probe for FISH analysis. Results for **A–F** (upper left through lower right), probe pattern first and then interpretation: **A:** Red, Red, Green, Green; normal, no evidence of abnormality; **B:** Red, residual Red, Green, Fusion; typical pattern for t(12;21)(p13;q22); **C:** Red, residual Red, Fusion; t(12;21) with loss of the *TEL* (*ETV6*) locus from the uninvolved 12; **D:** Red, Red, Green; no evidence of translocation, loss of one *TEL* (*ETV6*) signal consistent with del(12p); **E:** Red, Red, Red, Red, Red, Green, Green; no translocation, this pattern was found in a patient with high hyperdiploidy with five copies of chromosome 21; and **F:** A partial metaphase spread from a patient with no evidence of t(12;21) but multiple *AML1* (*RUNX1*) signals on a single marker chromosome (*AML1* amplification).

the probes is quite long (eg, the Vysis *RUNX1* probe is approximately 500 kb and spans the entire *RUNX1* locus on 21q). An abnormal cell with the translocation would show the *ETV6/RUNX1* fusion as yellow (red+green on the derivative chromosome 21), one green signal (uninvolved chromosome 12), one large red signal (uninvolved chromosome 21), and one small red signal (extra signal residual on the chromosome 12 involved in the translocation) (Figure 2B). Although the translocation itself is cryptic, it is often associated with cytogenetically visible structural and numeric abnormalities of the chromosomes. In a collaborative study of 169 children with t(12;21)-positive ALL, additional structural chromosomal abnormalities were reported in 89.7% of the karyotypes containing the cryptic translocation, and numeric abnormalities were seen in 47%.¹⁸

FISH is particularly useful for analysis of ALL cases with cytogenetically normal diagnostic studies. In addition to finding the expected 12;21 translocation in many of these patients, other abnormal patterns, with and without the *ETV6/RUNX1* fusion, are also observed.²⁰ For example, extra *RUNX1* signals without an *ETV6/RUNX1* fusion can be indicative of the presence of an undetected high hyperdiploid cell line or gene amplification (Figure 2, E and F). The finding of extra copies of *RUNX1* in an interphase FISH assay requires correlation with metaphase FISH or traditional cytogenetic studies for accurate interpretation (Figure 2F). The presence of five or more copies of *RUNX1* in an interphase nucleus can suggest gene amplification. Metaphase chromosome studies of

most cases of *RUNX1* amplification show a single copy of the gene on a normal chromosome 21 and the remainder of the *RUNX1* signals clustered on a duplicated 21, a marker chromosome, or a ring (Figure 2F). The important distinction between polysomy 21 (often seen in high hyperdiploidy with a good prognosis) and *RUNX1* amplification (often seen in a near diploid or a pseudodiploid karyotype and associated with a poor prognosis) is that with *RUNX1* amplification multiple signals are clustered on one or two, often structurally abnormal, chromosomes. Abnormal signal patterns for the *ETV6* probe can indicate loss of material from the chromosome 12 short arm (deletion 12p; Figure 2C) or rearrangement of 12p. Aberrations involving 12p have long been appreciated as a recurring abnormality in both childhood and adult ALL, occurring in 4 to 9% of patients.¹²

Dual-Color, Break-Apart Probe: *MLL*

Structural rearrangements of the mixed lineage leukemia (*MLL*) gene at 11q23 are well-documented recurring abnormalities and are observed in *de novo* and therapy-related hematologic disorders, including myelodysplastic syndromes, acute lymphoid, myeloid and biphenotypic leukemias, as well as secondary AML after treatment with topoisomerase II inhibitors.^{21–23} Hematologic malignancies with *MLL* rearrangement are particularly common in infants (~85% of infantile ALL and ~65% of infantile AML) and can also be observed in children and adults.^{24–26}

Figure 3. G-banded metaphase spread [47,XY,+11,t(11,21)(q23;q22)] on the right with **arrows** showing the two normal copies of chromosome 11 and derivative chromosomes 11 and 21. *MLL* break-apart probe FISH analysis on the same metaphase cell (left) showing the two normal copies of chromosome 11 (yellow), the red derivative chromosome 21 [der(21)] signal, and the derivative chromosome 11 (**arrow**).

