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A novel approach to quantitating leukemia fusion transcripts by qRT-PCR without the need for standard curves

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ABSTRACT

Acute myeloid leukemia patients with recurrent cytogenetic abnormalities including inv(16);*CBFB-MYH11* and t(15;17);*PML-RARA* may be assessed by monitoring the levels of the corresponding abnormal fusion transcripts by quantitative reverse transcription-PCR (qRT-PCR). Such testing is important for evaluating the response to therapy and for the detection of early relapse. Existing qRT-PCR methods are well established and in widespread use in clinical laboratories but they are laborious and require the generation of standard curves. Here, we describe a new method to quantitate fusion transcripts in acute myeloid leukemia by qRT-PCR without the need for standard curves. Our approach uses a plasmid calibrator containing both a fusion transcript sequence and a reference gene sequence, representing a perfect normalized copy number (fusion transcript copy number/reference gene transcript copy number; NCN) of 1.0. The NCN of patient specimens can be calculated relative to that of the single plasmid calibrator using experimentally derived PCR efficiency values. We compared the data obtained using the plasmid calibrator method to commercially available assays using standard curves and found that the results obtained by both methods are comparable over a broad range of values with similar sensitivities. Our method has the advantage of simplicity and is therefore lower in cost and may be less subject to errors that may be introduced during the generation of standard curves.

1. Introduction

Cancer cells are characterized by the acquisition of mutations in key regulatory genes involved in cell growth, division, and differentiation. These mutations include single nucleotide substitutions, small insertions/deletions, and large scale changes including chromosomal rearrangements. Chromosomal rearrangements can result in the expression of fusion transcripts that alter normal cellular processes either by constitutively activating or by inhibiting one of the fusion partners (De Braekeleer et al., 2014; Deininger et al., 2000). Many diseases, including acute myeloid leukemia (AML), can be defined and subclassified based on cytogenetic features, including the presence of recurrent chromosomal rearrangements (Byrd et al., 2002; Grimwade et al., 1998; Slovak et al., 2000). Detection and quantitation of the resulting fusion transcripts is important for diagnosis, prognosis, and disease monitoring (Chendamarai et al., 2012; Pigazzi et al., 2015; Yin et al., 2012).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a standard laboratory technique used to detect and quantitate fusion transcripts. Assays are performed by amplifying patient cDNA and plasmid standards of known concentration for both the fusion and reference genes (Chen et al., 2015; Gabert et al., 2003). The absolute copy numbers for both are calculated from the standard curves that are generated on each PCR run. The ratio of fusion to reference gene transcripts is then reported as a normalized copy number (NCN) (D'Haene et al., 2010; Mocellin et al., 2003). Patient NCN values are monitored over time to assess the effectiveness of treatment and for evidence of minimal residual disease (MRD) (Corbacioglu et al., 2010; van der Velden et al., 2003; Yin et al., 2012).

qRT-PCR assays are dependent upon testing external plasmid standards of known concentration. These can be expensive to produce or purchase. The additional PCR reactions required for a series of standards also increases the complexity of the overall workflow. This additional complexity increases the chance for errors that may impact the final result. Decreasing workflow complexity is desirable from a variety of standpoints, most importantly for decreasing the overall cost of a test and for maintaining test quality, reproducibility and reducing run-to-run variation. Novel approaches to reducing workflow complexity in *PML-RARA* qRT-PCR have been described, including a multiplex assay to detect and quantitate fusion transcripts (Chen et al., 2015) and loop mediated amplification (Spinelli et al., 2015). However the multiplex assay amplifies *PML-RARA* fusion transcripts in one reaction and *ABL1* in a second, which may negatively impact quantitation because of

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error. The loop mediated amplification reaction suffers from lower sensitivity, potentially resulting in false-negative results.

