RAPID COMMUNICATION

Improved Sensitivity of BCR-ABL Detection: A Triple-Probe Three-Color Fluorescence In Situ Hybridization System

By P.B. Sinclair, A.R. Green, C. Grace, and E.P. Nacheva

Chronic myeloid leukemia (CML) is a clonal disorder arising in early stem cells. In the chronic phase, the expanded clone typically consists of the more mature stages of the granulocytic lineage.1 Bone marrow metaphases from around 90% of CML patients are characterized by the Philadelphia (Ph) translocation [t(9;22)(q34;q11)].2 The same cytogenetic abnormality is seen at a lower frequency in other hematologic malignancies, in particular acute lymphoblastic leukemia (ALL).3 As a result of the Ph translocation, parts of the ABL and BCR genes become fused. Cytogenetic quantification of Ph+ metaphases can be used to monitor patient response to treatment but is of limited sensitivity and applies only to cycling cells. Fluorescence in situ hybridization (FISH) with probes from the BCR and ABL regions can also identify the Ph translocation in interphase cells. Established systems for the detection of fusion genes by FISH rely on colocalization of two different probes but are associated with a high rate of false-positive results. We have introduced a third probe labeled with a different fluorochrome to create a triple-probe/three-color system that permits identification of both the Ph chromosome and the derivative 9 chromosome in Ph+ cells. This system was used to determine the frequency of interphase cells carrying the BCR-ABL fusion gene in bone marrow and peripheral blood granulocytes from patients showing variable cytogenetic responses to interferon. Our data show that the triple-probe/three-color approach allows highly sensitive detection of residual disease. Moreover, this method is readily applicable to the analysis of other chromosome translocations.

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three-color system exhibits a very low false-positive rate, thus allowing the sensitive detection of residual disease.

**MATERIALS AND METHODS**

*Patient material and standard cytogenetics.* Slides were prepared from methanol/acetic acid-fixed standard cytogenetic preparations from five lymphoma patients with no morphologic evidence of bone marrow involvement and used as negative control samples. Stored fixed cytogenetic preparations from Ph⁺ CML patients treated with IFN on which standard G-banded cytogenetic analysis had been performed were used to make slides for FISH analysis. Granulocyte preparations (>95% granulocytes) were made according to standard protocols from peripheral blood taken concomitantly with the BM samples. A granulocyte negative control was prepared from a blood donor sample.

**DNA probes.** A Spectrum Green-labeled probe extending from between BCR exons 13 and 14 (b2 and b3) for approximately 300 kb in the 5’ direction and a Spectrum Orange-labeled probe extending for approximately 200 kb in the 3’ direction from between exons 4 and 5 on ABL were supplied by Vysis Inc (Downer’s Grove, IL; catalogue no. 32-190022). Cosmid clones D9S115, D9S62, D9S63, and ASS were kindly provided by M. Leversha (Sanger Centre, Hinxton Hall, Cambridge, UK). Probes were labeled with biotin using a BioNick Labelling system (GIBCO BRL, Gaithersburg, MD; catalogue no. 18247-015) or with Cy5 using a fluorolink Cy5 Nick translation kit (Amersham Life Science Inc, Arlington Heights, IL; catalogue no. PA35001). For single-color experiments, 100 ng of probe was preannealed with 10 μg of Cot-1 DNA (GIBCO-BRL) for 1 hour before hybridization. For three-color experiments, 100 ng Cy5-labeled probe was ethanol precipitated with 10 μg Cot-1 DNA and redissolved in Vysis LSI hybridization buffer (catalogue no. 30-804826) with Spectrum Green-labeled BCR probe and Spectrum Orange-labeled ABL probe. Probes were denatured at 73°C for 5 minutes and preannealed for 1 hour before hybridization.

**Hybridization and detection procedures.** FISH with biotinylated probes was performed as described previously.27 Hybridization procedures and washing for the triple probes were performed according to the manufacturer’s protocol for the BCR-ABL probes.

