Quantitative competitive allele-specific TaqMan duplex PCR (qCAST-Duplex PCR) assay: a refined method for highly sensitive and specific detection of *JAK2*V617F mutant allele burdens

*JAK2*V617F mutation is one of the key driver mutations in myeloproliferative neoplasms (MPN). Since its discovery, many diagnostic techniques have been applied in its detection. Unfortunately, there were significant discrepancies in the allele burden (AB) quantification when blinded samples were tested in a multicenter study.[1] The study also concluded by delineating the importance of using well-defined, accurate standards to refine *JAK2* quantitative assays.[1] Although DNA from *JAK2*-mutated UKE-1 and HEL cells are commonly used for this purpose,[2,3] none of these cells are considered ideal, as the HEL cells carry multiple copies of *JAK2*, and UKE-1 cells do undergo clonal evolution with increasing *JAK2* copies during *in vitro* cultures.[1,4]

To improve the accuracy of *JAK2*V617F detection and quantification, we made some refinements to the allele-specific real-time polymerase chain reaction (qPCR) assay and created a novel method. Firstly, we decided to select *JAK2* exon 21 as our reference control (Figure 1A). It improved the efficacy of copy number normalization by directly calibrating equal copies of target DNA with the reference control within the same gene. In the second step, the exon 21 control sequence, along with either one of the target regions [*JAK2* wild-type (WT) and V617F mutant], were cloned into a yT&A cloning vector (Yeastern Biotech, Taiwan) to generate two standard plasmids (*JAK2_WT_Ctrl_yT&A* and *JAK2_V617F_Ctrl_yT&A*, representing 0% and 100% mutant AB, respectively) (Figure 1B). The plasmid pair had the same size (3306 bps) and could be easily manipulated with identical copy numbers. They were used to establish quantification standards. Thirdly, allele-specific (AS) primers and a FAM-containing TaqMan probe (Bio-

