Title

Development of a fluorescence in-situ hybridization probe for detecting *IKZF1* deletion mutations in patients with acute lymphoblastic leukemia

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Intragenic deletion of *IKZF1* is a recurrent genomic alteration in acute lymphoblastic leukemia (ALL). The deletions are mediated by illegitimate V(D)J recombination via cryptic recombination signal sequences (RSSs). We developed a fluorescence in-situ hybridization (FISH) probe set that can detect any type of *IKZF1* deletion including the commonly deleted exon 4-7 region. The probe set consists of a designed probe for the commonly deleted region (Cy3, red) and a bacterial artificial chromosomes (BAC) clone probe for detecting the 3' flanking region (Spectrum Green). Intact *IKZF1* showed a fusion signal, and the deleted allele showed loss of the red signal (0R1G1F). The FISH probes worked correctly for human leukemic cell lines and clinical samples. One case showed an atypical break-apart signal (1R1G1F). Inverse PCR of the case revealed rearrangement of the excised *IKZF1* fragment into a legitimate RSSs site at immunoglobulin kappa on chromosome 2, suggesting a pathogenic role of this RAG1/2-mediated event. In this study, we established FISH probe detecting *IKZF1* deletion in a quick, quantitative and cost-effective manner, and the results provided a novel insight into B-cell receptor editing by rearrangement of a cryptic RSS-mediated genomic fragment in ALL pathology.

Keywords: FISH, illegitimate V(D)J rearrangement, *IKZF1*, ikaros, RAG1/2-mediated rearrangement

Introduction

IKZF1 codes Ikaros, which is a member of a family of zinc-finger nuclear proteins that is required for normal lymphopoiesis[1]. RAG1/2-mediated recurrent intragenic deletion of *IKZF1* in patients with B-cell acute lymphoblastic leukemia (B-ALL) has been reported [2]. *IKZF1* mutations in cases of B-ALL are associated with poor prognosis and high risk of relapse[3]. *IKZF1* mutations are found in about 15% to 20% of pediatric B-ALL cases[2, 4] and in more than 75% of pediatric *BCR-ABL* positive ALL cases[4, 5]. The incidences of *IKZF1* mutations in adults are about 50% in B-ALL cases[6, 7] and about 65% in *BCR-ABL* positive ALL cases[8, 9]. The presence of either *IKZF1* mutation or *BCR-ABL* has been reported to be an independent risk factor of poor prognosis for patients with B-ALL. However, despite the prognostic value and potential for risk stratification based on the presence of *IKZF1* mutations[10], there are no suitable testing methods for these mutations, and current clinical applications are therefore limited[3].

Most *IKZF1* mutations (94%) are deletion mutations and there are rare point mutations resulting in loss of function of Ikaros[5]. *IKZF1* deletion mutation can be detected by next-generation sequencing (NGS), array comparative genomic hybridization (aCGH), multiplex ligation-dependent probe amplification (MLPA) and multiplex PCR, but these methods are costly and time-consuming

to do in routine clinical practice. We therefore designed a FISH probe for a commonly deleted region of *IKZF1* and developed a FISH probe set that can detect various types of *IKZF1* deletion distinguished from a larger allelic loss.

Materials and Methods

Patient samples

Following institutional review board approval, Carnoy's fixed bone marrow samples from 22 cases of leukemia were obtained from Hokkaido University Hospital tissue registry. The 22 cases included 19 cases of lymphoblastic leukemia (9 cases of Philadelphia chromosome-positive ALL, 7 cases of Philadelphia chromosome-negative B-ALL, and 3 cases of lymphoid crisis of chronic myelogenous leukemia) and 3 cases of acute myeloid leukemia as *IKZF1* deletion negative controls. In addition, 10 normal peripheral blood samples were included as a negative control to establish normal cutoffs.

Cell lines

PALL2 (Philadelphia-positive B acute lymphoblastic leukemia), MY (Philadelphia-positive acute biphenotypic leukemia), NALM-6 (B-ALL), BALL-1 (B-ALL), and P30/OHK (non-T non-B ALL) cells were used for FISH analysis. PALL-2 and MY cells were purchased from Japanese Collection of Research Bioresources (JCRB). NALM-6, BALL-1, and P30/OHK cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All cells were cultured in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin in 5% CO2. Chromosome analysis was performed using standard procedures.

FISH probe design

IKZF1 deletion is mediated by cryptic recombination recognition signals (RSSs) located in an intron of the *IKZF1* gene. Four types of deletions depending on the choice of RSSs have so far been reported (Figure 1A). A probe set was developed to identify an intragenic deletion of *IKZF1* at Chr7 (Figure 1B). This probe set includes an *IKZF1*-deleted region probe, labeled in Cy3 that consists of a commonly deleted part of *IKZF1,* and a 3' downstream region probe, labeled in Spectrum Green that consists of RP11-248J17 (chr7: 50,503,964 - 50,673,819, 170 kb). The *IKZF1* commonly deleted region was amplified using LongAmp Taq DNA polymerase (NEW ENGLAND BioLabs, Ipswich, MA) with primer sets: *IKZF1*ΔaF: 5'-GAGATCATGGCAAATCAGAGG-3' and *IKZF1*ΔaR: 5'-CGTACAGTACAAAGACTGCTGC-3', *IKZF1*ΔbF: 5'-GCAGCAGTCTTTGTACTGTACG-3' and *IKZF1*ΔbR: 5'-CCTCTTGCTTGTCATAAGAAGC-3', *IKZF1*ΔcF: 5'-AGAGGCAGGACTGTCTTCAG-3' and *IKZF1*ΔcR: 5'-

CCTTCACAAGGACTCCATAAGG-3', *IKZF1*ΔdF: 5'-CTTGTCTGTCAGTGTTAGGCTG-3' and *IKZF1*ΔdR: 5'-GTACCACCTTTGTCTCCAGG-3', *IKZF1*ΔeF: 5'-CATGCTAACCAGTGGTCTAGG-3' and *IKZF1*ΔeR: 5'-GAGCCTCAGAATAGCTCTGG-3', as 5 fragments (9-10 kb each, total of 49 kb, Supplemental Figure1). Amplified PCR products were gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Bacterial artificial chromosomes (BAC) were obtained from the BACPAC Resource Center (https://bacpacresources.org, last access on Dec. 28, 2017). DNA was isolated from bacterial cultures following a standard protocol using a PhasePrep BAC DNA Kit (Sigma-Aldrich, St. Louis, MO). The purified PCR products and extracted BAC DNA were fluorescently labeled via nick translation with Cy3-dUTP (GE Healthcare, Chicago, IL) and Green-dUTP (Abbot Molecular, Des Plaines, IL), respectively. The labeled probes were mixed with sonicated salmon sperm DNA (STRATAGENE, La Jolla, CA) and cot-1 DNA (Invitrogen, Carlsbad, CA).