All FISH images were analyzed using a Smart Capture-View Point multi-color imaging FISH station (Digital Scientific, Ltd, Cambridge, UK) composed of a fluorescence microscope (Zeiss Axio phot), equipped with a quadriband pass (fluorescein isothiocyanate [FITC], CY3, Cy5, and DAPI) filter set (Chroma, Battleboro, VT), 50 W Mercury lamp, and a CCD camera (Photometrics, Tucson, AZ). The analysis of the four-color images was performed on merged files containing data from all channels, with DAPI being presented by a contor that marks the end of density gradient (the boundaries of a nucleus).

**RESULTS**

*Selection and assessment of probes.* Directly fluoresceinated probes from the 3’ region of ABL and 5’ region of BCR (Vysis) were selected as the basis for a triple-probe/three-color detection system because they produced consistently bright signals in our hands and could be used for the detection of both MBCR-ABL and mBCR-ABL. We then looked for additional probes from the other side of each break point. A significant part of the genome immediately 3’ of BCR consists of sequences repeated on both sides of the gene.26 Because cross-hybridization of FISH probes to these repeats could lead to ambiguous signals, we chose to study four cosmids thought to lie within 1.5 Mb upstream of ABL exon 1b. The published order of the four cosmids in relation to ABL is centromere, D9S115, D9S62/D9S63, ASS, ABL.27 The cosmids were biotinylated and hybridized to normal metaphase chromosomes. After detection with avidin FITC, each cosmid gave a discrete signal on band q34 of chromosome 9 and, when pooled, they produced a single localized signal. However, on interphase cells, the pool of all four cosmids did not consistently produce a single localized signal. We therefore studied ASS, the probe closest to ABL. The ASS cosmid was isolated with the probe ASSg²8,29 derived from the Argininosuccinate synthetase (ASS) gene and had previously been used in FISH mapping studies.27 All further studies were performed using the ASS cosmid labeled with Cy 5, in combination with the existing 3’ ABL probe labeled with Spectrum Orange and 5’ BCR probe labeled with Spectrum Green.

**Extended DNA preparations.** Extended DNA preparations were obtained by cytopsin of purified granulocytes.

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**Fig 1.** Fluorescence signals resulting from hybridization of ABL (in red) and ASS (in blue) probes hybridized to extended chromatin from normal cells at different stages (a, b, and c) of condensation.

**Fig 2.** Fluorescence signals from a Ph⁺ bone marrow metaphase hybridized with the triple-probe/three-color system; ABL (in red), BCR (in green), and ASS (in blue); yellow arrows, Ph translocation t(9;22)(q34.1;q11.2); white arrows, normal 9 and 22 homologues.

**Fig 3.** Diagrammatic presentation of fluorescent signals obtained using conventional two-probe system (A, B, and C) or triple-probe/three-color system (a, b, and c) hybridized to interphase cells: red, ABL; green, BCR; blue, ASS. The figure shows the signal patterns obtained from normal cells (A and a), BCR-ABL⁺ cells (B and b), and as a consequence of coincidental colocalization of BCR and ABL in normal cells (C and c).

**Fig 4.** Representative examples of the triple-probe/three-color system hybridized to interphase cells: red, ABL; blue, ASS; green, BCR. (a) Normal cells displaying two isolated green signals (BCR gene) marking the normal 22s and two colocalized red and blue (or purple) signals (ASS and ABL genes) from the normal 9s. (b) Ph⁺ cells displaying a typical localized red/green (or yellow) (BCR-ABL fusion gene) marking the Ph chromosome together with a single blue signal (ASS gene) from the derivative 9 chromosome with blue/red and isolated green signals from the normal 9 and 22 homologues. (c) Cells displaying a cluster of red/blue/green signals (arrow) marking coincidental colocalization of either ASS/ABL and BCR (false-positive) or BCR/ABL and ASS (false-negative). Note that cells displaying this signal pattern have not been scored. (d) A cell displaying a typical Ph⁺ signal pattern but without fusion of the BCR and ABL signals. Note that cells displaying this signal pattern have not been scored. (e) Cell displaying less than 6 signals have not been scored.
previously treated with hypotonic solution. Interphase cells prepared in this way were three or four times the size of conventionally treated granulocytes. After hybridization, the ABL probe gave rise to a series of discrete signals in some cells (Fig 1a). The ASS cosmid probe gave either a single unbroken signal (Fig 1a and c) or a closely associated doublet (Fig 1b). The ABL probe is composed of several discrete clones covering approximately 200 kb. From the results shown in Fig 1, the physical distance between the ASS and ABL probes can be estimated as 200 to 400 kb. Because the 5’ end of the Vysis ABL probe was approximately 200 kb downstream of ABL exon 1b, the distance between exon 1b and the ASS cosmid is not more than 200 kb. This figure is in agreement with a previous estimate. 27

