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

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Supplemental Information

Supplemental Content

Effects of in vitro Culture on the JAK2 Mutant Allele Burden of HEL and UKE-1 Cells and Results on Sample Analyses when Employing Cell-line DNA as Standards

To evaluate copy number variations in cultured HEL and UKE-1 cells, we collected their genomic DNA from different passages and employed the qCAST-Duplex PCR method to quantify their *JAK2*V617F copy numbers. Content dilution with equal amount of healthy donors' DNA to a theoretical 50% mutant allele burden was also performed and subjected to qPCR analyses. The *JAK2*V617F AB ranged from 85-110% in the undiluted UKE-1 DNA from various passages (Supplemental Figure 1A, upper panel), and the variations were similarly seen in half-diluted samples. In the HEL cells, the discrepancies in copy numbers across different passages were also significant (Supplemental Figure 1A, lower panel). Furthermore, the HEL samples that were half-diluted persistently showed way much higher AB than expected, suggesting the presence of very high copy numbers in these cells. Next, we used various DNA dilutions from either HEL or UKE-1 cells to create standard curves for *JAK2*V617F AB quantification. In both the HEL-*HCK* and the UKE-1-*HCK* methods, there was prominent inconsistency across different experiments, as the shifts between the standard curves of two individual tests using different batches of cells were apparent (Supplemental Figure 1B; left panel: HEL cells; right panel: UKE-1 cells). On analyses using the same five clinical samples (four *JAK2*-mutated [M3, M4, M11, and M15] and one *JAK2*-unmutated [M34]), there were huge inter-assay (HEL-*HCK*, UKE-1-*HCK*, HEL-*JAK2*, and UKE-1-*JAK2*) and intra-assay variations (Supplemental Figure 1C).

To assess whether false positive results could occur in various qPCR assays, DNA

from 6 replicates of *JAK2*V617F-negative HL-60 cells and 28 adult healthy controls was subjected to analyses with qCAST-Duplex PCR and the HEL-*HCK* method (Supplemental Figure 1D). All samples were tested as negative for *JAK2*V617F mutation in the qCAST-duplex PCR assay (left lower quadrant, Supplemental Figure 1D). These samples all had FAM Ct values significantly higher than that of the 0.01% mutant standard, and the accuracy of these PCR reactions was confirmed by adequate amplification of the HEX-emitting control sequence (data not shown). On the other hand, sample NP9 had a false-positive result in the HEL-based assay (left upper quadrant, Supplemental Figure 1D). The sample had a FAM Ct value too close to that of the 0.01% mutant standard in the HEL-*HCK* method. When the HEX Ct value of the reference control in NP9 unexpectedly exceeded that of the 0.01% mutant standard, the ΔCt ($Ct._{FAM}$- $Ct._{HEX}$) value decreased and a false-positive result was obtained (data not shown).

We next obtained DNA samples from 32 MPN patients (25 of them *JAK2*-mutated) and tested them with various assays. Supplemental Figure 1E demonstrated the correlation between test results, comparing different cell-based methods with the qCAST-Duplex PCR assay. There was apparent over-estimation of mutant AB when HEL cells were used as the DNA standards (Supplemental Figure 1E, left panel). The UKE-1-*HCK* method showed better correlation (Supplemental Figure 1E, right panel), but there were still quite a few outliers with this assay. These results provide strong evidence showing that both HEL and UKE-1 cells are unreliable sources for being useful genomic DNA standards for *JAK2*V617F AB quantification.

Supplemental Methods

Study Population and Sample Collection, and DNA Extraction

Patients with MPN who were followed at our institutes were enrolled in this study. Adult healthy individuals were also included as control. The study was approved by the Institutional Review Board of Chang-Gung Memorial Hospital. All participants provided an informed written consent in accordance with the Declaration of Helsinki for study sample collection. After blood sampling, peripheral blood (PB) granulocytes from the patients and healthy individuals were harvested using Ficoll-Hypaque gradient centrifugation and cryopreserved. Prior to mutational analysis, DNA was extracted and diluted to a final working concentration of 100ng/μL.