FISH protocol

After drying the sample on a slide glass, the probe was applied to the marked area and co-denatured with the target DNA at 80 C for 2 min and then hybridized at 37 C overnight in an incubator（HYBrite, VYSIS, Downers Grove, IL）. The first washing was done with 0.4xSSC (Invitrogen)/0.3% CA-630 (Sigma Aldrich) at 76 C for 40 sec. After the second washing in 2xSSC/0.1% CA-630 at 76 C for 15 sec, 3 times dilution of 10% DAPI –II counterstain (Vysis) was applied. Slides were examined using an Olympus BX50 microscope equipped with appropriate filter sets for visualizing Spectrum Green, Spectrum Orange, and DAPI counterstains (Leica Microsystems, Buffalo, NY). Images were captured using CytoVision (Applied Imaging Corporation, Santa Clara, CA). For each sample, 100 interphase cells were counted to calculate the percentages of cells with a positive signal.

Genome PCR and sequencing

Genome samples were purified using a QIAamp DNA Mini Kit (Qiagen). The multiplex PCR was done to identify type of *IKZF1* deletion using previously reported primer sets[4]. PCR reactions were carried out with EmeraldAmp PCR Master Mix (Takara Bio, Kusatsu, Japan) under the following conditions: 94 C for 2 min; for 40 cycles, 94 C for 30 s, 62 C for 30 s, and 72 C for 30 s; 72 C for 7 min. The amplified PCR product was sequenced by the conventional Sanger method.

Inverse PCR

For a case with an atypical break-apart signal (UPN1), the integration site of the *IKZF1*-deleted fragment was analyzed by inverse PCR. After BamHI digestion of the genome, ligation was done using a DNA Ligation Kit (Takara Bio). Nested PCR was done using the primer set, 5'-joint of

*IKZF1*Δ fragment 1st PCR: 5'-GAGATCATGGCAAATCAGAGG-3' and 5'-GATTCAGTTTGTGCTTTGGAGAC-3', 2nd PCR: 5'-ACTCTAGGTTTGGAGCTCAGG-3' and 5'-CTGAGTAATATGTTAATAAGCCTGC-3', 3'-joint of *IKZF1*Δ fragment 1st PCR: 5'-TTGAGGATGTTGGCCTGTTG-3' and 5'-GTTGCTTGAATGTAAAGTCCAATC-3', 2nd PCR: 5'-GACAGAGTCTATGGTCTTGGG-3' and 5'-AGTGAGCCAGTAGATGCTGC-3'. The PCR amplified product was purified and sequenced to verify the junction of the integration site.

Results

The proximity of the red (R) and green (G) signals in non-deleted *IKZF1* results in a yellow fusion (F) signal. A typical *IKZF1*-deleted cell shows 1 yellow signal from the wild-type allele and 1 green signal from loss of the red signal by deletion (0R1G1F). This probe set was designed to detect any type of 4 major *IKZF1* deletions, which involved deletion of exons 4–7 (Figure 1). Two carboxy-terminal zinc fingers (exon 8) are responsible for dimerization with other IKAROS family members. Four amino-terminal zinc fingers (exons 4-6) mediate DNA binding. Loss of 2 or more amino-terminal zinc fingers encoded by exons 4-6 with deletion of the binding domain but retention of the dimerization domain results in a dominant-negative form of *IKZF1*[11]. Loss of exon 2 with the ATG start codon or loss of exon 8 with the dimerization domain results in loss of the function of *IKZF1* [12].

The probe set was validated using a sample from a healthy individual. The normal karyotype showed 2 fusion signals without an isolated red or green signal (0R0G2F) (Figure 2A). Fusion signals were identified on chromosome 7 without non-specific signals (Supplemental Figure 2). *IKZF1* deletion was detected as a loss of the red signal resulting in 1 green signal and 1 fusion signal (0R1G1F) (Figure 2B). Additional 10 normal control samples were analyzed to calculate normal ranges for false-positive signal patterns. Based on 500 nuclei scored, 0.4-1.6% false-positive nuclei were observed, thus normal cutoff was established to be 1.50% by calculating the upper boundestablished of a one-sided 95% confidence interval analysis by the binomial distributionto be 1.6%. Then we analyzed 5 cell lines and 22 clinical samples. Their characteristics and FISH results for each sample are summarized in Table 1. *IKZF1* deletion was detected in 2 Philadelphia (Ph)-positive ALL cell lines but not in 3 Ph-negative ALL cell lines. In clinical samples, *IKZF1* deletion was frequent in Ph-positive ALL (7/9, 77.8%) but relatively rare in CML lymphoid crisis (1/3, 33.3%) and Ph-negative ALL (2/7, 28.6%). On the other hand, *IKZF1* deletion was not detected in any of the AML samples. For the cases with *IKZF1* deletion, the exact deletion type could be identified by multiplex PCR. The joint sequence of each deletion was verified, and they showed a typical RAG1/2-mediated V(D)J recombination signature that represented 1) the consensus heptamer-like sequence located precisely at the recombination site, 2) nontemplated nucleotides added at the junction site, reminiscent of ‘‘N”-region addition, and 3) variable number of nucleotides

deleted from both ends, similar to the exonucleolytic ‘‘nibbling’’ seen at normal antigen receptor coding joints (Table 2). We identified 13 unique *IKZF1* deletions in 12 cases. *IKZF1*Δ4-7 (5/13) and *IKZF1*Δ4-8 (5/13) were equally frequent, followed by *IKZF1*Δ2-7 (3/13). *IKZF1*Δ2-8 was not detected in our series.

There were several atypical FISH signal patterns. Monosomy of chromosome 7 resulted in 0R0G1F pattern reflecting loss of the entire *IKZF1* region (Figure 2C). On the other hand, a hyperploidy sample showed extra signals reflecting ploidy of the region, as 0R0G4F pattern in AML with 92,XXYY (Figure 2D). One case showed 0R2G0F pattern that suggested bi-allelic *IKZF1* deletion (Figure 2E). Multiplex PCR analysis revealed 2 unique *IKZF1* deletions (Δ4-8, Δ2-7) in this sample, suggesting 2 independent deletion events can occur in a single ALL clone. The biological significance of bi-allelic *IKZF1* deletions compared to a heterozygous *IKZF1* deletion is not clear.