Application of triple probe to normal cells and Ph+ cells. A representative Ph+ metaphase is shown in Fig 2. Colocalization of red and blue signals (ABL and ASS) was seen on the normal chromosome 9 homologue and a single green signal (BCR) on the normal chromosome 22. By contrast, colocalization of red and green signals identified the Ph chromosome and an isolated blue signal was observed on the derivative chromosome 9.

The triple probe was also hybridized to interphase normal and Ph+ bone marrow cells. The signal patterns observed with a conventional two-probe system and with the triple-probe/three-color system are shown diagramatically in Fig 3. Representative interphase cells hybridized with the triple-probe/three-color system are presented in Fig 4. Normal interphase cells were characterized by a pattern of two isolated green signals and two colocalized red and blue signals (Fig 4a). Although not all red and blue signals were associated closely enough to give a region of overlap, only 2% to 4% were separated by more than a signal width. In Ph+ cells, a single red/blue colocalization (normal chromosome 9), a red/green colocalization (BCR-ABL), a single green signal (normal 22), and single blue signal were seen (Fig 4b). In the majority of cells, colocalization of red and green signals resulted in a yellow region of overlap.

Quantification of false-positive results. To assess the sensitivity of the triple-probe/three-color system, interphases from disease-free bone marrow samples obtained from lymphoma patients and granulocytes from a normal donor were analyzed. Signals were captured and scored by two observers independently in the following way. For each sample, 200 cells were scored in which two signals from each of the three probes were present. A cell was considered to have a BCR-ABL colocalization if a red signal and a green signal were touching or overlapping to give a yellow region. A cell was considered to show loss of colocalization of ABL and ASS if one pair of red and blue signals was separated by more than a signal width. A cell was scored as positive for a BCR-ABL fusion gene if one ABL signal showed colocalization with BCR and also loss of colocalization with ASS. Cells displaying a triple (green-red-blue) BCR-ABL-ASS colocalization (Fig 4c) were considered to represent coincidental colocalization. Cells displaying loss of colocalization of ABL and ASS but without overlapping or touching red and green signals (Fig 4d) were also not scored as positive for the BCR-ABL fusion gene. Cells in which one or more signals were lost (Fig 4e) were excluded from the 200 cells scored but constituted between 5.2% and 25.7% (mean, 13.9%) of the total number of cells captured.

The results of this analysis are presented in Table 1. Of 1,200 cells scored, none were BCR-ABL+. According to the criteria given above, Colocalization of BCR and ABL was seen in 119 cells (9.9%), but in all of these ABL also colocalized with ASS to give a triplet composed of red, green, and blue signals (Fig 4c). Loss of colocalization of ABL and ASS was seen in 34 cells (2.8%), but without colocalization of BCR and ABL in any of them. A theoretical predicted false-positive rate can be calculated for each negative control from the number of cells showing colocalization of BCR and ABL and loss of colocalization of ABL and ASS. Calculated false-positive rates varied between 0.065% and 0.27%, with a mean and SD of 0.15% and 0.08%.

Quantification of residual disease. Bone marrow cells and peripheral blood granulocytes were obtained from patients exhibiting varying degrees of cytogenetic response to interferon as assessed by G-banding. These samples were hybridized with the triple probe and scored for numbers of normal and BCR-ABL+ cells (Table 2). Two patients were in complete cytogenetic remission. One of these showed no evidence of BCR-ABL+ cells in either the bone marrow or peripheral blood granulocyte preparations (patient no. 1) in