The majority of *MLL* rearrangements occur as a result of an established chromosomal translocation involving 11q23; however, cryptic insertions and complex abnormalities that can mask its involvement are not uncommon. Occasionally, *MLL* is rearranged by partial tandem duplication and subsequent self-fusion, often in association with trisomy 11 or with a normal karyotype. Conventional cytogenetic analysis identifies the principal 11q23 translocations [eg, t(4;11)(q21;q23)], but it can neither discriminate cryptic *MLL* translocations from del(11)(q23) nor confirm the involvement of the *MLL* gene in cases with undefined or rare 11q23 translocation partners. The accurate detection and characterization of *MLL* rearrangements often requires the use of more than one technique. Conventional cytogenetics and FISH are the first choice for describing the aberrant chromosomes, and *MLL* assessment may be complemented by molecular methods, particularly when cytogenetic results are discrepant or incomplete.

Given the promiscuity of the *MLL* gene (it is involved in >80 different translocations), the most effective and appropriate FISH probe for detecting the *MLL* rearrangements is a dual-color break-apart probe made of differentially labeled (red and green) DNA segments located on either side of the *MLL* breakpoint cluster region. The separation of red and green signals indicates *MLL* break for the 3' and 5' regions of the gene. In normal cells, the two probes co-localize to produce two yellow fusion signals, whereas in the presence of a translocation involving the *MLL* gene, one of the fusion signals splits, resulting in a characteristic 1red-1green-1yellow fusion signal pattern. The advantage of this kind of FISH assessment is that it can detect all recurrent and possibly novel *MLL* rearrangements in a single experiment. For cases with complex *MLL* rearrangements, FISH on metaphase cells often provides clues to understanding the underlying mechanism and

allows for correct identification of the chromosome abnormality.

Occasionally, an atypical pattern is observed in which the 3' telomeric red signal is lost. This 0red-1green-1fusion pattern has been assumed to indicate the presence of an *MLL* rearrangement because the two signals have separated. The loss of the 3' red signal (20% of *MLL* rearrangements) is thought to arise from a concurrent deletion event, in which the retention of 5' *MLL* is consistent with the preservation of the more important of the two fusion products.^{27,28} Other types of variant signal patterns, such as deletion of the 5' *MLL* region (Figure 3), have also been observed. However, because of the rarity of such variants, the clinical significance is not clear.

Conclusions

FISH has now become an invaluable tool in defining and monitoring acquired chromosome abnormalities associated with hematologic and other neoplasias. The implementation of FISH into the routine diagnostic laboratory requires rigorous attention as to when it is appropriate to apply the technology, a very systematic approach to the validation of probes and technical procedures involved in FISH, and the training of individuals that will perform the testing, and a comprehensive, but understandable, means of reporting out results. As the number of critical loci involved in neoplastic chromosome rearrangements or numeric abnormalities continues to expand, the diversity of FISH probes and unique probe sets will undoubtedly improve. FISH has become an important means both for definition of the initial chromosome changes in a disease process, as well as a reliable means for the

ongoing monitoring of response to therapy and disease remission.