Another case showed an atypical “break-apart signal” with separated red and green signals other than 1 fusion signal (1R1G1F) (Figure 2F). Inverse PCR revealed that the *IKZF1*-deleted region was integrated into chromosome 2 on abParts of immunoglobulin light chain (kappa) in which there was a 45-kbp deletion (Figure 3A). The *IKZF1* excision fragment retained the entire cryptic RSSs at both ends, and the integrated acceptor site had genuine RSSs, which were replaced by random “N” nucleotide adding (Figure 3B). This finding indicated that the *IKZF1* excision fragments were cleaved and then integrated at another legitimate V(D)J recombination site (Figure 3C). Integration of the *IKZF1*-deleted fragment region into chromosome 2 was also confirmed by metaphase FISH (Supplemental Figure 3). In this case, *IKZF1* Δ4-7 was sequence verified (Table 2). Thus, a rare break-apart signal event of our probe represents *IKZF1* deletion event. We tried to verify integration of the *IKZF1* exon inserted into the immunoglobulin kappa transcript by RT-PCR using exon primer set flanking inserted *IKZF1* fragment, but we could not find a chimeric transcript.

Discussion

Intragenic deletion of *IKZF1* is mediated by V(D)J recombination. V(D)J recombination is initiated by cleavage at specific recombination signal sequences (RSSs) that flank V (variable), D (diversity), and J (joining) segments, which encode a variable region of an immunoglobulin (Ig) or T-cell receptor (TCR) protein. These RSSs consist of a highly conserved heptamer (consensus sequence 5’ -CAC(A/T)GTG-3’). The RAG1/2 complex recognizes these RSSs and cleaves them to excise the intervening genomic DNA segments as circular DNA. V(D)J recombination is essential for the proper development of the mammalian immune system. However, mistakes in normal V(D)J recombination via cryptic RSSs can lead to intrachromosomal interstitial deletions[13]. Recent advances in sequence technology have revealed many targets of such “illegitimate V(D)J recombination” including *TAL1*[14], *IKZF1*[2, 5], *BTG1*[15, 16], *CDKN2A*[17], *SLX4IP*[18], *CD200/BTLA*[19], *ERG*[20], and *PTEN*[21] in B or T-ALL. Of these deletion targets, *IKZF1* deletion is the most frequent

and the most extensively analyzed. Detection of an *IKZF1* deletion is important because the existence of a mutation is an independent poor prognostic factor in ALL. Previously, *IKZF1* deletion was only detected through next-generation sequencing (NGS), array comparative genomic hybridization (aCGH), multiplex ligation-dependent probe amplification (MLPA) or multiplex PCR. However, most of these techniques were not available in daily clinical practice due to the high cost or need for special equipment. FISH analysis is widely used to detect cytogenetic abnormalities such as a fusion gene or aneuploidy in hematological malignancy. FISH analysis is a quick and quantitative method that is available in most clinical laboratories. Most of the currently available FISH probes were developed using BAC clones. Coverage of a BAC clone probe is approximately 150kb or larger, so there was no suitable BAC clone that specifically annealed to the commonly deleted region of *IKZF1* due to size limitation. There is a commercially available FISH probe covering entire *IKZF1* gene, which designed for detection of gain or loss of the entire locus or translocations, however intragenic *IKZF1* deletion could not be detected correctly by the probe [22]. Thus, we designed and cloned an ideal probe and combined the probe with another BAC clone that annealed the 3' region of *IKZF1*. The probe set worked effectively for cell lines and clinical samples, and various types of deletions were detected. In all cases with *IKZF1* deletion that were FISH-positive, the joint sequence was identified by multiplex PCR. Furthermore, the FISH method can detect whole gene deletions that are not detected by the multiplex PCR method. The multiplex PCR approach could miss an atypical deletion with a rare RSS. Our *IKZF1* deletion FISH can detect such an atypical deletion pattern. Relative quantitative-PCR (RQ-PCR) detection of *IKZF1* was reported to be a sensitive MRD marker[23]. In that study, only cases with Δ4-7 deletion were validated. In the RQ-PCR method, the primer set should be changed depending on the deletion type. Our *IKZF1* deletion FISH can detect any of the 4 types of deletion spanning *IKZF1* exon 4 to 7. Limitation of our probe is that the probe could not detect relatively rare but recurrent deletion spanning exons 2-3 [12].

In the V(D)J recombination process, the excision fragment is known to form circular DNA. In T cell development, the cleaved V(D)J recombination fragment forms a T-cell receptor excision circle (TREC). The TREC is not integrated into another chromosome and is not replicated in the cell division process, and thus it is eventually diluted and lost by cell proliferation. TREC quantification has been used to assess thymic output in several clinical settings, such as congenital immunodeficiency, human immunodeficiency virus (HIV) infection, autoimmune disease and immune reconstitution after hematopoietic stem cell transplantation[25, 26]. Most cases with *IKZF1* deletion showed the same pattern of 0R1G1F and an excised red signal was not detected in the cell. However, 1 case (UPN 1) had an atypical break-apart signal showing 1R1G1F pattern in all leukemic cells. We determined the precise insertion site and joint sequence by inverse PCR. The excised *IKZF1* fragment including cryptic RSSs was integrated into chromosome 2 immunoglobulin

kappa abPart at the legitimate RSSs which lost by the insertion event (Figure 2A-C). Each joint had an "N" nucleotide adding, which is a hallmark of V(D)J recombination and is mediated by terminal deoxynucleotidyl transferase (TdT) activity. In such a case, the *IKZF1* deletion is difficult to identify by the NGS or MLPA method, because the event did not cause a copy number alteration of the deleted region. FISH is cytogenetic analysis, which maintains genome integrity of the sample, so the result is clear-cut and such an atypical *IKZF1* deletions never missed. Although we could not detect a chimeric kappa chain transcript, recent studies have shown similar events in which a foreign templated sequence insertion into immunoglobulin loci resulted in a chimeric transcript. A partial fragment of the *LAIR1* gene on chromosome 19 was integrated into the immunoglobulin heavy chain locus on chromosome 14, and broadly reactive antibodies against malaria variant antigens were generated[27, 28]. However, it was not mentioned whether the fragment was deleted in a RAG1/2-mediated manner due to cryptic RSSs. Another recent study showed insertions of RAG1/2-mediated DNA fragments into an artificial DNA double-strand break site[29]. These results indicated the possibility of exchange of V(D)J-mediated deletion fragments between distinct chromosomes. Our results are the first description showing that the insertion of a RAG1/2-mediated fragment can occur between genuine and cryptic RSSs in ALL. RAG1/2-mediated genome exchange could play a role in leukemogenesis by causing cancer-associated insertion that results in disruption of the expression of a tumor suppressor. In normal lymphocytes, RAG1/2-mediated genome shuffling might be an additional mechanism of antibody diversification.

