False-negative rates for *MYC* fluorescence *in situ* hybridization probes in B-cell neoplasms

As *MYC* rearrangements in high-grade B-cell neoplasms are associated with poor prognosis, interphase fluorescence *in situ* hybridization (FISH) testing for *MYC* has become part of the routine clinical evaluation of high grade B-cell lymphomas (HGBCL), diffuse large BCL (DLBCL) and plasma cell neoplasms (PCN).[1-5] This study examined BCL and PCN cases in which FISH for *MYC* rearrangements was evaluated by concurrent MYC breakapart (BAP) and *MYC*/IGH dual fusion (D-FISH) probe sets (both commercially available), to assess whether a screening strategy using MYC BAP or D-FISH alone is sufficient for detecting MYC rearrangements. Our results indicate high false negative (FN) rates using either approach alone (4.1% MYC BAP and 22.1% MYC-IGH), suggesting that the use of both FISH probe sets is superior for detection of MYC rearrangements in BCL and PCN than either individual probe. Additionally, mate-pair sequencing (MPseq) uncovered multiple cryptic and complex mechanisms providing the underlying genomic architecture and potential mechanisms that may ultimately lead to improved methods of detection.

BCL with *MYC* and *BCL2* and/or *BCL6* rearrangements has been included as a distinct entity in the 2017 World Health Organization (WHO) classification (HGBCL with MYC and BCL2 and/or BCL6 rearrangements).[4] These patients have a poor prognosis and require more intensive therapy.[5,6] *MYC* rearrangement alone also portends poor prognosis in some studies.[2,7] In plasma cell myeloma (PCM), secondary *MYC* rearrangement is associated with aggressive disease.[8] Thus, it has become standard-of-care in the workup of DLBCL, HGBCL, and PCM to perform FISH evaluating for *MYC* rearrangement.

Fluorescence *in situ* hybridization testing for *MYC* on formalin-fixed, paraffin-embedded tissue (FFPET) is the preferred and most widely used clinical laboratory

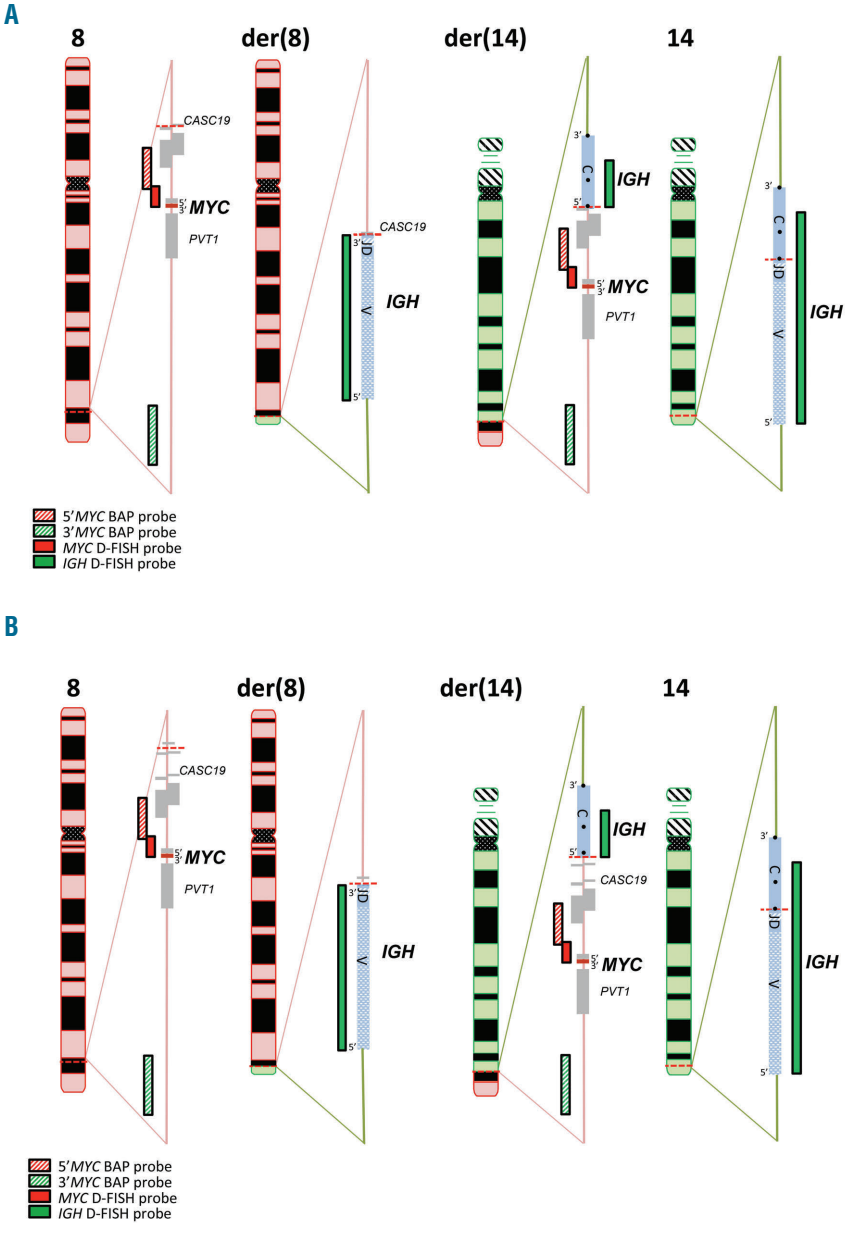
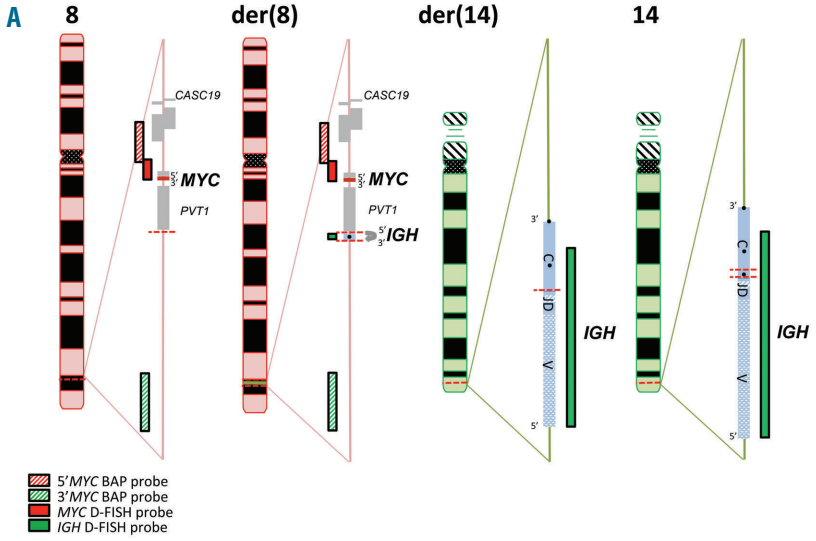