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References

- Jaffe ES, Harris NL, Stein H, Vardiman JW (Eds): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, IARC Press, 2001
- Dewald G, Stallard R, Alsaadi A, Arnold S, Blough R, Ceperich TM, Rafael Elejalde B, Fink J, Higgins JV, Higgins RR, Hoeltge GA, Hsu WT, Johnson EB, Kronberger D, McCorquodale DJ, Meisner LF, Micale MA, Oseth L, Payne JS, Schwartz S, Sheldon S, Sophian A, Storto P, Van Tuinen P, Wenger GD, Wiktor A, Willis LA, Yung JF, Zenger-Hain J: A multicenter investigation with D-FISH BCR/ABL1 probes. *Cancer Genet Cytogenet* 2000, 116:97-104
- Ferrant A, Labopin M, Frasson F, Prentice HG, Cahn JY, Blaise D, Reiffers J, Visani G, Sanz MA, Boogaerts MA, Lowenberg B, Gorin NC: Karyotype in acute myeloblastic leukemia: prognostic significance for bone marrow transplantation in first remission: a European Group for Blood and Marrow Transplantation study. *Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT)*. *Blood* 1997, 90:2931-2938
- Schad CR, Hanson CA, Paietta E, Casper J, Jalal SM, Dewald GW: Efficacy of fluorescence in situ hybridization for detecting PML/RARA gene fusion in treated and untreated acute promyelocytic leukemia. *Mayo Clin Proc* 1994, 69:1047-1053
- Döhner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, Döhner K, Bentz M, Lichter P: Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000, 343:1910-1916
- Shanafelt TD, Geyer SM, Kay NE: Prognosis at diagnosis: integrating molecular, biologic, insights into clinical practice for patients with CLL. *Blood* 2004, 103:1202-1210
- Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel L, Chesi M, Davies FE, Drach J, Greipp PR, Kirsch HR, Kuehl WM, Hernandez JM, Minvielle S, Pilarski LM, Shaughnessy JD, Stewart AK, Avet-Loiseau H: Genetics and cytogenetics of multiple myeloma: a workshop report. *Cancer Res* 2004, 64:1546-1558
- Test and Technology Transfer Committee, American College of Medical Genetics: Technical and clinical assessment of fluorescence in situ hybridization: an ACMG/ASHP position statement. I. Technical considerations. Test and Technology Transfer Committee. *Genet Med* 2000, 2:356-361
- Dewald G, Stallard R, Al Saadi A, Arnold S, Bader PI, Blough R, Chen K, Elejalde BR, Harris CJ, Higgins RR, Hoeltge GA, Hsu WT, Kubic V, McCorquodale DJ, Micale MA, Moore JW, Phillips RM, Scheib-Wixted S, Schwartz S, Siembieda S, Strole K, VanTuinen P, Vance GH, Wiktor A, Wise L, Yung JF, Zenger-Hain J, Zinsmeister A: A multicenter investigation with interphase fluorescence in situ hybridization using X- and Y-chromosome probes. *Am J Med Genet* 1998, 76:318-326
- Wiktor AE, Van Dyke DL, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majorowicz JR, Dewald GW: Preclinical validation of FISH assays for clinical practice. *Genet Med* 2006, 8:16-23
- Stupca PJ, Meyer RG, Dewald GW: Using controls for molecular cytogenetic testing in clinical practice. *J Assoc Genet Technol* 2005, 31:4-8
- Mrózek M, Heerema NA, Bloomfield CD: Cytogenetics in acute leukemia. *Blood Rev* 2004, 18:115-136
- Shaffer LG, Tommerup N (Eds): ISCN 2005: An International System for Human Cytogenetic Nomenclature. S. Karger, Basel, 2005
- Tefferi A, Dewald GW, Litzow ML, Cortes J, Mauro MJ, Talpaz M, Kantarjian HM: Chronic myeloid leukemia: current application of cytogenetics and molecular testing for diagnosis and treatment. *Mayo Clin Proc* 2005, 80:390-402