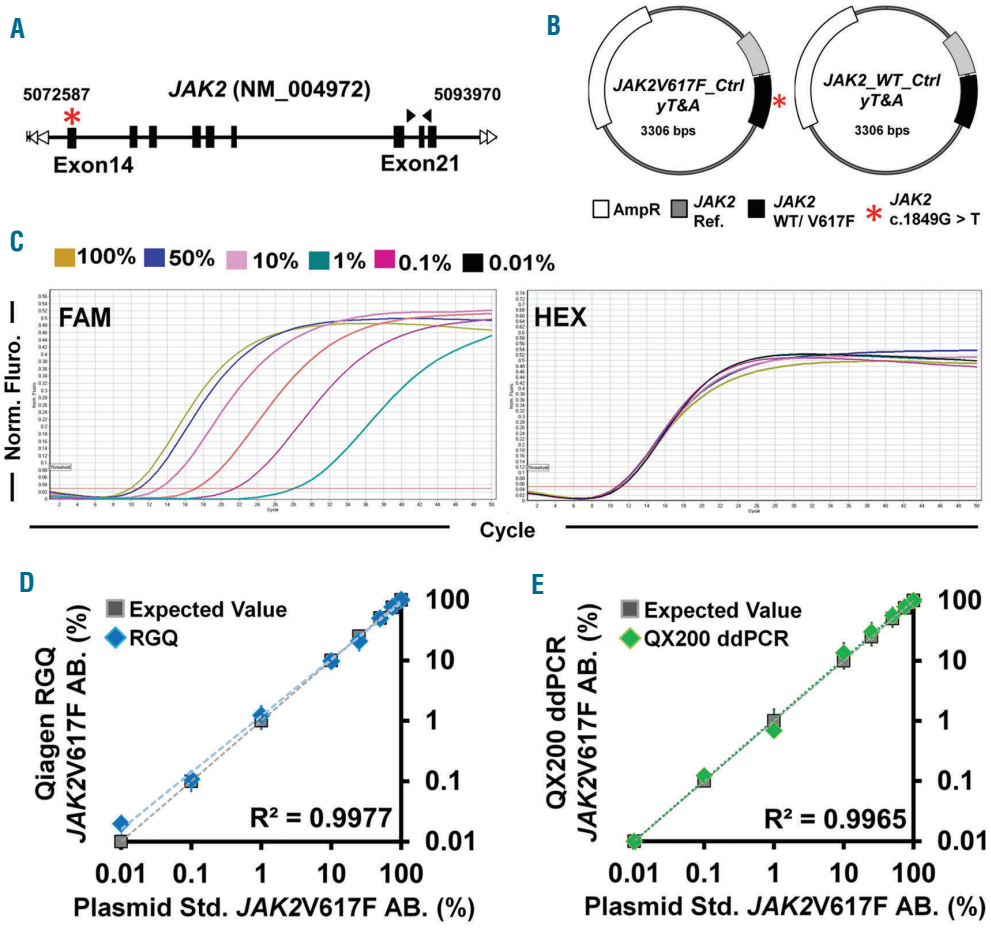
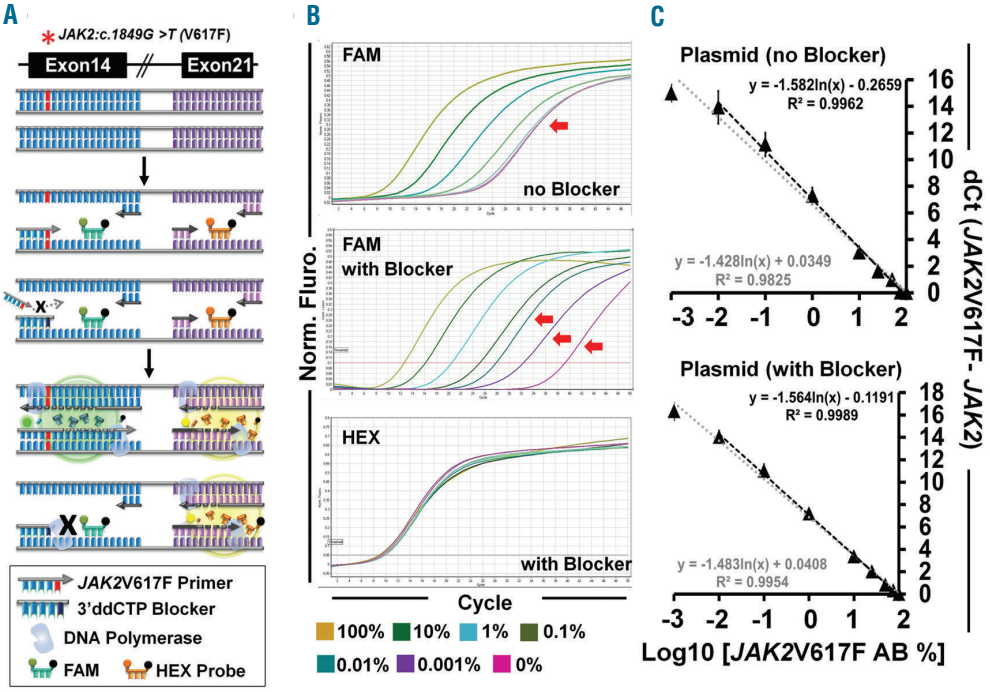
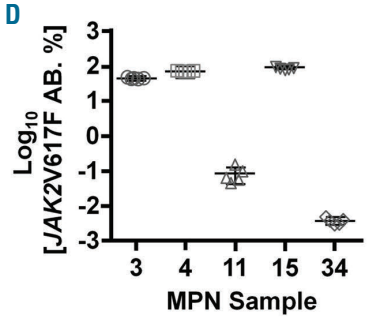


