Sensitive and Specific Detection of *JAK2* V617F Using Droplet Digital PCR

Lisa Haley, Stacy Riel, K Beierl, E Adams, G Zheng, Ming-Tseh Lin, James Eshleman, Christopher D. Gocke
Departments of Pathology and Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

Introduction: Detection of *JAK2* V617F mutation is important in the diagnosis and prognostic risk assessment of myeloproliferative neoplasms (MPNs). Methods designed to detect this variant have also been used to monitor patient response to therapy. This study evaluates the use of droplet digital PCR (ddPCR) for low level detection of *JAK2* V617F in blood and bone marrow to assess disease progression and therapeutic response.

Methods: ddPCR technology incorporates end-point and emulsion based PCR techniques resulting in highly sensitive detection of somatic variants. Utilizing RainDance ddPCR technology, DNA and reagents are distributed into as many as ten million usable droplets per specimen allowing for direct data analysis that does not require the application of Poisson statistics. Each droplet containing a single molecule of fragmented DNA undergoes PCR amplification with fluorescently labelled probes designed to detect either wild type *JAK2* sequence or the mutant V617F sequence. After amplification, droplets expressing either wild type fluorescence, variant fluorescence or no fluorescence are counted. The accompanying analysis software then quantitates the level of *JAK 2* V617F expression.

Results: Over 80 DNA specimens from whole blood or bone marrow were tested, including 50 samples that had been previously assessed for *JAK2* V617F via alternate methods, 15 samples from healthy donors, and multiple samples from individual patients to assess this method's ability to monitor therapeutic response. In addition, a *JAK2* V617F positive cell line HEL, and a *JAK2* wild type cell line MV4-11 were used to determine the limit of detection and limit of blank for V617F with ddPCR. This approach is capable of detecting *JAK2* V617F with high sensitivity, specificity, and repeatability down to detection levels of <0.02% mutant allele frequency (MAF). ddPCR has qualitative concordance with alternative methods including: Sanger sequencing of RNA from plasma, next generation sequencing (NGS) methodologies, and qPCR; as well as quantitative concordance with NGS and qPCR. Retrospective analysis of DNA from patients with specimens from multiple time points over the course of treatment and disease reveals that ddPCR can provide a sensitive method for tracking disease course and therapeutic response.

Conclusions: ddPCR can be used to detect *JAK2* V617F in DNA from blood and bone marrow with high sensitivity below 0.02% MAF for template inputs of 50 ng. This method does not require a standard curve for quantitation and has minimal non-specific background fluorescence to mask low level variant detection. When additional factors such as speed, ease of use, and cost are considered, ddPCR technology provides a highly sensitive, accurate, fast, and cost effective method for detection of *JAK2* V617F.

Background

- *JAK2* V617F variant occurs in over 95% of polycythemia vera (PV) patients, ~ 50% of patients with either essential thrombocythemia (ET) or primary myelofibrosis (PMF), and results in activation of the tyrosine phosphorylation pathway
- Disease severity in PV, survival estimation in PMF, and likelihood of thrombotic events in ET can all be correlated to mutant allele burden of *JAK2* V617F
- *JAK2* V617F MAF levels as low as 1% detected at 28 days post bone marrow transplant for PMF can predict a higher risk of relapse and lower overall survival when compared to patients with no detectable JAK2 V617F at this time point

Materials & Methods

Methods:

- Quantitative real time polymerase chain reaction (qPCR) – Gold Standard method
  - Limit of detection (LOD) 0.01-0.1%*
  - Limit of blank (LOB) 0.004-0.014%* *Limits stated for at least 10,000 genomic copies per specimen
- Droplet digital polymerase chain reaction (ddPCR)
  - LOD 0.02% with 50ng input (≤ 0.001% variant with >1µg input)
  - LOB 0.00002%

Materials:

- HEL - V617F mutant positive cell line; MV-4-11 – wild type cell line
- DNA extracted from peripheral blood (PB) and bone marrow (BM) from 15 normal donors and over 80 specimens from patients diagnosed with PV, ET or PMF.

Droplet DigitalPCR:

Forward *JAK2* primer sequence: 5'-CAGCAAGTATGATGAGCAA-3'
Reverse *JAK2* primer sequence:5'-AGCCTGTAGTTTACTTACTC-3'
Wild type probe:5'-/5TET/CTCCAC+AGA/ZEN/+CAC+ATACTCC/3IABkFQ/ -3'
V617F probe 5'-/56-FAM/CTCCAC+AGA/ZEN/+AAC+ATACTCC/3IABkFQ/ -3'
The ddPCR was run on the RainDance Instrument System consisting of the RainDrop Source for droplet generation of up to 10 million droplets per specimen and the RainDrop Sense for droplet detection and counting.

Quantitative Real Time PCR: The Ipsogen *JAK2* MutaQuant Kit (Qiagen, Cat#67513) was used according to manufacturer's specifications for qPCR with 25ng DNA template per reaction. qPCR was run on the QuantStudio 6 Flex Real Time Thermal Cycler with run conditions as specified by the MutaQuant assay.