Cell Lines and Construction of Plasmids

Human erythroleukemia cell line HEL and essential thrombocythemia (ET)-transformed AML cell line UKE-1 were purchased from American Type Culture Collection (VA, USA) and Coriell Cell Repositories, Coriell Institute (NJ, USA), respectively. All cells were maintained according to the distributors' recommendation.

To minimize the copy number variation and increase the accuracy of *JAK2*V617F AB determination, we constructed a pair of plasmids as reference standards. With few somatic mutations existed in the exon 21 of the *JAK2* gene, we selected this region as our reference control (Figure 1A). Using the *H_JAK2_clon_exon21_F&R* primer pair (Supplementary Table 2), a specific sequence of *JAK2* Exon 21 was amplified to obtain 264-bp amplicons. Purified PCR products were ligated into a cloning vector (yT&A cloning kit, Yeastern Biotech, Taipei, Taiwan) according to the manufacturer's protocol. Vectors containing the control DNA region (*JAK2_Ctrl_yT&A)* were purified and validated by Sanger sequencing. Next, we used another primer pair containing two

restriction enzyme sites (*Sal*I-h*JAK2*_WT/V617_F and *BamH*I-h*JAK2*_WT/V617F_R, Supplementary Table 1) to amplify the two target regions (*JAK2* wild-type and V617F mutant DNA, both 323 bps in size). The HEL genomic DNA was used as the PCR template to clone the *JAK2*V617F mutation region and the wild-type sequence was amplified from the granulocytic DNA of normal control individuals. Following *Sal*I and *Bam*HI restriction enzyme digestion, purification, and ligation, the respective PCR products were cloned into the *JAK2_Ctrl_yT&A* vector to generate two standard plasmids (*JAK2_WT_Ctrl_yT&A* and *JAK2_V617F_Ctrl_yT&A,* Figure 1B). Validation of both was confirmed by Sanger sequencing and, upon adjustment to identical DNA concentrations, these two plasmids represented 0% and 100% *JAK2*V617F mutant allele burdens, respectively.

Creation of Standard Curves Using Artificial Plasmids, UKE-1 cells, and HEL cells

By mixing these two artificial plasmids at different compositions, a handful of mixtures of various *JAK2*V617F mutant AB contents [e.g. 75μl of *JAK2_V617F_Ctrl_yT&A* (0.104pg/μl) + 25μl of *JAK2_WT_Ctrl_yT&A* (0.104pg/μl) = 75% *JAK2*V617F allele burdens] could be obtained and subjected to standard curve creation. In cell lines, both HEL and UKE-1 cells are homozygous for *JAK2*V617F mutation but carry different *JAK2* copy numbers.[1, 2] In general, the UKE-1 cells harbor only one copy of homozygous *JAK2*V617F mutation. Therefore, DNA extract from UKE-1 can be directly diluted with various proportions of wild-type DNA from normal healthy control donors' granulocytes (GC) [e.g. 100ng/μl, 50μl of UKE-1 DNA (100ng/μl) + 50μl of normal GC DNA = 50% *JAK2*V617F allele burdens] to obtain mixtures of various *JAK2*V617F mutant AB contents for standard curve creation.[3] Two reference genes, the human hematopoietic cell kinase (*HCK*) gene[4] and the total *JAK2* gene (represented by PCR amplification at the aforementioned exon 21 region), were selected as reference

PCR controls in separate experiments. On the other hand, the HEL cells may have varied *JAK2*V617F copy numbers ranging from 10 to 18 copies.[5, 6] As a result, pre-analysis dilution should be done to normalize the copy number variation between *JAK2* and *HCK* when using *HCK* as the reference control gene. A mixture of 10% HEL + 90% normal GC was defined as 100% *JAK2*V617F mutant AB,[6] and the 100% mixture was then subjected to further dilution with normal GC DNA to create the standard curve. It is noteworthy that when the total *JAK2* gene is selected as the reference PCR control, the first dilution of HEL DNA is not mandatory due to the equivalent copy numbers between *JAK2*V617F and the total *JAK* gene.