Figure 1. Chromosomal rearrangement mechanisms for patients 1 and 2 with discordant *MYC* BAP and *MYC*/IGH D-fluorescence *in situ* hybridization (FISH) results. Reciprocal translocations in case 1 (A) and case 2 (B) (breakpoints indicated by horizontal dashed red lines) had atypical breakpoints located centromeric to the *MYC* BAP footprints (striped red and green rectangles located on chromosome 8). However, a single *MYC*/IGH fusion on the derivative chromosome 14 was observed by the *MYC*/IGH D-FISH probe set (solid red rectangle located on chromosome 8 and solid green rectangle located on chromosome 14) in both cases. The normal *MYC* BAP FISH result is explained in both cases since the chromosome 8 breakpoint is centromeric to the FISH probe. Variable (V), diversity (D), joining (J) and constant (C) regions of the IGH locus are indicated.

method. In approximately 60% of *MYC*-rearranged BCL cases, *MYC* is translocated to an immunoglobulin gene (IG), either the heavy chain (IGH) (most common), lambda light chain (IGL), or kappa light chain (IGK).[4,6] Therefore, MYC rearrangements are detectable either by a BAP or D-FISH probe strategy looking specifically for a *MYC*/IGH (or -*IGL*, -*IGK*) translocation. The *MYC* breakpoint can vary widely, particularly if the partner gene is not IGH.[9] Thus, it is not surprising that *MYC* BAP probes have been shown to be more sensitive than *MYC* D-FISH probes and are often used in isolation for screening.[10,11] Here we conducted a large retrospective analysis of cases in which FISH was performed concurrently using both *MYC* BAP and *MYC*/IGH D-FISH to evaluate for rearrangements detectable with one probe but with a normal result for the other probe. We then used MPseq to analyze a subset of cases in which *MYC* BAP was falsely negative, in order to understand more fully the biological mechanism of these unusual *MYC* rearrangements.

From January 2006 to June 2011, we performed both the *MYC* BAP and *MYC*/IGH D-FISH probe sets on 2518 FFPET and 971 fresh liquid [peripheral blood (PB), bone marrow (BM), and body fluids] specimens, yielding a total of 3489 cases in our study. Commercial MYC BAP and *MYC*/IGH D-FISH probe sets (Abbott Molecular, Des Plaines, IL, USA) are used in our laboratory. All specimens were subjected to standard specimen-specific laboratory protocols (*Online Supplementary Appendix 1*).