Results

Fig. 1 Mass input study – Detection of *JAK2* V617F by ddPCR

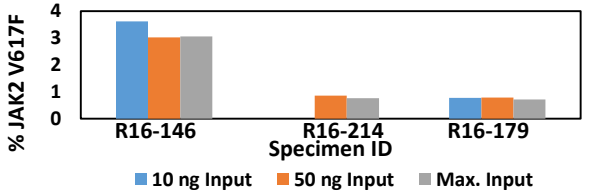


Fig. 2 Impact of tissue source on detection of *JAK2* V617F by ddPCR

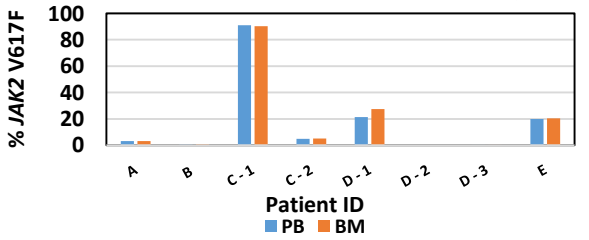
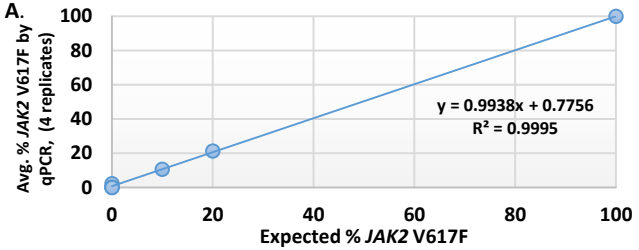


Fig. 3 Determination of LOD- Serial dilution of HEL - V617F mutant line into MV-4-11 - V617V WT line



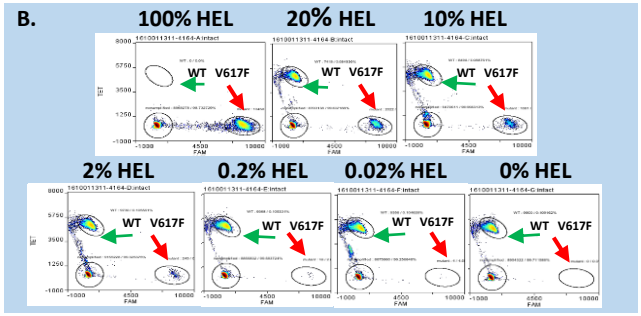


Fig. 4 Concordance between ddPCR and qPCR for detection of *JAK2* V617F

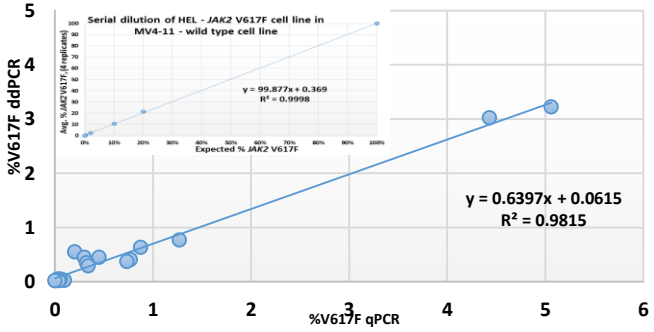


Fig.5 Concordance between ddPCR and NGS for detection of *JAK2* V617F

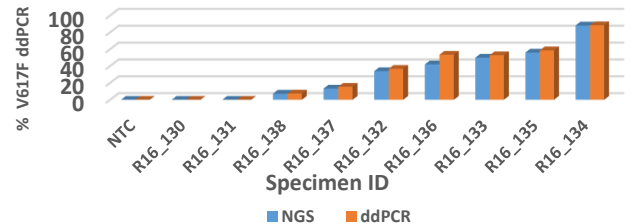


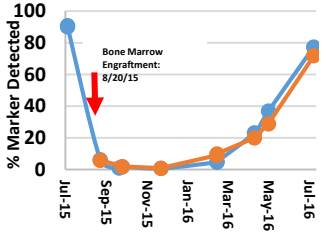
Table 1 Sensitivity and Specificity of ddPCR assay for Low JAK2 V617F MAF qPCR

ddPCR	Positive	Negative
Positive	18	1
Negative	0	15

Sensitivity 100% Specificity 94%

Fig. 6 Efficacy of ddPCR - monitoring disease course and therapeutic response

Pat. A Myeloproliferative Disorder



Pat. B PV and MF converting to AML

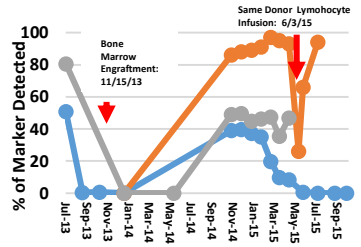


Table 2 Decision matrix for *JAK2* V617F testing

Method	DNA/RNA Input Required	LOD of V617F	LOB	Assay Complexity	Std. curve needed for quantitation	Total Run Time	Hands-on time	Reagent Cost/specimen
Digital Droplet PCR	50ng DNA	0.02%*	0.00002%	Low	No	10 hours	45 min.	$50.00
qPCR	50ng DNA (25ng for WT and 25ng for Mut)	>0.014%	0.014%	Medium	Yes	4.5 hours	1.5hours	$200.00
NGS	0.2 -3µg	2%	1%	High	No	2-4 days	12 hours	$750.00
National Ref. Lab Sanger Sequencing	RNA from Plasma	10%	-------	Send-out Test	No	9 days	-------	$300.00 est.
National Ref. Lab Quantitative Pyrosequencing	DNA	1%	-------	Send-out Test	No	4 days	-------	$600.00 est.

Conclusions

- ddPCR technology applied to the detection of *JAK2* V617F provides a robust and reproducible assay capable of detecting the variant in both blood and bone marrow across a wide range of DNA template input levels.
- The LOB for the assay is 0.00002%.
- Th LOD for inputs of 50ng is 0.02% MAF
- Sensitivity 100%, Specificity 94% when compared to qPCR
- ddPCR does not require a standard curve for quantitation of *JAK2* V617F MAF
- ddPCR requires less hands on time and has lower reagent cost than qPCR