PCR Condition

Real-time quantitative PCR was performed on a Qiagen Rotor-Gene Q system (Qiagen, Germany). The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Determination of Test Sensitivity among Various Assays

To detect the sensitivity of cell-based assays including HEL-*HCK*, HEL-*JAK2*, UKE-1-*HCK*, and UKE-1-*JAK2*, the two artificial plasmids were mixed in serial dilutions to obtain various mutant DNA contents ranging from 100% to 0.001%. Pure *JAK2_WT_Ctrl_yT&A* plasmid (representative of 0% mutant AB) was also included in the analysis. The detection limit of a specific assay was defined as the lowest dilution of the *JAK2* mutant that yielded unequivocal and consistent results on repetitive experiments when employing the same method.

Commercial Kits for JAK2V617F AB Quantification

For the purpose of comparison, *JAK2*V617F AB quantification was also performed by

employing other commercial assays, the *JAK2* RGQ PCR KIT (Qiagen, Germany), competitive allele-specific TaqMan PCR assay (CAST PCR, Applied Biosystems, Thermo Fisher, USA) and PrimePCR™ ddPCR™ Mutation Detection Assay Kit for droplet digital PCR (QX200 ddPCR, Bio-Rad, USA). These assays were performed according to the manufacturer's protocol.

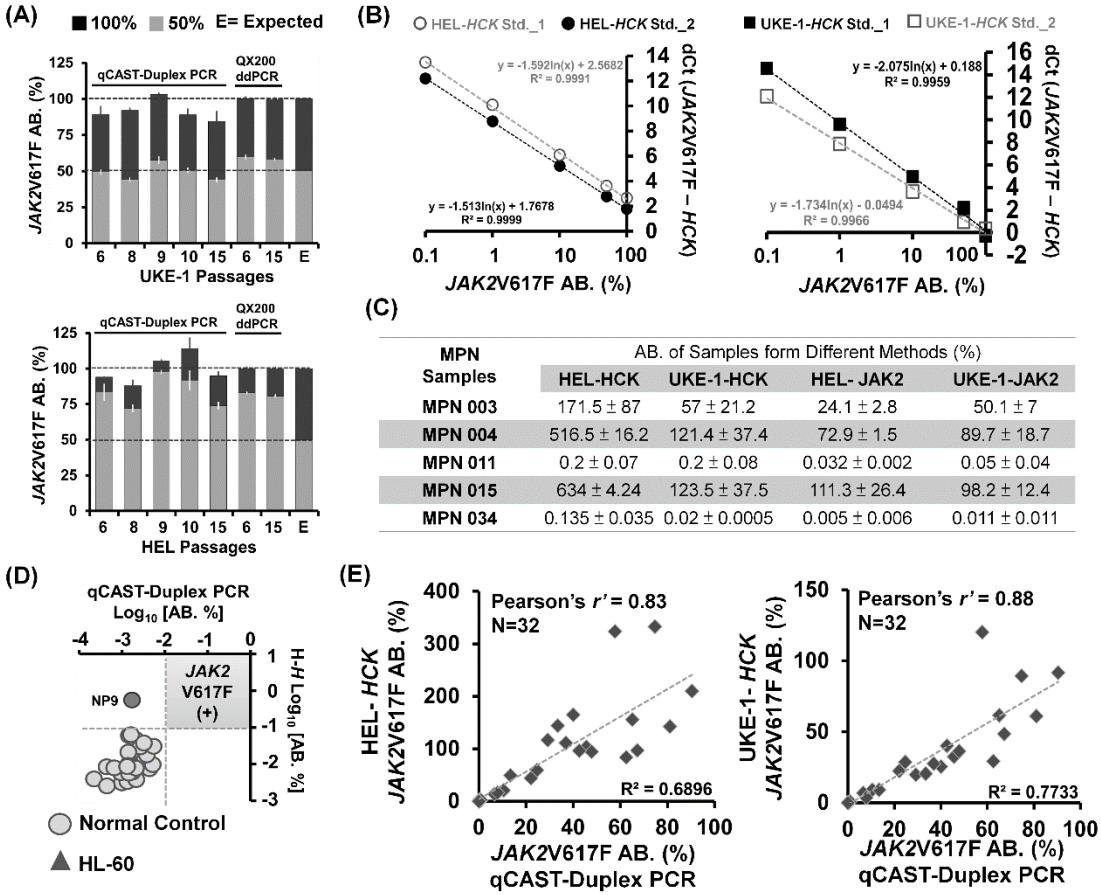
Multiplex PCR Amplicon Sequencing

Exon sequences of 35 target genes (including *JAK2*) were downloaded from UCSC Genome browser (GRCh37/hg19). We designed primers for these genes. Each of them contained a respective adaptor, and the expected maximal PCR product size was set at less than 300bps. In larger exons, the sequences of PCR products might overlap. We employed the micro-fluid-based method to construct the sequence library for each sample (Access Array™ system, Fluidigm). Illumina HiSeq2500 was used to perform the paired-end 150-bp cycle sequencing. After data processing, mapping and variant calling were performed under the CLC Genomics Workbench (Qiagen). *JAK2*V617F mutation was identified with the criteria of more than 50 coverage and an allelic frequency of more than 1%.

Statistical methods

The intra- and inter-assay variation was obtained by calculating the coefficients of variation on allelic burden values. All calculations were performed using the Statistical Package of Social Sciences software (version 17.0; SPSS, Inc.). The level of statistical significance was set at 0.05 for all tests.

Supplemental Figure 1



MPN Samples	AB. of Samples form Different Methods (%)			
	HEL-HCK	UKE-1-HCK	HEL- JAK2	UKE-1-JAK2
MPN 003	171.5 ± 87	57 ± 21.2	24.1 ± 2.8	50.1 ± 7
MPN 004	516.5 ± 16.2	121.4 ± 37.4	72.9 ± 1.5	89.7 ± 18.7
MPN 011	0.2 ± 0.07	0.2 ± 0.08	0.032 ± 0.002	0.05 ± 0.04