Conclusion

We developed FISH analysis that can detect an *IKZF1* deletion mutation in ALL. The FISH analysis detecting *IKZF1* is a simple, fast, sensitive, quantitative and cost-effective method. Our *IKZF1* deletion probe set can detect all types of common variations of *IKZF1* deletion, and results obtained by using the probe set indicated that a V(D)J-mediated excised fragment can integrate into another RSS site.

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Author contributions

J.H., M.O. and K.O. designed the study, analyzed the data and wrote the manuscript. J.H., S.F., S.O., and M.T. performed experiments. M.N., D.H., K.K., T.K., and C.S., provided critique to the manuscript. T.T. revised and approved the manuscript.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. FISH probe design schematic.

A) *IKZF1* gene: Four types of *IKZF1* deletion are shown. Triangles represent 4 RSSs that are located in intron 1 (+ strand), intron 3 (+ strand), intron 7 (- strand) and 3'UTR (- strand). Each RSS showed 5/7 to 7/7 match to the consensus CAC(T/A)GTG. The probe (red bar) was designed to detect a commonly deleted region in all 4 types of *IKZF1* deletion. A blue arrow represents primers for PCR amplification and sequence the joint of each *IKZF1* deletion.

B) *IKZF1* and flanking genes: The *IKZF1* deletion probe set consists of a probe that identifies a commonly deleted region (red bar) and a BAC clone probe (RP11-248J17) that identifies a 3' flanking region of *IKZF1* (green bar). This probe set identifies intact *IKZF1* as a yellow fusion signal. *IKZF1* gene deletion resulted in loss of the yellow fusion signal into green.

Figure 2. Representative interphase FISH signal patterns that may occur for the *IKZF1* deletion probe set.

A: Normal nuclei with a 0R0G2F pattern.

B: Typical *IKZF1* deletion signal pattern of 0R1G1F.

C: Entire *IKZF1* region loss resulting in 0R0G1F (CML-LC with 7 monosomy, UPN18).

D: Increased number of *IKZF1* alleles resulting in 0R0G4F in cases with hyperdiploidy (AML with 92,XXYY, UPN22).

E: *IKZF1* deletion with an atypical signal (0R2G0F, UPN7).

F: *IKZF1* deletion with an atypical signal (1R1G1F, UPN1).

Figure 3. A case with *IKZF1* deletion showing an atypical signal (UPN1).

A) Inverse PCR revealed insertion of the deleted *IKZF1* fragment (51 kb) into chromosome 2 immunoglobulin kappa abParts region, where a 45-kb region was excised in a V(D)J-mediated manner. Triangles represent RSSs.

B) Detailed sequence of the *IKZF1* and chromosome 2 joints is shown. The inserted *IKZF1* fragment retains entire RSSs (yellow triangles), but chromosome 2 acceptor site has lost RSSs (green triangles). Each junction was connected by random "N" nucleotides adding.

C) Schematic image of V(D)J-mediated genome shuffling. Chromosome 7 *IKZF1* deletion site and insertion of the deleted fragment into chromosome 2 was sequence confirmed. Chromosome 2-

deleted fragment could form an excised circle, which could not be detected anymore due to dilution by cell division.

Table 1. Type of *IKZF1* deletion and FISH results for ALL cell lines and clinical cases.
ALL, acute lymphoblastic leukemia; Ph (+), Philaelphia chromosome positive; Ph (-), Philadelphia chromosome negative; UPN, unique patient number; CML-LC, chronic myelogenous leukemia-lymphoid crisis; AML, acute myeloid leukemia; R, red; G, green; F, fusion; NA, not analyzed
Abnormal FISH findings and representative *IKZF1* deletion or aneuploidy were shown in bold.

Table 2. Verified joint sequence of *IKZF1* deletion.
The underline represents cryptic RRSs resembling consensus CAC(A/T)GTG.

Supplemental materials

Supplemental Figure 1. Probe design for *IKZF1* deletion
IKZF1 deletion probe, labeled in Cy3 that consists of a commonly deleted part of *IKZF1,* was developed by using 5 separately amplified PCR fragments (*IKZF1*Δa, *IKZF1*Δb-, *IKZF1*Δc , *IKZF1*Δd , and *IKZF1*Δe, ~~is~~ 9-10 kb in size each, total of 49 kb).

Supplemental Figure 2. Validation of *IKZF1* deletion FISH probe on metaphase from health individual
Two s~~S~~pecific fusion signals (yellow arrow) were observed on chromosome 7.

Supplemental Figure 3. Atypical signal pattern of *IKZF1* deletion on metaphase (UPN1)
Isolated red signal was observed on chromosome 2 (red arrow) whereas a green signal was retained on chromosome 7 (green arrow).