Figure 1. The reference sequence, plasmid construction, and validation of the accuracy of artificial plasmid mixtures as quantification standards. (A) Physical map of *JAK2* exon 14-22, including the mutation position on exon 14 and the 264-bp reference control region on exon 21. The asterisk indicates the *JAK2*V617F mutation position (cDNA 1849 G>T). The arrow heads show the primers which amplify the reference control sequence. (B) Physical map of our standard plasmids. The plasmids contain an ampicillin resistance gene (white), the reference control region on exon 21 (gray) and either V617F mutant or wild-type DNA sequence on exon 14 (both black). (C) Results of qPCR analyses on various mixtures of these two plasmids with differential mutant allele burdens. (Left) Amplification of mutant DNA templates with allele-specific primers which was quantified by a FAM-emitting probe. Distinct allele burdens are represented in different colors. (Right) Amplification of the reference control templates was measured with a HEX-containing probe. The considerably overlapping curves suggest unequivocal qPCR results with the accelerated amplification of the control sequence initiating at nearly identical cycles of reactions. To further prove the accuracy of these standard dilutions, the allele burdens of each plasmid mixture were quantified with (D) the Qiagen RGQ commercial kit and (E) the Bio-Rad droplet digital PCR assay. The gray rectangles indicate the expected mutant AB of each plasmid mixture, and the blue (RGQ) or green (ddPCR) rhombuses plotted the observed values with the respective method.

Rad, USA) were used for *JAK2*V617F detection, whereas primers for the control gene were mixed with a HEX-containing probe to quantify *JAK2* exon 21 (Figure 1C), and the calculation of mutant AB was determined by the ΔCt method. The designs allowed us to perform duplex PCR (including mutant-specific assay and copy-number-normalizing control assay) in one tube and quantify two PCR products concurrently, which also helped minimize sampling bias and decrease the consumption of genomic DNA. The latter could be a major but unexpected benefit when some rare samples of limited quantity are examined. Lastly, but most importantly, we designed an oligonucleotide (ON) WT template blocker containing a di-deoxycytidine at its 3' end (3'-ddCTP). It competed with the mutant allele-specific primer and preferentially annealed to WT templates with high affinity, which impeded their non-specific amplification. With these refinements, we designated our novel method as quantitative competitive allele-specific TaqMan Duplex PCR (qCAST-Duplex PCR) assay. The sequences and working concentrations of all primers, probes and the blocker are listed in *Online Supplementary Table S1*. Other relevant information is also available in the *Online Supplementary Appendix*.

Before quantifying mutant AB, the artificial plasmid pair were mixed at various proportions to obtain a series





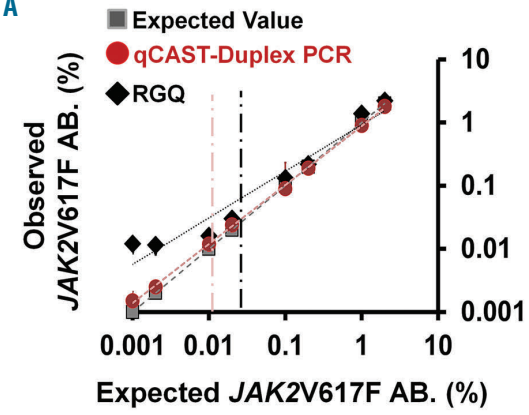
MPN Samples	*JAK2*V617F AB. (%)	
	qCAST-Duplex	QX200 ddPCR
M03	45 ± 0.02	43.7 ± 0.4
M04	72 ± 0.035	75.4 ± 0.7
M11	0.085 ± 0.0	0.07± 0.0
M15	92 ± 0.041	97.12 ± 0.9
M34	0.0041± 0.0	0.004 ± 0.0