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Cell Type</th>
<th>Normal</th>
<th>BCR-ABL+</th>
<th>Colocalization of ABL-ASS With BCR</th>
<th>Separation of ASS and ABL With no BCR-ABL Colocalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM</td>
<td>170 (85.0%)</td>
<td>0</td>
<td>23 (11.5%)</td>
<td>7 (3.5%)</td>
</tr>
<tr>
<td>2</td>
<td>BM</td>
<td>179 (89.5%)</td>
<td>0</td>
<td>17 (8.5%)</td>
<td>4 (2.0%)</td>
</tr>
<tr>
<td>3</td>
<td>BM</td>
<td>173 (86.5%)</td>
<td>0</td>
<td>21 (10.5%)</td>
<td>6 (3.0%)</td>
</tr>
<tr>
<td>4</td>
<td>BM</td>
<td>183 (91.5%)</td>
<td>0</td>
<td>13 (6.5%)</td>
<td>4 (2.0%)</td>
</tr>
<tr>
<td>5</td>
<td>BM</td>
<td>177 (88.5%)</td>
<td>0</td>
<td>18 (9.0%)</td>
<td>5 (2.5%)</td>
</tr>
<tr>
<td>6</td>
<td>PBG</td>
<td>165 (82.5%)</td>
<td>0</td>
<td>27 (13.5%)</td>
<td>8 (4.0%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1047</td>
<td>0</td>
<td>119</td>
<td>34</td>
</tr>
</tbody>
</table>

Mean (%): 87.3 ± 3.25, SD: 9.9 ± 2.43, 2.8 ± 0.82

Two hundred cells were analyzed from each sample. Individual cells were categorized as either normal (Fig 4a), BCR-ABL+ (Fig 4b), showing a triple BCR-ABL-ASS colocalization (Fig 4c), or showing separation of ASS and ABL with no BCR-ABL colocalization (Fig 4d). Cells not showing the expected six FISH signals, two from each probe (Fig 4e), were not included in this analysis.

Abbreviations: BM, bone marrow; PBG, peripheral blood granulocyte.
the second patient (patient no. 2), four bone marrow cells (2%) and eight peripheral blood granulocytes (4%) were scored as positive. Because no false-positive cells were seen among 1,200 normal nuclei, these figures strongly suggest the presence of a low level of residual disease that was not detected by G-banding.

Two other patients (patients no. 3 and 5) exhibited a partial cytogenetic response, as assessed by G-banding, and triple-probe/three-color FISH detected similar levels of Ph+ cells in both bone marrow cells and peripheral blood granulocytes. In patients no. 4 and 6, all of the metaphases were Ph+ by G-banding analysis of bone marrow cells. When hybridized to bone marrow cells, the triple probe produced similar results, with 99% and 96% of cells scored as BCR-ABL+. However, in both patients no. 4 and 6, a significantly lower proportion of peripheral blood granulocytes (69% and 67%) were BCR-ABL+ (x² = 40.62 and 27.89, respectively; P < .01 in each case). Independent experiments produced similar levels of positive granulocytes in both these patients. This finding is unlikely to be a technical artefact, because preparation of and hybridization to granulocytes was performed in an identical manner in the six patients and numbers of unscorable cells were similar. Instead, these results imply preferential release of cytogenetically normal granulocytes from the bone marrow in some patients.

In the three cases showing predominantly BCR-ABL+ bone marrow interphases, cells showing separation of one ABL-ASS pair but no colocalization of BCR and ABL (Fig 4d) can be considered false-negative results. Levels of cells displaying this signal pattern in the bone marrow and granulocytes, respectively, were 7.5% and 2.7% in patient no. 4, 3.6% and 2.7% in patient no. 5, and 6.5% and 4.4% in patient no. 6.

DISCUSSION

Results from this study show that the introduction of a third differentially labeled probe greatly increased the sensitivity of a conventional two-probe/two-color FISH system. The triple-probe/three-color system produced a very low incidence of false-positive cells and therefore permitted highly sensitive detection of residual disease.