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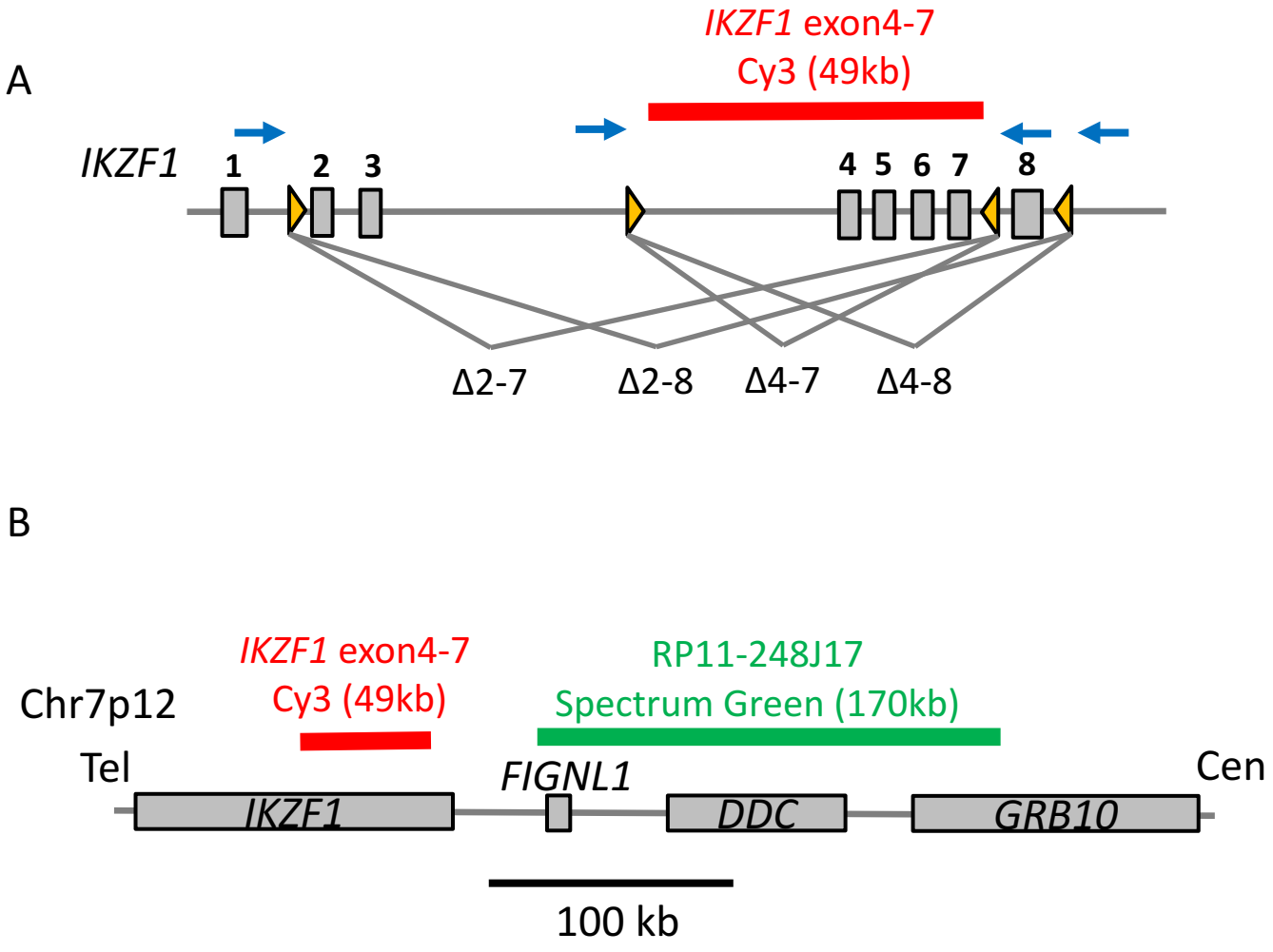
Figure 1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IKZF1 exon4-7
Cy3 (49kb)
IKZF1
1
2
3
4
5
6
7
8
Δ2-7
Δ2-8
Δ4-7
Δ4-8
B
IKZF1 exon4-7
Cy3 (49kb)
RP11-248J17
Spectrum Green (170kb)
Chr7p12
Tel
Cen
FIGNL1
IKZF1
DDC
GRB10
100 kb