A total of 266 out of 2518 (10.5%) FFPET and 98 out of 971 (10.1%) liquid specimens were positive for *MYC*/IGH fusion using the D-FISH probes. Of these, 246 FFPET and 72 liquid specimens also had *MYC* BAP results, yielding a total of 318 cases that had successful results for both probe sets. *MYC* BAP FISH was negative in 9 out of 246 (3.7%) FFPET and 4 out of 72 (5.6%) liquid specimens in which *MYC*/IGH fusion was detected, resulting in a FN rate overall for the *MYC* BAP of 4.1% (13 out of 318). Of these 13 cases with negative MYC BAP, 10 showed two intact *MYC* signals, and 3 demon-



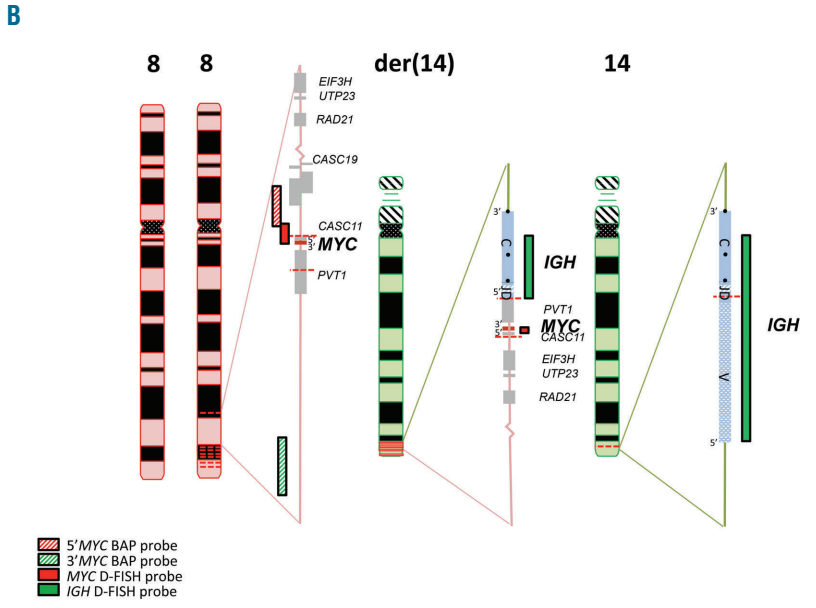


Figure 2. Chromosomal rearrangement mechanisms for patients 3 and 4 with discordant *MYC* BAP and *MYC*/IGH D-fluorescence *in situ* hybridization (FISH) results. (A) In case 3, a 212 Kb genomic segment of the IGH constant region was inserted in an inverted orientation distal to the PVT1 gene downstream of *MYC*, thus producing a single fusion by the *MYC*/IGH D-FISH probe set. (B) In case 4, a 245 Kb genomic segment of the *MYC* gene and a portion of the *PVT1* gene is duplicated and inserted into chromosome 14 distal to the IGH constant region and diversity and joining gene segments. Numerous additional portions of chromosome 8 were translocated into the derivative chromosome 14 distal to the *MYC* gene. Due to the size and/or complexity of the rearrangement, and the lack of clone coverage at the rearrangement breakpoints, neither of these structural abnormalities would be detected by the *MYC* BAP FISH probe. Variable (V), diversity (D), joining (J) and constant (C) regions of the IGH locus are indicated.

strated three intact signals due to gain of chromosome 8 or the MYC region when using the *MYC* BAP. Of the 318 cases, we evaluated 175 cases with a BCL2 BAP probe and 39 (22.3%) demonstrated a rearrangement; 115 cases were evaluated with a BCL6 BAP probe and 8 (7%) demonstrated a rearrangement. One case had a concurrent BCL2 and BCL6 rearrangement.

When the indication for FISH is DLBCL/HGBCL, an Ig gene partner is detected in approximately 60% of MYC-rearranged cases and non-IG partner in 40%.[6] Of the *MYC*-Ig cases, 75% have *MYC*/IGH fusion, while 25% show fusion with either IGL or IGK.[6] Therefore, if 4.1% of *MYC*/IGH fusion cases are associated with FN *MYC* BAP probe results, and MYC/IGH fusion accounts for only 45% of MYC rearranged cases, these data suggest there may be additional FN results associated with non-IGH partners.