We have previously described a novel approach to quantitative allele-specific PCR for point mutations, such as *JAK2 V617F* and *BRAF V600E*, without the need for standard curves (Szankasi et al., 2013). This method utilizes a heterozygous calibrator plasmid at a single concentration that contains both the targeted mutation and the wild-type sequence. This calibrator plasmid has a mutant allele fraction of exactly 0.5, regardless of plasmid concentration. The allele fraction in patient samples is then calculated by extrapolation from the single calibrator. Here we describe a method that applies the same concept to the quantitation of mRNA fusion transcripts. We quantified *CBFB-MYH11* fusion transcripts in cases of AML with inv(16);*CBFB-MYH11* and *PML-RARA* fusion transcripts present in cases with t(15;17);*PML-RARA* as proof-of-concept examples. Fusions transcript were quantified relative to the reference gene *ABL1*. The plasmid calibrator standard that we used carries copies of both a *CBFB-MYH11* or *PML-RARA* fusion and an *ABL1* reference cDNA fragment. Thus, the calibrator has a NCN of exactly 1.0, regardless of the concentration. The assay is designed to detect and quantitate *CBFB-MYH11* types A, D, and E and *PML-RARA* bcr 1/2 and 3 fusion transcripts. We show that this novel approach to quantitation of *CBFB-MYH11* and *PML-RARA* fusion transcripts has equivalent performance to standard curve-based, commercially available qRT-PCR assays. The assays we describe are suitable for detecting and monitoring *CBFB-MYH11* and *PML-RARA* fusion transcripts at a reduced cost and with a simplified workflow.

2. Materials and methods

2.1. Patient specimens and controls

De-identified patient samples that had undergone testing for *CBFB-MYH11* or *PML-RARA* fusion transcripts were obtained from the ARUP Molecular Oncology laboratory with the approval of the University of Utah Institutional Review Board. We identified 20 specimens expressing *CBFB-MYH11* type A, 25 expressing *PML-RARA* bcr 1 or 2, and 32 expressing *PML-RARA* bcr 3. Twenty specimens negative for *PML-RARA* and *CBFB-MYH11* fusion transcripts were also evaluated. RNA was isolated from 1×10^7 WBCs using the Maxwell 16 LEV simplyRNA Blood Kit following the manufacturer's instructions (Promega, Madison, WI). Ten μL of RNA was reverse transcribed into cDNA with random primers using the Ipsogen RT Kit (Qiagen, Inc., Valencia, CA) and stored at −20 °C.

2.2. Plasmid construction

Five plasmid calibrators (p*Fusion*:*ABL1*), each containing a distinct fusion sequence and reference gene sequence, were constructed (Fig. 1). For the generation of the 3 p*CBFB-MYH11*:*ABL1* plasmid calibrators, a cDNA fragment covering either the *CBFB-MYH11* fusion breakpoint type A, D, or E was combined with a cDNA fragment of the *ABL1* exon 2–3 junction. For generation of the two p*PML-RARA*:*ABL1* plasmid calibrators, a cDNA fragment covering either the *PML-RARA* bcr 1 or bcr 3 was combined with a cDNA fragment of the *ABL1* exon 2–3 junction. Since bcr 1 and bcr 2 *PML-RARA* rearrangements have relatively close breakpoints within *PML* gene, they can be detected by the same assay. Of note the *ABL1* cDNA fragment in both p*PML-RARA*:*ABL1* and p*CBFB-MYH11*:*ABL1* plasmid calibrators is longer than the fusion sequence, in order to equalize amplification efficiency for both fusion and reference gene sequences. The five sequence templates were provided to Integrated DNA Technologies (Coralville, IA) for synthesis and cloned into the pIDTBlue vector. The resulting plasmids were transfected into *Escherichia coli*, isolated and sequence verified prior to use. Each plasmid calibrator carried a single copy of the fusion gene fragment containing portions of *CBFB* exon 5 fused to *MYH11* exon 28, 29, or 33 (previously known as exons 7, 8, and 12, respectively) (van Dongen et al., 1999) followed by the *ABL1* exon 2–3 junction sequence or *PML* exons 3 or 6 fused to *RARA* exon 3 followed by the *ABL1* exon 2–3 junction sequence. Calibrator plasmids were diluted in 10 mM Tris-HCl pH 7.5 and stored at −20 °C.