Figure 2

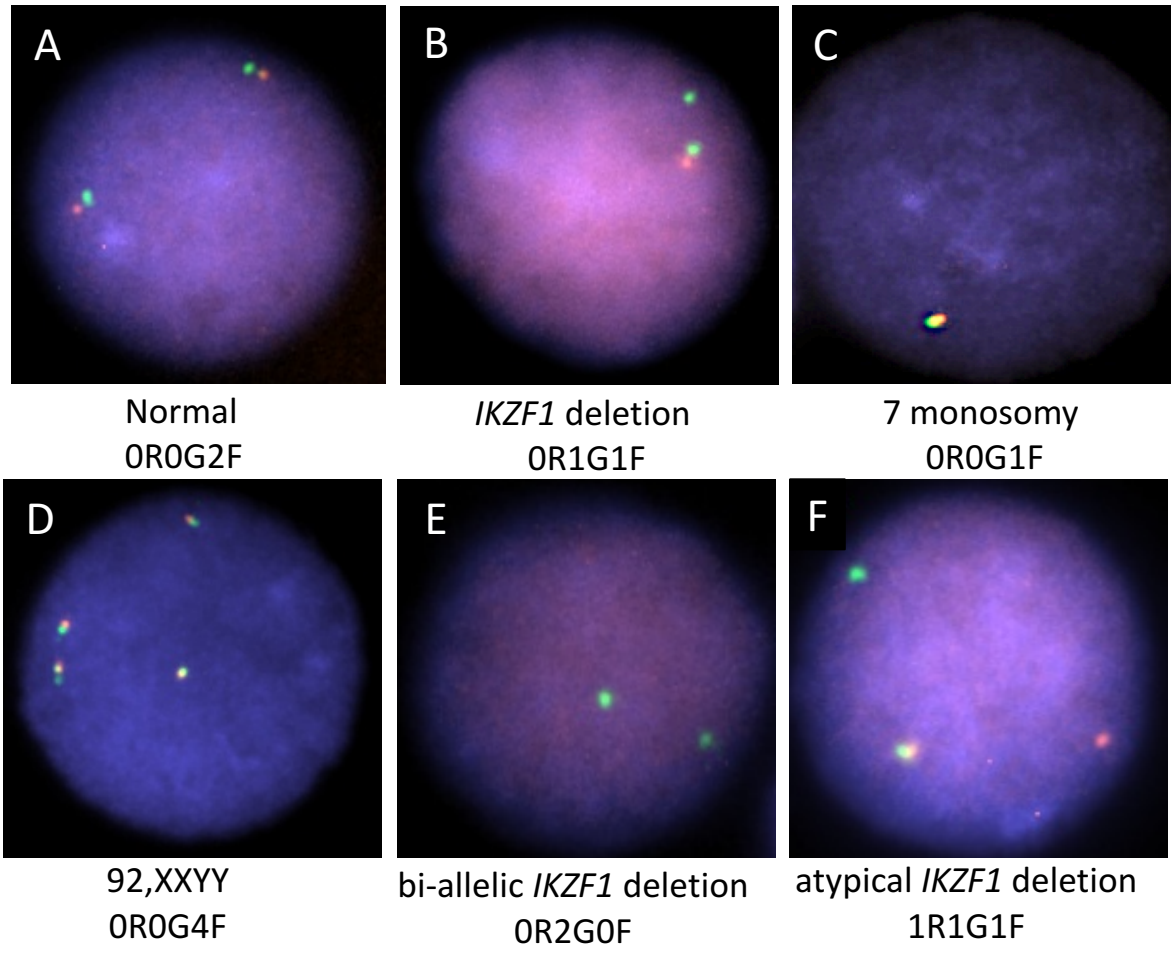
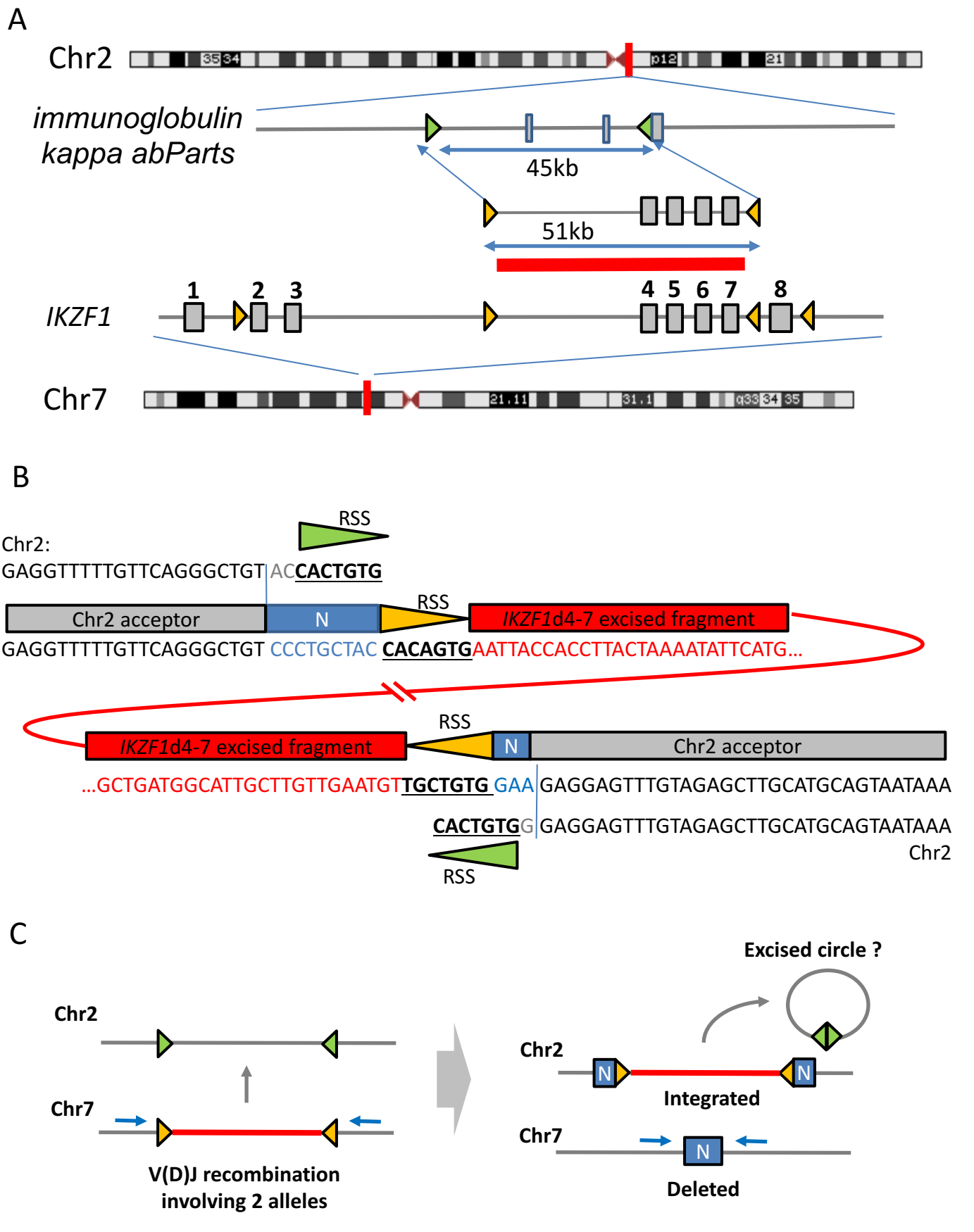

A
B
C
Normal
0R0G2F
IKZF1 deletion
0R1G1F
7 monosomy
0R0G1F
D
E
F
92,XXYY
0R0G4F
bi-allelic *IKZF1* deletion
0R2G0F
atypical *IKZF1* deletion
1R1G1F

Figure 3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Chr2
35 34
p12
21
immunoglobulin kappa abParts
45kb
51kb
IKZF1
1 2 3
4 5 6 7 8
Chr7
21.11
31.1
q33 34 35
B
RSS
Chr2:
GAGGTTTTTGTTCAGGGCTGT ACCACTGTG
Chr2 acceptor
N
RSS
IKZF1d4-7 excised fragment
GAGGTTTTTGTTCAGGGCTGT CCCTGCTAC CACAGTGAATTACCACCTTACTAAAATATTCATG…
RSS
IKZF1d4-7 excised fragment
N
Chr2 acceptor
…GCTGATGGCATTGCTTGTTGAATGTTGCTGTG GAA GAGGAGTTTGTAGAGCTTGCATGCAGTAATAAA
CACTGTGG GAGGAGTTTGTAGAGCTTGCATGCAGTAATAAA
Chr2
RSS
C
Chr2
Chr7
V(D)J recombination involving 2 alleles
Excised circle ?
Chr2
N
N
Integrated
Chr7
N
Deleted

Table1 Type of IKZF1 deletion and FISH results for ALL cell lines and clinical cases