- Ravandi F, Kadkol SS, Ridgeway J, Bruno A, Dodge C, Lindgren V: Molecular identification of CBFbeta-MYH11 fusion transcripts in an AML M4Eo patient in the absence of inv16 or other abnormality by cytogenetic and FISH analyses—a rare occurrence. *Leukemia* 2003, 17:1907-1910
- Dewald GW, Schad CR, Christensen ER, Tiede AL, Zinsmeister AR, Spurbeck SL, Thibodeau SN, Jalal SM: The application of fluorescent in situ hybridization to detect Mbcra/abl fusion in variant Ph chromosomes in CML and ALL. *Cancer Genet Cytogenet* 1993, 71:7-14
- Dewald GW, Wyatt WA, Juneau AL, Carlson RO, Zinsmeister AR, Jalal SM, Spurbeck JL, Silver RT: Highly sensitive fluorescence in situ hybridization method to detect double BCR/ABL fusion and monitor response to therapy in chronic myeloid leukemia. *Blood* 1998, 91:3357-3365
- Raynaud SD, Dastugue N, Zoccola D, Shurtleff SA, Mathew S, Raimondi SC: Cytogenetic abnormalities associated with the t(12;21): A collaborative study of 169 children with t(12;21)-positive acute lymphoblastic leukemia. *Leukemia* 1999, 13:1325-1330
- Ferrando AA, Look AT: Clinical implications of recurring chromosomal and associated molecular abnormalities in acute lymphoblastic leukemia. *Semin Hematol* 2000, 37:381-395
- Jabber Al-Obaidi MS, Martineau M, Bennett CF, Franklin IM, Goldstone AH, Harewood L, Jalali GR, Prentice HG, Richards SM, Roberts K, Harrison CJ: Medical Research Council Adult Leukaemia Working Party: ETV6/AML1 fusion by FISH in adult lymphoblastic leukemia. *Leukemia* 2002, 16:669-674
- Thirman MJ, Gill HJ, Burnett RC, Mbangkollo D, McCabe NR, Kobayashi H, Ziemer-van der Poel S, Kaneko Y, Morgan R, Sandberg AA, Chaganti RSK, Larsen RA, LeBeau MM, Diaz MO, Rowley JD: Rearrangement of the MLL gene in acute lymphoblastic and acute myeloid leukemias with 11q23 chromosomal translocations. *N Engl J Med* 1993, 329:909-914
- Broeker PL, Super HG, Thirman MJ, Pomykala H, Yonebayashi Y, Tanabe S, Zeleznik-Le N, Rowley JD: Distribution of 11q23 breakpoints within the MLL breakpoint cluster region in de novo acute leukemia and in treatment related acute myeloid leukemia: correlation with scaffold attachment regions and topoisomerase II consensus binding sites. *Blood* 1996, 87:1912-22
- Pui CH, Relling MV: Topoisomerase 2 inhibitor-related acute myeloid leukemia. *Br J Haematol* 2000, 109:13-23
- Pui CH, Kane JR, Crist WM: Biology and treatment of infant leukemias. *Leukemia* 1995, 9:762-769
- Ziemer-van der Poel S, McCabe NR, Gill HJ, Espinosa III R, Patel Y, Harden A, Rubinelli P, Smith SD, Le Beau M, Rowley JD, Diaz MO: Identification of a gene MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. *Proc Natl Acad Sci USA* 1991, 88:10735-10739
- Ludwig WD, Rieder H, Bartram CR, Heinze B, Schwartz S, Gassmann W, Löffler H, Hossfeld D, Heil G, Handt S, Heyll A, Diedrich H, Fischer K, Weiss A, Volkers B, Aydemir U, Fonatsch C, Gokbuget N, Thiel E, Hoelzer D: Immunophenotypic and genotypic features, clinical characteristics, and treatment outcome of adult pro-B acute lymphoblastic leukemia: results of the German multicenter trials GMALL 03/87 and 04/89. *Blood* 1998, 92:1898-1909
- Kolomietz E, Al Maghrabi J, Brennan S, Karaskova J, Minkin S, Lipton J, Squire JA: Primary chromosomal rearrangements of leukemia are frequently accompanied by extensive submicroscopic deletions and may lead to altered prognosis. *Blood* 2001, 97:3581-3588
- König M, Reichel M, Marschalek R, Haas OA, Strehl S: A highly specific and sensitive fluorescence in situ hybridization assay for the detection of t(4;11)(q21;q23) and concurrent submicroscopic deletions in acute leukemias. *Br J Haematol* 2002, 116:758-764