Quantitation of residual disease in CML has major clinical implications. Several trials have now reported that the degree of cytogenetic response to IFN has independent prognostic value.20-22 Furthermore, the level of residual disease after allogeneic bone marrow transplantation indicates the likelihood of relapse.23 G-banded analysis of metaphase chromosomes has been widely adopted as a means of quantifying residual disease. However, relapse may occur after a complete cytogenetic response and the continued monitoring of Ph+ patients with more sensitive techniques allows early detection of relapse and intervention.12 Southern and Western blots have been used to measure levels of the BCR-ABL gene or protein, respectively, but lack sensitivity.13 Reverse transcription-polymerase chain reaction (RT-PCR) techniques can detect a single BCR-ABL+ cell among 106 normal cells.24 However, RT-PCR is not without drawbacks; in particular, quantification is technically challenging and currently not widely applied.

FISH allows the analysis of individual cells for the presence of the fusion gene and offers many of the advantages of G-banded analysis while overcoming its limitations.14 Importantly, interphase FISH can be applied to blood samples, thus obviating the need for multiple bone marrow aspirates. The role of FISH in monitoring residual disease is currently limited by the sensitivity with which it can detect Ph+ cells and by the number of cells that can be scored in practice. The latter constraint will be minimized by the development of suitable automated image analysis systems. However, new approaches are needed to increase the sensitivity of FISH by reducing the frequency of false-positive cells.

Conventional systems for the detection of BCR-ABL by FISH rely on differentially labeled probes from 3′ of ABL and 5′ of either MBCR or mBCR.15-17,22,27,38 False-positive levels among negative control samples were reported to vary between 1.5% and 6.5% in the most extensive published study.22 Colocalization of BCR and ABL among our negative control samples was higher, between 6.5% and 13.5%, con-

Table 2. Analysis of CML Patient Samples Using Triple-Probe/Three-Color FISH System

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Initials</th>
<th>Age* / Sex</th>
<th>Ph+/Total Metaphases†</th>
<th>Interphase Cells Scored‡</th>
<th>BCR-ABL§</th>
<th>Interphase Cells Scored¶</th>
<th>BCR-ABL∥</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K.S.</td>
<td>42/M</td>
<td>0/17</td>
<td>200</td>
<td>0</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>D.W.</td>
<td>58/M</td>
<td>0/20</td>
<td>200</td>
<td>4 (2%)</td>
<td>200</td>
<td>8 (4%)</td>
</tr>
<tr>
<td>3</td>
<td>S.L.</td>
<td>46/F</td>
<td>4/10 (40%)</td>
<td>100</td>
<td>39 (39%)</td>
<td>42</td>
<td>16 (38%)</td>
</tr>
<tr>
<td>4</td>
<td>S.L.</td>
<td>46/F</td>
<td>10/10 (100%)</td>
<td>100</td>
<td>99 (99%)</td>
<td>100</td>
<td>64 (64%)</td>
</tr>
<tr>
<td>5</td>
<td>R.C.</td>
<td>47/M</td>
<td>9/10 (90%)</td>
<td>100</td>
<td>93 (93%)</td>
<td>100</td>
<td>98 (98%)</td>
</tr>
<tr>
<td>6</td>
<td>G.D.</td>
<td>69/M</td>
<td>10/10 (100%)</td>
<td>100</td>
<td>96 (96%)</td>
<td>100</td>
<td>67 (67%)</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; PBF, peripheral blood granulocyte.

* Age at time of analysis.
† As assessed by G-banding.
‡ For this analysis, only cells that were normal (Fig 4a) or BCR-ABL+ (Fig 4b) were scored. All other patterns (Figs 4c, d, and e) were excluded from the analysis.
§ As defined by the presence of a green-red (BCR-ABL) colocalization and separation of one red-blue (ASS-ABL) doublet. See text for details.
consistent with a previous study making use of the Vysis probes. The observed difference in false-positive rates is likely to reflect the relatively large size of the Vysis probes compared with the probes used in earlier studies. Data have also been published for two dual-color FISH systems, both of which used a large probe spanning one of the translocation break points. Seong et al generated a probe by inter-Alu PCR from a somatic cell hybrid containing 5 Mb of human DNA from the ABL region. Using this probe and a cosmid from 5′ BCR, a false-positive rate of between 0.19% and 0.85% (mean, 0.53%) in different samples was achieved. A similar system made use of a YAC containing the BCR gene and a 3′ ABL probe and resulted in an average false-positive rate of 0.1%. However, systems of this type are associated with several technical problems. Firstly, interpretation of results can be difficult, because use of large probes results in FISH signals occupying a high proportion of the cell area. Secondly, hybridization conditions suitable for simultaneous use of small and large probes usually represent a compromise. Thirdly, YAC clones and somatic cell hybrids are frequently unstable when propagated in culture.