Cell line or Patient	Age / Sex	Diagnosis	Karyotype	*IKZF1* intragenic deletion	Interphase *IKZF1* FISH signal pattern	*IKZF1* intragenic deletion positive (%)	Blast (%)
PALL2	45/M	Ph (+) B-ALL	NA	***IKZF1*Δ4-7**	**0R1G1F**	100%	100.0%
MY	52/F	Ph (+) B-ALL	NA	***IKZF1*Δ4-8**	**0R1G1F**	100%	100.0%
NALM-6	19/M	Ph (-) B-ALL	NA	(-)	0R0G2F	0%	100.0%
BALL-1	75/M	Ph (-) B-ALL	46,XY	(-)	0R0G2F	0%	100.0%
P30/OHK	11/M	non-T, non-B ALL	46,XY	(-)	0R0G2F	0%	100.0%
UPN1	37/M	Ph (+) B-ALL	46,XY,t(9;22)(q34;q11.2)	***IKZF1*Δ4-7**	**1R1G1F**	91%	97.8%
UPN2	61/F	Ph (+) B-ALL	46,XX,add(2)(p21),-9,dic(9;9)(9qter→9p12::9p12→9q34::22q11.2→22qter), add(16)(p13.3),-20,der(22)t(9;22)(q34;q11.2),+mar	***IKZF1*Δ4-7**	**0R1G1F**	97%	98.0%
UPN3	53/M	Ph (+) B-ALL	46,XY,t(9;22)(q34;q11.2)	***IKZF1*Δ4-8**	**0R1G1F**	100%	95.2%
UPN4	41/M	Ph (+) B-ALL	46,Y,del(X)(q26),add(7)(p15),t(9;17)(p13;q25), t(9;22)(q34;q11.2),t(11;13)(p11.2;p13)	***IKZF1*Δ4-8**	**0R1G1F**	100%	99.0%
UPN5	39/F	Ph (+) B-ALL	45,XX,-8,add(9)(p13),der(9)add(9)(p13)t(9;22)(q34;q11.2), -13,add(19)(p13),-20,der(22)t(9;22),+2mar	***IKZF1*Δ2-7**	**0R1G1F**	100%	95.6%
UPN6	65/F	Ph (+) B-ALL	46,XX,t(9;22)(q34;q11.2)	***IKZF1*Δ2-7**	**0R1G1F**	50%	30.0%
UPN7	60/F	Ph (+) B-ALL	46,XX,t(9;22)(q34;q11.2)	***IKZF1*Δ2-7**	**0R2G0F**	10%	28.2%

				***IKZF1*Δ4-8**			
UPN8	80/M	Ph (+) B-ALL	44,XY,**-7**,der(9)del(9)(p2212)t(9;22)(q34;q11.2),-20	(-)	**0R0G1F**	0%	46.0%
UPN9	59/F	Ph (+) B-ALL	48,XX,t(2;10)(p21;q26),+8,t(9;22)(q34;q11.2),+der(22)t(9;22)	(-)	0R0G2F	0%	95.4%
UPN10	21/M	Ph (-) B-ALL	45,XY,add(6)(q15),der(19;20)(p10;p10)	***IKZF1*Δ4-7**	**0R1G1F**	100%	94.2%
UPN11	58/M	Ph (-) B-ALL	46,XY,del(17)(p11.2)	***IKZF1*Δ4-8**	**0R1G1F**	92%	97.0%
UPN12	48/F	Ph (-) B-ALL	45,XX,add(1)(q21),-9,-11,+mar1	(-)	0R0G2F	0%	95.6%
UPN13	68/M	Ph (-) B-ALL	46,XY	(-)	0R0G2F	0%	92.0%
UPN14	54/M	Ph (-) B-ALL	46,XY,add(X)(q13),del(6)(q?),del(7)(q?),add(9)(p11)	(-)	0R0G2F	0%	69.2%
UPN15	62/F	Ph (-) B-ALL	46,XX,t(1;3)(q21;q25),add(2)(p13),der(6)t(6;9)(p25;q34), -9,add(9)(p22),add(13)(q32),-16,+20,+mar	(-)	0R0G2F	0%	97.6%
UPN16	30/F	Ph (-) B-ALL	47,XX,+X,t(2;8)(p13;p11.2),t(13;17)(q13;q23)	(-)	0R0G2F	0%	99.4%
UPN17	63/M	CML-LC	46,XY,t(9;22)(q34;q11.2)	***IKZF1*Δ4-7**	**0R1G1F**	97%	65.4%
UPN18	43/M	CML-LC	46,XY,t(3;6)(q21;p21.3),**-7**,add(7)(p11.2), t(9;22)(q34;q11.2),add(18)(p11.2),+mar	(-)	**0R0G1F**	0%	56.6%
UPN19	37/F	CML-LC	46,XX,t(9;22)(q34;q11.2)	(-)	0R0G2F	0%	73.0%
UPN20	49/F	AML	46,XX	(-)	0R0G2F	0%	96.4%
UPN21	65/F	AML	46,XX	(-)	0R0G2F	0%	85.8%
UPN22	20/M	AML	**92**,XXYY,t(8;21)x2	(-)	**0R0G4F**	0%	88.2%