Figure 2. The quantitative competitive allele-specific TagMan Duplex PCR (qCAST-Duplex PCR) assay. (A) Simplified animation of the qCAST-Duplex PCR assay. Blue: bases in exon 14; purple: bases in the reference exon 21 sequence; red: *JAK2* c.1849 G>T mutation; dark blue: 3'-ddCTP modified. FAM- and HEX-containing probes are used to detect amplification in mutant and reference templates, respectively. The blocker impedes the binding of the allele-specific mutant primer to the wild-type (WT) template, thereby reducing non-specific amplification and increasing the sensitivity of this assay. (B) Amplification curves of qCAST-Duplex PCR with and without (no) the blocker. Various plasmid mixtures in log-scale dilutions ranging from 100% to 0.001% as well as 0% mutant allele burdens were tested. In the absence of a blocker, the amplification curves of 0.01%, 0.001%, and 0% mutant templates largely overlapped. With the addition of the 3'-dideoxy blocker, the curves of those three diluents were explicitly separated in the amplification of mutant templates (visualized with FAM-containing probes), whereas the curves of HEX-emitting control templates were not affected. (C) Creation of standard curves with or without the blocker. Please note the shift of the standard curve (dashed black) towards the expected line (dashed gray) upon the addition of the blocker. (D) Measurements of 5 clinical samples using the qCAST-Duplex PCR assay. The same 5 clinical samples were run in triplicates using DNA standards derived from 5 different batches of plasmids. The average allele burdens, along with their standard deviations, of the 5 measurements for each sample were listed in the table on the right-hand side. To validate these data, the 5 samples were also verified by ddPCR and the data were listed side by side with the results obtained from qCAST-Duplex PCR. MPN: myeloproliferative neoplasms.

of diluents (containing mutant AB ranging from 100% to 0%) to create standard curves. We employed a commercial kit (*JAK2* RGQ KIT, Qiagen) and the droplet digital PCR system (QX200 ddPCR, Bio-Rad) to assess these standard dilutions and prove their accuracy. Figure 1D and E demonstrates the respective plotting curves of the expected mutant AB (gray rectangles) and the calculated values using either the kit (blue rhombuses) or the ddPCR assay (green rhombuses). These superimposed curves suggested an excellent correlation between the calculated and the expected AB, which affirmed the unequivocal accuracy of using these plasmid mixtures as quantification standards.

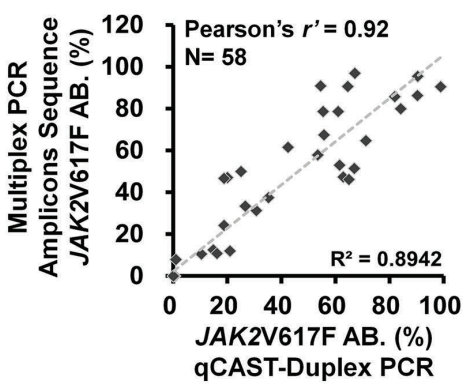
The roles of AS primers, the 3'-ddCTP blocker, and the

A



Blind Test Samples	JAK2V617F AB. (%)		
	Exp.	qCAST-Duplex	RGQ
S1	1	0.9 ± 0.08	1.38 ± 0.04
S2	0.1	0.09 ± 0.01	0.131 ± 0.03
S3	0.01	0.0119± 0.00	0.016 ± 0.01
S4	0.001	0.0013 ± 0.00	0.012 ± 0.01
S5	2	1.8 ± 0.08	2.2 ± 0.01
S6	0.2	0.19 ± 0.00	0.218 ± 0.01
S7	0.02	0.024 ± 0.001	0.029 ± 0.0
S8	0.002	0.0025 ± 0.0	0.0115 ± 0.0

B



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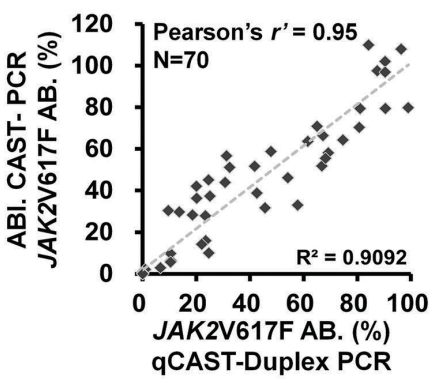

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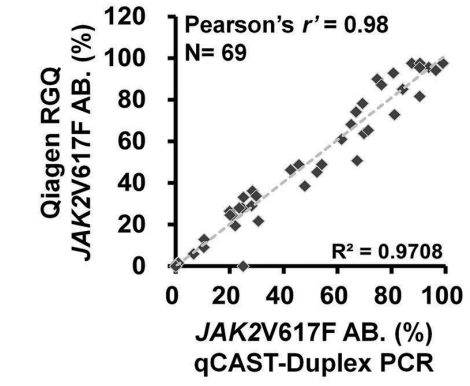