To address the opposite scenario, in which *MYC* rearrangements were identified by the *MYC* BAP probe but had a signal pattern not indicative of fusion using the *MYC*/IGH D-FISH probe, we identified an additional 131 FFPET cases from 2016. Of these 131 cases with a positive MYC BAP probe result and a negative MYC/IGH fusion result, 102 cases (77.9%) had additional MYC signals in relation to centromere 8 FISH probe signals using the *MYC*/IGH probe, suggesting a *MYC* rearrangement with a non-IGH partner. Of these 102 cases, 9 had *MYC*/IGK fusion, 26 had MYC/IGL fusion, and 67 were interpreted as MYC/non-Ig fusion positive. The remaining 29 cases (5 with *MYC*/IGK fusion, 1 with *MYC*/IGL fusion, and 23 with *MYC*/non-Ig fusion per subsequent analysis) had both a positive *MYC* BAP result and a "normal" *MYC* result using the *MYC*/IGH probe set (no evidence of *MYC*/IGH/IGH fusion nor additional MYC signals in relation to centromere 8), resulting in a 22.1% (29 out of 131) FN rate for *MYC* using the *MYC*/IGH probe set.

We sought to resolve the genomic rearrangements of four discordant FISH cases (normal MYC BAP/abnormal *MYC*/IGH) using MPseq, a novel next-generation sequencing-based technique that allows the detection of structural rearrangements with higher resolution compared to FISH, thus enabling characterization of precise rearrangement breakpoints[12,13] (*Online Supplementary Appendix 1*).

Cases 1 (Figure 1A) and 2 (Figure 1B) uncovered similar reciprocal translocations placing the MYC gene distal to the IGH region on the derivative chromosome 14. While the chromosome 8 breakpoints for both cases were centromeric to the 5'*MYC* BAP footprint resulting in a FN *MYC* result using the *MYC* BAP probe, a single fusion signal was observed using the *MYC*/IGH D-FISH probes due to the proximity of the IGH and *MYC* genes. In case 3 (Figure 2A), MPseq characterized a 212 Kb genomic segment of the IGH variable and diversity regions inserted in an inverted orientation within 8q24.21 immediately distal to the *PVT1* gene downstream of the *MYC* gene. Because the inverted segment of IGH was inserted distal to *MYC*, the 5' and 3' *MYC* BAP footprints remained intact on both the normal and derivative chromosome 8. This insertional mechanism would be cytogenetically "cryptic" and also explains the presence of a single *MYC*/IGH fusion signal. Case 4 (Figure 2B) also demonstrated a translocation between the variable and diversity segments of the *IGH* gene and a region telomeric to the *MYC* gene within the *PVT1* gene. A tandem duplication of a 245 kb portion of 8q24.21 containing a portion of CASC11, *MYC* and a portion of the *PVT1* genes was also observed. These results provide detailed visualization of three different genomic mechanisms resulting in the translocation of portions of IGH in close proximity to MYC, resulting in *MYC*/IGH fusion, yet eluding detection with a *MYC* BAP probe. Common breakpoints near or within long non-coding RNA sequences surrounding the *MYC* gene[8] were observed in each case (*Online Supplementary Appendix 2*).

Factors such as cost, efficiency, and a desire for information about the translocation partner all impact the *MYC* FISH strategy chosen by a given pathologist or clinician. Literature review, as well as anecdotal experience, suggest that screening with a *MYC* BAP alone is the most widely used method in both BCL and PCM, and that IG D-FISH probes, if evaluated, are performed only if *MYC* BAP reveals a positive result.[1,2,4,5] Precise FN rates using the *MYC* BAP alone are not widely publicized, although our results are similar to previous reports.[14,15] As our laboratory uses the most sensitive commercial *MYC* BAP, these estimates of FN rates may actually be conservative.[10] Furthermore, determining the significance of extra *MYC* signals seen with a *MYC*/IGH D-FISH probe used in isolation (*MYC* rearrangement in which the partner is not IGH *vs.* extra intact copies of *MYC*) requires the concurrent use of a robust *MYC* BAP probe.[10]

In summary, given the importance of detecting *MYC* rearrangements in BCL, awareness of the FN rate of the commonly used detection method is of critical importance to practising hematologists, pathologists, and geneticists. Our results indicate an at least 4% FN rate when screening with *MYC* BAP alone, which can be mitigated by performing *MYC*/IGH D-FISH. *MYC*/IGH D-FISH alone is also inadequate due to high FN rates (22.1%) and the significant proportion of non-IGH *MYC* translocations. Although only available on a small subset of cases, and limited by the lack of clinical information available to us, MPseq reveals a genomic complexity, and this fresh insight could lead to improved methods of detection.

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