Design Verification Testing of a Highly Sensitive Multiplex qPCR Assay That Detects the *JAK2* V617F Variant Commonly Present in Myeloproliferative Neoplasms

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Summary

- Testing for the *JAK2* V617F variant is routine in diagnosing myeloproliferative neoplasms (MPNs) and is increasingly important for minimal residual disease (MRD) monitoring.
- In response to the need for greater sensitivity and workflow efficiency, we developed a highly sensitive multiplex qPCR assay capable of detecting the V617F mutation alongside an endogenous control target in a single-tube reaction.
- Here we show results from verification studies, including evaluation of limits of detection, quantification, and blank (LoD, LoQ and LoB), linearity, and precision.

Introduction

Approximately 95% of patients with polycythemia vera (PV) and 60% of patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF) are positive for the *JAK2* V617F variant, the most common driver mutation in MPNs. Testing for V617F is routine in MPN diagnosis and increasingly relevant in MRD monitoring, including assessment of molecular response to interferon-based therapies and prediction of outcomes after allogeneic transplantation.

We aimed to characterize sensitivity and accuracy when detecting the V617F variant in whole blood and bone marrow aspirate DNA samples using a multiplex, single-tube qPCR assay.

Methods

Study-specific sample panels were generated using 37 unique V617F-positive and negative whole blood, bone marrow aspirate, and cell line samples. DNA was isolated from blood and bone marrow using the QIAamp® DNA Blood Mini Kit (Qiagen). Additional DNA was isolated from blood using the Wizard® Genomic DNA Purification Kit (Promega) and MagNA Pure 24 Total NA Kit and System (Roche). DNA was quantified using a Nanodrop spectrophotometer. Verification studies for the QuantideX® qPCR *JAK2* Kit[1] included assessments of analytical sensitivity, linearity, sample type and isolation method, and within-lab precision. Testing was performed on the ABI 7500 Fast Dx, QuantStudio™ 5 Dx, and QuantStudio 7 Pro Dx instruments. Data was analyzed with the QuantideX® *JAK2* Analysis Software Module. This software plots the standard curve for calibrators, reports Cq values for all reactions, and reports variant allele frequencies (VAF) for controls and samples.

Workflow

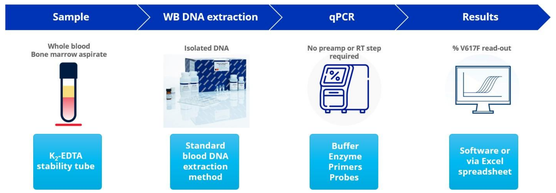

Figure 1. QuantideX® qPCR *JAK2* Kit Workflow. The workflow is streamlined from sample-to-answer and can be performed in less than 2.5 hours with less than 40 minutes of total hands-on time.

Results

Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) of 0.0126%. LOD and LOQ were determined by testing four V617F-positive samples with target inputs of 165 ng and VAF values of approximately 0.01% in quintuplicate using two kit lots in three separate runs for a total of 120 measurements on each supported qPCR platform (7500 Fast, QuantStudio 5, and QuantStudio 7 Pro). V617F was detected in 350 out of 360 reactions for an overall percent positive agreement of 97.2%. Analysis based on the classical nonparametric approach described in CLSI document EP17-A2 was used to determine LOD and LOQ. The LOD for 165 ng of DNA input is 0.0126%, which is the maximum LOD value obtained across all qPCR platforms. Because the assay provided quantitative results with acceptable precision at the LOD, LOQ = LOD = 0.0126%.

Platform	Lot 1 % Detected	Lot 2 % Detected	N Detected (Percent)	VAF SD Range
7500	98.3%	93.3%	115/120 (95.8%)	0.17-0.31
QS5	98.3%	96.7%	117/120 (97.5%)	0.20-0.29
QS7	100%	96.7%	119/120 (99.2%)	0.20-0.34
Overall	**98.8%**	**95.6%**	**350/360 (97.2%)**	

Table 2. Summary of Limit of Blank (LOB) Results. LOB was determined by testing four presumed V617F-negative whole blood samples at a target input of 165 ng per reaction in quadruplicate using two kit lots in three runs for a total of 95 measurements on each supported qPCR platform (7500 Fast, QuantStudio 5, QuantStudio 7 Pro). V617F was undetected in 284 of 285 V617F-negative sample reactions, across all qPCR platforms. LOB analysis resulted in values of zero for both kit lots on all three platforms. The overall LOB was zero.