MPN 015	634 ± 4.24	123.5 ± 37.5	111.3 ± 26.4	98.2 ± 12.4
MPN 034	0.135 ± 0.035	0.02 ± 0.0005	0.005 ± 0.006	0.011 ± 0.011

Supplemental Figure Legends

Figure 1. Inconsistent measurements of *JAK2*V617F allele burdens while employing cell-based DNA standards. (A) Quantification of *JAK2* mutant allele burdens in various passages of UKE-1 (upper panel) and HEL (lower panel) cells. The cellular DNA extract was assumed to contain 100% mutant AB, and a 1:1 mixture with healthy control DNA supposedly had the AB reduced by half. Samples were measured by the qCAST-Duplex PCR and ddPCR. The undiluted DNA was colored in black, and the diluted samples were shown in light gray color. The numbers on the horizontal axis denoted the passages of the cells, whereas the letter "E" indicated the expected results. The two dash lines represented 100% and 50% AB, respectively. **(B)** Generation of standard curves with different cell lines and reference genes. In the HEL-*HCK* method (left panel), the standard curves were created by employing the HEL-generated standards with *HCK* gene being the reference control. The two dash lines represented the creation of 2 standard curves using two different batches of HEL cells as standard preparation. There was an apparent shift between the standard curves from two individual tests. In the UKE-1-*HCK* method (right panel), identical experiments were repeated but the UKE-1 cells were used as standards instead. **(C)** Measurement of five clinical samples with four different cell-based methods (HEL-*HCK*, UKE-1-*HCK*, HEL-*JAK2*, and UKE-1-*JAK2*). Samples were analyzed by the HEL and UKE-1 -generated standard with either *HCK* or *JAK2* gene being the reference control. All samples were quantified in triplicates with three different batches of cell-line DNA as standard preparation. Data were represented as mean ± standard deviations. **(D)** DNA from 28 healthy controls and 6 replicates of HL-60 cells were measured by the qCast-Duplex PCR and the HEL-*HCK* method. The gray zone, outlined by the differential sensitivities of various assays, indicated the *JAK2*V617F-positive region. False positive detection of *JAK2* mutant was noted in NP9 when HEL-*HCK* generated standard was

employed. Normal samples were plot in gray circles and HL-60 DNA was represented by dark triangle. H-*H* indicated the HEL-*HCK* method. **(E)** The results of *JAK2*V617F allele burden quantification in 32 MPN patients employing the qCAST-Duplex PCR assay were plotted against those obtained from HEL-*HCK* method (left panel) and UKE-1- *HCK* method (right panel). The "*r*" was calculated with the *Pearson's* correlation, and R^2 indicated the regression analysis. AB. = Allele burden.