Table2 Verified joint sequence of *IKZF1* deletion

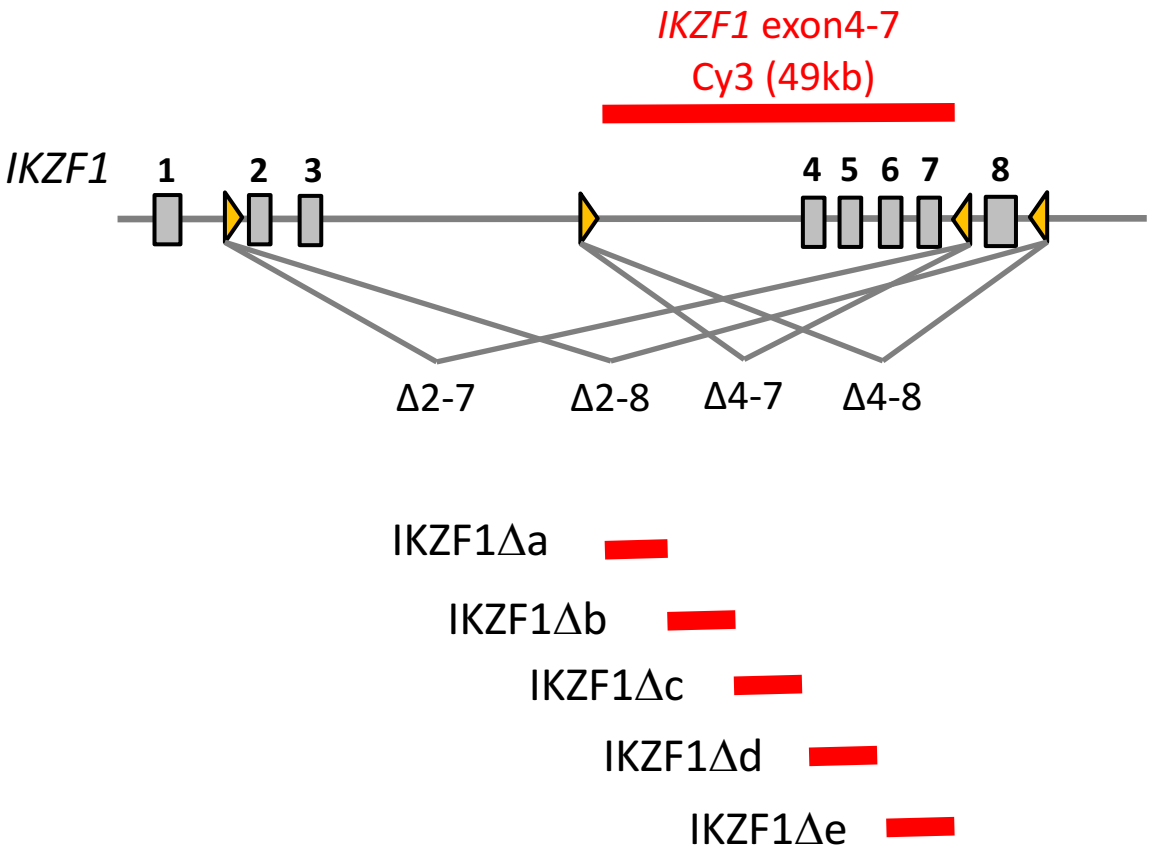
IKZF1 Δ4-7	5′	N	3′
Reference sequence	5′- CAGGGATCTCAGAAATTATTAGTACATCC<u>CACAGTG</u>		<u>TGCTGTG</u>GAAACATCAAGTCTAGTGTAACTGTTTCT -3′
PALL2	5′- CAGGGATCTCAGAAATTATTAGTACATC	GCC	GAAACATCAAGTCTAGTGTAACTGTTTCT -3′
UPN1	5′- CAGGGATCTCAGAAATTATTAGTA	GGG	CATCAAGTCTAGTGTAACTGTTTCT -3′
UPN2	5′- CAGGGATCTCAGAAATTATTAGTACATCC	T	AAACATCAAGTCTAGTGTAACTGTTTCT -3′
UPN10	5′- CAGGGATCTCAGAAATTATTAGTACA	ATTACGGA	AAACATCAAGTCTAGTGTAACTGTTTCT -3′
UPN17	5′- CAGGGATCTCAGAAATTATTAGTACATC	GGTTGG	AACATCAAGTCTAGTGTAACTGTTTCT -3′

IKZF1 Δ4-8	5′	N	3′
Reference sequence	5′- CAGGGATCTCAGAAATTATTAGTACATCC<u>CACAGTG</u>		<u>CAAGGTG</u>TGGGCTGACATGCTGGCTCTCTTCCCTGT -3′
MY	5′- CAGGGATCTCAGAAATTATTAG	G	GGGCTGACATGCTGGCTCTCTTCCCTGT -3′
UPN3	5′- CAGGGATCTCAGAAATTATTAGTAC	TTTCC	TGGGCTGACATGCTGGCTCTCTTCCCTGT -3′
UPN4	5′- CAGGGATCTCAGAAATTATTAGTAC	CCATG	GGGCTGACATGCTGGCTCTCTTCCCTGT -3′
UPN7	5′- CAGGGATCTCAGAAATTATTAGT	CCTGCCGCCA	TGGGCTGACATGCTGGCTCTCTTCCCTGT -3′
UPN11	5′- CAGGGATCTCAGAAATTATTAGTACATCC	TA	TGGGCTGACATGCTGGCTCTCTTCCCTGT -3′

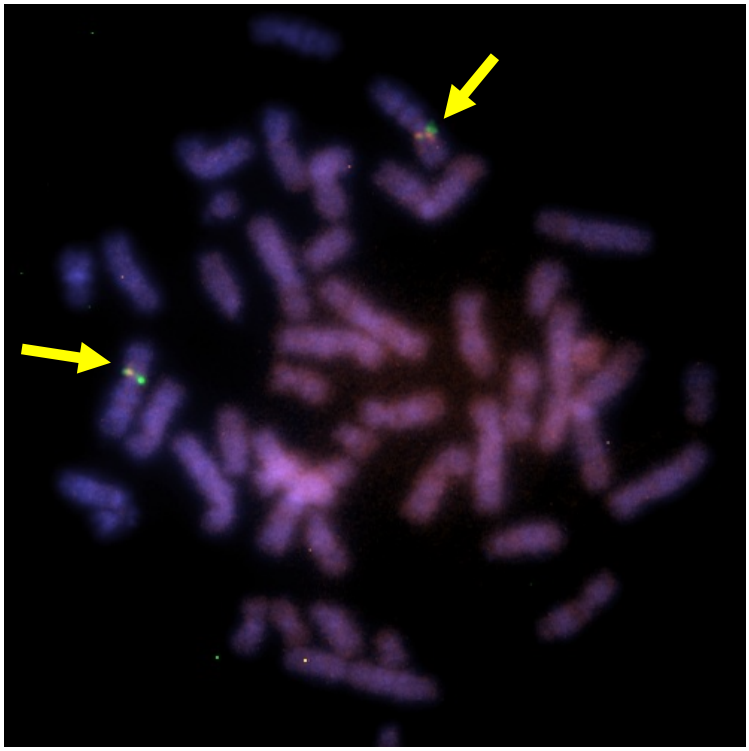
IKZF1 Δ2-7	5′	N	3′
Reference sequence	5′- ACGTAGAGTTTCAGAGGATCAGCATTATA<u>CACACTG</u>		<u>TGCTGTG</u>GAAACATCAAGTCTAGTGTAACTGTTTCT -3′
UPN5	5′- ACGTAGAGTTTCAGAGGATCAGCATTATA	GG	ACATCAAGTCTAGTGTAACTGTTTCT -3′

UPN6	5′- ACGTAGAGTTTCAGAGGATCAGCATT	CCCCAAGG	CATCAAGTCTAGTGTAACTGTTTCT -3′
UPN7	5′- ACGTAGAGTTTCAGAGGATCAGCAT	ATATGGGGC	ACATCAAGTCTAGTGTAACTGTTTCT -3′

Supplemental Figure1



Supplemental Figure 2



Supplemental Figure 3