Platform	Lot	N Detected	N Undetected	Total N	% Undetected
7500	1	1	47	48	97.9
7500	2	0	47	47	100
QS5	1	0	48	48	100
QS5	2	0	47	47	100
QS7	1	0	48	48	100
QS7	2	0	47	47	100

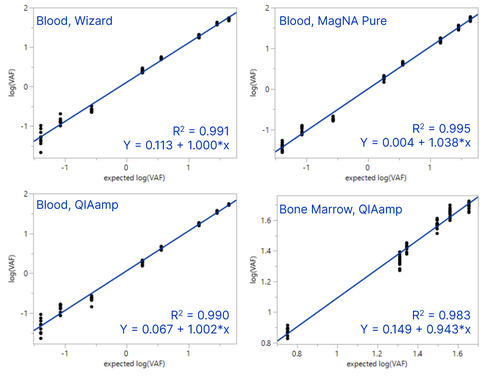

Figure 2. QuantideX® qPCR *JAK2* Kit log(VAF) Measurements Plotted Against Average Expected log(VAF) by Sample Type and Isolation Method. Variant allele frequencies (VAF) determined by an orthogonal method (Bio-Rad droplet digital PCR) were used as the expected VAF values. All R^2 values were >0.980, demonstrating strong correlation between qPCR and expected results for all sample types and isolation methods. The QuantideX® qPCR *JAK2* Kit is compatible with DNA extracted from whole blood or bone marrow aspirates collected in K_2EDTA tubes. The recommended methods for extracting DNA from whole blood are the QIAamp® DNA Blood kit (Qiagen), MagNA Pure 24 Total NA Isolation Kit on the MagNA Pure 24 System (Roche), or Wizard® Genomic DNA Purification kit (Promega).

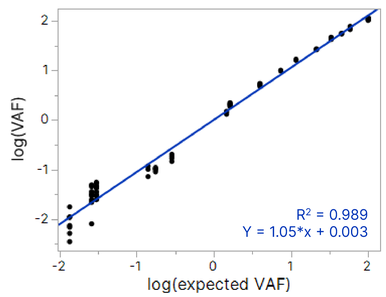

Figure 3. The QuantideX® qPCR *JAK2* Kit Showed Linearity from the LOD/LOQ of 0.0126% to 100% VAF. Linearity was assessed on the QuantStudio 5 using V617F-positive whole blood samples and mixes of V617F-positive and negative cell line DNA with VAF values ranging from 0.01% to 100% across the full sample set. replicates for each of the three lowest VAF samples and replicates for the highest VAF sample were tested; four replicates were tested for all other samples. V617F was detected in all 80 sample reactions. The *JAK2* log(VAF) measurements were plotted against log (expected VAF) values. The assay satisfied linearity requirements across the full quantifiable range of 0.0126% to 100% VAF.

Table 3. Within-Lab Precision Data Indicated Acceptable Precision Spanning the Full Linear Range on the QuantStudio 5. Within-lab precision was evaluated using six V617F-positive whole blood samples and two contrived cell line mixtures prepared by combining V617F-positive and V617F- negative cell lines. Together, these samples spanned a target VAF range of 0.05% – 90%. Testing included two kit lots, two operators, and three separate run days to generate 287 sample measurements. All results met the acceptance criteria, indicating that performance is similar across runs performed by different operators using different kit lots.

Sample	N	Mean VAF (%)	VAF CV (%)	Percent Detected
Cell line mix 1	36	90.8	6.0	100%
Cell line mix 2	36	74.2	6.1	100%
Blood 1	36	49.8	7.6	100%
Blood 2	35	28.7	6.3	100%
Blood 3	36	11.6	10	100%
Blood 4	36	2.57	7.5	100%
Blood 5	36	0.130	19	100%
Blood 6	36	0.0538	38	100%

Conclusions

- The multiplex qPCR assay enables quantification of the *JAK2* V617F variant and an endogenous control in both blood and bone marrow samples, with detection capability at <0.1% VAF in a single reaction.
- With a total time <2.5 hours from sample to result, this high sensitivity assay has the potential to aid in reliable identification of the V617F variant associated with MPNs and is suitable for MRD research.
- The QuantideX® qPCR *JAK2* Kit is harmonized with other QuantideX qPCR HemOnc portfolio kits (BCR-ABL and PML-RARA) allowing mixed batching on a plate, for additional lab workflow efficiencies.
- The QuantideX® qPCR *JAK2* Kit is ready-to-use with all necessary qPCR reagents, controls, calibrators, and analysis software.

All authors have the financial relationship to disclose: Employment by Bio-Techne Diagnostics.

[1]For Research Use Only. Not for use in diagnostic procedures.