Supplemental Table 1. List of Primer Sequences

Primer	Gene	Sequence (5'-3')	Final Conc.
H_JAK2_clon_exon21_F	**HUMAN JAK2 (NM_004972)**	CAGTATAATATGGCAGAGTAAAACATTA	**200nM**
H_JAK2_clon_exon21_R		CCTTTATTATCTATGAAAACGTCTAGATGA	
*Sal*I-h*JAK2*_WT/V617F_F		GATATGGATCCGGACCAAAGCACATTGTATCCTCAT	**200nM**
*Bam*HI-hJAK2_WT/V617F_R		ATATAGTCGACGTCGACCTGACACCTAGCTGTGA	
H_JAK2_ref_ex21_F		GGAATATTTACCATATGGAAGTTTACGAGTACT	**150nM**
H_JAK2_ref_ex21_R		CAACACGGTTGCTTCATCTACAGCA	
H_JAK2_ref_probe_HEX		HEX-ACGGATAGATCACATAAAACTTCTGCAGTACACA -IABkFQ	**200nM**
H_JAK2_V617_F		TTATGGACAACAGTCAAACAACAATTC	**150nM**
H_JAK2_V617_R		CTTACTCTCGTCTCCACAAAA	
H_JAK2_Probe_FAM		FAM-TTGTACTTTTTTTTTTCCTTAGTCTTTCTTTGAAGCAGCA-IABkFQ	**200nM**
H_JAK2_WT_blocker		TACTTACTCTCGTCTCCACAAA-ddC	**15nM**
H_HCK_F	**Human HCK (NM_002110)**	TATTAGCACCATCCATAGGAGGCTT	**25nM**
H_HCK_R		GTTAGGGAAAGTGGAGCGGAAG	**15nM**
H_HCK_Probe		HEX-TAACGCGTCCACCAAGGATGCGAA-IABkFQ	**200nM**

Supplemental Table 2

Comparison of *JAK2*V617F quantification using qCAST-Duplex PCR and other methods.

*JAK2*V617F AB.[a] (%)	qCAST-Duplex PCR	QX200 ddPCR[b]	Qiagen RGQ[b]	ABI. CAST PCR[b]	Multiplex PCR Amplicon seq.[b]
MPN Patients	**70**	**70**	**69**	**70**	**58**
***JAK2*V617F (+)**	55	(55/55)	(49/55)	(50/55)	(32/36)
>50 %	23	(23/23)	(22/22)	(23/23)	(17 /18)
10-50 %	20	(20/20)	(20/20)	(19/20)	(13/13)
1-10%	4	(4/4)	(4/4)	(5/4)	(1/1)
0.1-1%	1	(2/1)	(1/2)	(2/1)	(0/1)
0.01-0.1%	7	(6/7)	(2/7)	(5/6)	(0/3)
***JAK2*V617F (-)**	15	(15/15)	(21/15)	(20/15)	(26/22)
Healthy Adults	**30**	**8**	**4**	**25**	**n.a.**
>0.1%	0	0	0	0	n.a
>0.01%	0	0	0	0	n.a
None	30	8	4	25	n.a

[a] AB: allele burden

[b] Assays used for comparison: Bio-Rad QX200 droplet digital PCR, Qiagen RGQ PCR kit, ABI CAST PCR assay, and multiplex PCR Amplicon sequencing.

Number in parentheses denotes (Detected case number/expected case number). Since the results with qCAST-Duplex PCR and ddPCR were identical, the measurements from both assays were used to represent the correct allele burden

With limited availability in some of the assays, the numbers of samples analyzed with various assays were different.

Supplemental Reference

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