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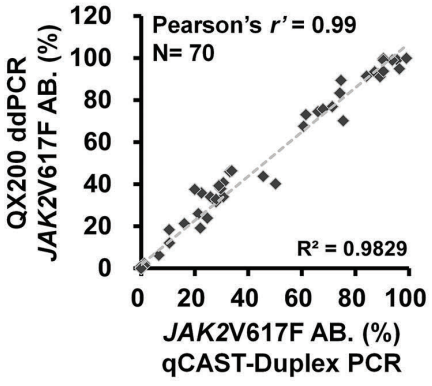

Figure 3. Correlation between analyses with the qCAST-Duplex PCR and with various assays. (A) Assessment of sensitivity of the qCAST-Duplex PCR assay. A random set of clinical sample mixtures with low AB (2%, 1%, 0.2%, 0.1%, 0.02%, 0.01%, 0.002%, and 0.001%, respectively) were prepared and subjected to analyses. Each sample was run in triplicates with the qCAST-Duplex PCR, and in duplicates with the RGQ kit. The gray rectangles indicate the expected mutant AB of each mixture, whereas the red circles (qCAST-Duplex PCR) and black rhombuses (RGQ) plot the observed values with the respective method. The calculated standard deviations were depicted as error bars, and the black and red dash lines indicated the sensitivity of the RGQ kit and qCAST-Duplex PCR, respectively. (B-E) Correlation curves between qCAST-Duplex PCR and various assays. DNA samples from 70 MPN patients (55 of them *JAK2*-mutated) were analyzed for mutant AB. The measured allele burdens with the qCAST-Duplex PCR assay were plotted against those obtained from (B) Multiplex PCR Amplicon Sequencing, (C) ABI- CAST -PCR, (D) QIAGEN RGQ *JAK2*V617F detection kit, and (E) Bio-Rad QX200 ddPCR, respectively. With limited availability in some of the assays, the numbers of samples analyzed with various assays differed. The *r'* was calculated with Pearson correlation, and R2 indicates the regression analysis. AB: allele burden.

dual TaqMan probes are shown in Figure 2A. Plasmid mixtures were subjected to qPCR with or without the blocker. Due to non-specific amplification, the amplification curves of standard dilutions with 0.01%, 0.001% and 0% mutant AB overlapped with each other in the absence of the blocker (Figure 2B, upper panel). Addition of the blocker significantly improved their discrimination (Figure 2B, middle panel), and the standard curve created with the ON blocker (Figure 2C, lower panel) was more closely superimposed with the expected line; this is important because an accurate standard curve is critical in our quantification. Without the blocker (Figure 2C, upper panel), the curve shifted at its end and led to a fluctuation in mutant AB measurements, especially in those samples with low AB (*data not shown*).

We next used our method to quantify mutant AB of 5 samples in 5 independent experiments. As shown in Figure 2D, the assay was shown to be highly sensitive and consistent in all *JAK2*-mutated samples (M3, M4, and M15) as well as in the sample with very low AB (M11). Furthermore, in the sample deemed *JAK2*V617F-negative (M34), the mutant AB was unequivocally below 0.01%. The variations in the measurements of each sample were minimal. Importantly, consistent results could be obtained with ddPCR (Figure 2D, right column). These data indicate that our qCAST-Duplex PCR assay can yield reproducible and affirmative results.