In this study, the problems listed above are avoided by using three pools of cosmids to create a triple probe system. Moreover, the three-color approach is less prone to false-positive results because positive cells are distinguished by two characteristics: firstly by the presence of a red/green colocalized signal marking BCR-ABL and secondly by loss of colocalization of ABL and ASS to leave an isolated blue signal marking the derivative chromosome 9. By contrast, the use of a single-color probe spanning BCR or ABL relies on the appearance of a third signal to mark the derivative chromosome in positive cells, a scenario that is easily mimicked by fluorescent debris. Our data show that no false-positive results were seen among 1,200 scorable cells using a triple-probe/three-color system, suggesting a false-positive rate of the order of 0.1% can be achieved. Based on numbers of cells showing either colocalization of BCR and ABL or loss of colocalization of ABL and ASS, a theoretical false-positive rate of 0.14% was calculated. However, this is likely to be an overestimate, because smaller cells that are most prone to coincidental colocalization of ABL and BCR are also less likely to show accidental loss of colocalization of ABL and ASS. Automated image capture and scoring will be needed to determine the precise frequency of false-positive cells, which may be considerably lower. It is also worth emphasising that the potential for false-positive results could be further reduced by replacing the ASS cosmid with a larger probe closer to ABL.

One of the major advantages of interphase FISH is that it permits analysis of peripheral blood cells. Previous studies of small numbers of patients have suggested that unfraccionated blood cells or purified neutrophils contain similar or higher levels of BCR-ABL+ cells than bone marrow. It was therefore intriguing to find two patients in whom the percentage of BCR-ABL+ granulocytes was significantly lower than the percentage of BCR-ABL+ bone marrow cells. To our knowledge, this is the first observation of this phenomenon that implies preferential retention and/or destruction of Ph+ granulocytes in the bone marrow in some patients. It is not clear whether this is related to abnormalities of progenitor cell adhesion to stroma that have been implicated in the pathogenesis of CML. However, the observation is reminiscent of the suggestion that granulocytes containing a 20q deletion may also be preferentially retained or destroyed in the bone marrow.

It is also important to compare the results of metaphase analysis by G-banding with interphase FISH. Previous studies have reported significant numbers of BCR-ABL+ metaphases in samples displaying only Ph+ metaphases. However, our data show levels of Ph+ metaphases correlated closely (within 5%) with numbers of BCR-ABL+ bone marrow interphases. A possible explanation for this discrepancy is that introduction of a third probe not only reduces the false-positive rate but also the false-negative rate. In some Ph+ cells, the BCR and ABL signals are close but do not touch (Fig 4d). The precise proportion of such cells reflects the position of the ABL break point and thus varies from patient to patient. These cells are therefore scored as negative for the fusion gene by two-probe two-color FISH. With the triple-probe/three-color system, the same cells are only scored as normal if both pairs of ABL and ASS signals colocalize (Fig 4a). In a population of cells that is predominantly BCR-ABL+, the number of cells displaying both loss of colocalization of BCR and ABL and of one ABL-ASS pair can be used to estimate the frequency of coincidental BCR-ABL separation or false-negative rate. The three bone marrow samples with a high proportion of positive cells displayed this pattern of signals in 7.5%, 3.6%, and 6.4% of cells. Levels of false-negative cells were lower, ie, 2.7%, 2.7%, and 4.4% in the corresponding granulocyte preparations. The relatively small numbers of false-negative granulocytes, which if more relaxed scoring criteria were applied could be scored as positive, do not account for the difference in levels of positive cells between bone marrow and granulocytes seen in patients no. 4 and 6.

These data show for the first time that the use of more than two differently labeled probes from a specific chromosome translocation enhances the sensitivity of residual disease detection. Two-color FISH has been used not only for the detection of BCR-ABL+ disease, but also for the detection of other leukemia-associated translocations. It is worth emphasising that the principle behind the triple-probe/three-color FISH system is potentially applicable to the analysis of any balanced translocation.

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