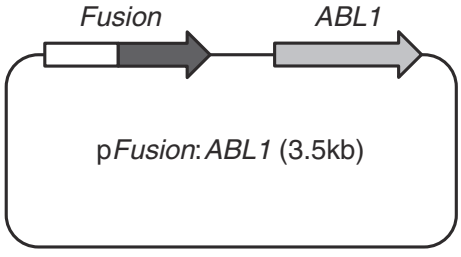

Fig. 1. Schematic illustration of the plasmid calibrator design. Plasmids contain *CBFB* exon 5 and *MYH11* exon 33 (type A), 29 (type D), or 28 (type E) or *PML* exon 6 (bcr 1/2) or 3 (bcr 3) and *RARA* exon 3 with *ABL1* exons 2–3 junction.

2.3. Quantitative real time PCR

PCR primers were designed based upon NCBI Reference Sequences for *ABL1* (NM_005157.4), *CBFB* (NM_022845.2), *MYH11*(NM_002474.2), *PML* (NM_033238.2) and *RARA* (NM_001145301.2). PCR primers and probes (sequences in Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). The *ABL1* hydrolysis probe was labeled with a Cy5 fluorophore to avoid spectral overlap with *CBFB-MYH11* or *PML-RARA* 6-FAM-labeled probes. Commercial qRT-PCR kits (Qiagen, Inc.) were available for detection of the *CBFB-MYH11* type A, the *PML-RARA* bcr 1/2, and the *PML-RARA* bcr 3 fusions. We therefore focused on these rearrangements for test comparisons.

In our assay each fusion transcript was analyzed in combination with the *ABL1* reference transcript in a multiplex reaction. All patient specimens and plasmid calibrator control reactions were assayed in duplicate. For *CBFB-MYH11* testing, 20 μL multiplex reactions contained 0.1 μM forward and reverse *ABL1* primers, 0.4 μM forward and reverse *CBFB-MYH11* primers, 0.05 μM *ABL1* and *CBFB-MYH11* hydrolysis probes, and 3 μL template in 1× Quantitect Multiplex (No ROX) Mastermix (Qiagen, Inc.). Quantitative PCR was performed on an LC480 instrument (F. Hoffmann-La Roche AG, Basel, CH) with a pre-incubation at 37 °C for 2 min followed by 95 °C for 15 min then 45 cycles of 10 s at 95 °C and 1 min at 60 °C (with a single fluorescence acquisition). For *PML-RARA* testing, 20 μL multiplex reactions contained 0.1 μM forward and reverse

Table 1
Primer and probe sequences.

Target	Sequence (5′–3′)
CBFB-MYH11 assays	
CBFB exon 5	AGG TCT CAT CGG GAG GAA
MYH11 exon 28	GCC TCC AGC TTC TTC TTC TTA TG
MYH11 exon 29	GTG TCC TGG AGC TGG GAA
MYH11 exon 33	ACT TCC AGC CGC AGT TTG
ABL1 exon 2	CGA AGG GAG GGT GTA CCA
ABL1 exon 3	GAT AAT GGA GCG TGG TGA TGA G
MYH11 exon 7 probe	6-FAM/ACG CTG GAG/ZEN/AAA GAG AAC GCA GAC/IABkFQ
MYH11 exon 8 probe	6-FAM/TGA AGT TGA/ZEN/GAG CGT CAC AGG GAT/IABkFQ
MYH11 exon 12 probe	6-FAM/TGG AGA CCC/ZEN/AGA TGG AGG AGA TGA A/IABkFQ
ABL1 exon 3 probe	Cy5/ACA CTG CTT CTG ATG GCA AGC TCT/IAbRQSp
PML-RARA assays	
PML exon 3	GCT GGT GCA GAG GAT GAA GT
PML exon 6	GGA AGG TCA TCA AGA TGG AG
RARA exon 3	AGG GCT GGG CAC TAT CTC TT
ABL1 exon 2	GCA ATG CCG CTG AGT ATC
ABL1 exon 3	GGG ACA CAC CAT AGA CAG TG
RARA exon 3 probe	6-FAM/AAC TGC TGC/ZEN/TCT GGG TCT CAA TGG/IABkFQ
ABL1 exon 3 probe	Cy5/ACA CTG CTT CTG ATG GCA AGC TCT/IAbRQSp

ZEN — internal quencher IABkFQ — Iowa BlackFQ IAbRQSp — Iowa Black RQ.