To delineate the sensitivity of our novel method, we then prepared low mutant AB DNA mixtures from *JAK2*-mutated patient samples that were serially diluted with adult healthy donors' DNA. The mixtures were assessed with qCAST-Duplex PCR and the Qiagen RGQ kit. Our novel method yielded quantification results that were more closely related to the expected values (Figure 3A). We also used multiplex PCR amplicon sequencing, ABI CAST-PCR kit, Qiagen RGQ kit, and ddPCR to measure the mutant AB in 55 *JAK2*-mutated and 15 *JAK2*-unmutated MPN samples and compared the results with those obtained from our qCAST-Duplex PCR assay. Good correlations were observed between qCAST-Duplex PCR and either one of these methods, suggesting that the performance of our novel assay was comparable (Figure 3B-E and *Online Supplementary Table S2*). Importantly, the results were especially consistent between measurements using qCAST-Duplex PCR and ddPCR, one of the most reliable quantification methods (Figure 3E). Furthermore, DNA from 30 healthy adults was also assessed with our qCAST-Duplex PCR and some other assays, and none of the healthy adults was tested *JAK2*V617F-positive in any of the assessments (*Online Supplementary Table S2*). These data further illustrate the assay's remarkable accuracy and low false positive rate.

On the other hand, we also used our novel method to refute the idea of using either HEL or UKE-1 DNA as the quantification standards because of their high copy number variation, inconsistent data acquisition, and possibly false-positive results (*Online Supplementary Figure S1*).

In the refined assay, 100ng of genomic DNA templates, estimated to contain 2.9×10^4 DNA copies, were added. Equal DNA copies of plasmids (~0.104pg) were used for standard preparation. The equivalent copy numbers helped minimize variations in mutant quantification. Furthermore, this translated into 2.9 copies of *JAK2*V617F in a plasmid standard with 0.01% mutant AB. We reasoned that samples with higher Ct values than that of the 0.01% AB standard should be considered *JAK2*V617F-negative since it would be impossible to have amplicons from less than one template in the qPCR reactions. Furthermore, we did not think the blockers could completely occupy all WT templates and false-positive amplification would still be possible. Therefore, we set 0.01% of mutant AB as the detection limit of our assay.

Our refined method represents an advance in sensitive *JAK2*V617F quantification. One might question the necessity of such an exceptional sensitivity and high accuracy, but the *JAK2*V617F AB actually carries important pathogenetic and clinical significances in MPNs. Researchers have demonstrated that *JAK2*V617F homozygosity could drive a phenotypic switch from essential thrombocythemia (ET) to polycythemia vera (PV) in mice models.[5] In clinical studies, the mutant AB is higher in PV than in ET,[6] and MPN patients with higher *JAK2*V617F AB are more likely to suffer from major thrombosis.[7] Based on the published guidelines, the *JAK2*V617F detection assays should be able to detect a mutant AB as low as 1-3%.[8] A sensitive assay is important to detect minimal residual disease in patients with primary meylofibrosis (PMF) who received allotransplant.[2] It is also indispensable in the assessment of treatment efficacy of novel therapies such as ruxolitinib and longer-acting interferon, as both have been shown to effectively reduce AB in some of the treated patients.[9,10]

The clinical significance of detecting *JAK2*V617F in healthy individuals remains controversial.[8] Nevertheless, it has been reported that, among the general population, *JAK2*V617F (mostly of low allele burden) is one of the most commonly identified mutations in age-related clonal hematopoiesis.[11,12] Importantly, the identified clonal hematopoiesis is significantly associated with an increased risk of developing hematologic cancers and atherosclerotic diseases.[13] In a recent study, we also demonstrated that low *JAK2*V617F AB was frequently detected in a specific portion of stroke patients who lacked predisposing factors.[14] Furthermore, in a large, population-based study, increased *JAK2*V617F AB in healthy adults was positively correlated with MPN progression, even among those with a mutant burden below 2%.[15] These data highlight the increasing need for precise *JAK2*V617F quantification and sensitive detection of low mutant AB.

In summary, our qCAST-Duplex PCR method uses dual AS-qPCR reactions in a single tube, a WT template blocker, and an artificial plasmid pair as standards to refine the quantification of *JAK2*V617F AB. The assay is effectively validated against ddPCR and some other reliable methods. It yields highly accurate and reproducible results. Our innovation represents a significant advance in the molecular diagnostics of MPN.

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