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High-resolution melting assay for rapid, simultaneous detection of *JAK2*, *MPL* and *CALR* variants

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ABSTRACT

Aims Identification of recurrent genetic alterations in *JAK2*, *MPL* and *CALR* remains crucial in the diagnosis of Philadelphia-negative myeloproliferative neoplasms (MPNs). Current laboratory testing algorithms may entail batching and/or sequential testing, involving multiple testing modalities and sometimes send-out testing that increase the technical and economic demands on laboratories while delaying patient diagnoses. To address this gap, an assay based on PCR and high-resolution melting (HRM) analysis was developed for simultaneous evaluation of *JAK2* exons 12–14, *MPL* exon 10 and *CALR* exon 9, embodied in the HemeScreen® (hereafter 'HemeScreen') MPN assay.

Methods The HemeScreen MPN assay was validated with blood and bone marrow samples from 982 patients with clinical suspicion for MPN. The HRM assay and Sanger sequencing were performed in independent Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories with Sanger sequencing (supported by droplet digital PCR) serving as the gold standard.

Results HRM and Sanger sequencing had an overall concordance of 99.4% with HRM detecting 133/139 (96%) variants confirmed by sequencing (9/10 MPL, 25/25 CALR, 99/104 JAK2), including 114 single nucleotide variants and 25 indels (3–52 bp). Variants consisted of disease-associated (DA) variants (89%), variants of unclear significance (2%) and non-DA variants (9%) with a positive predictive value of 92.3% and negative predictive value of 99.5%.

Conclusions These studies demonstrate the exquisite accuracy, sensitivity and specificity of the HRM-based HemeScreen MPN assay, which serves as a powerful, clinically applicable platform for rapid, simultaneous detection of clinically relevant, somatic disease variants.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Identification of recurrent mutations is crucial for the diagnosis of myeloproliferative neoplasms (MPNs), yet current laboratory workflows often result in sequential, potentially costly testing algorithms that can delay patient diagnosis.

WHAT THIS STUDY ADDS

⇒ This study demonstrates the ability of an assay based on PCR and high-resolution melting (HRM) analysis to effectively and concurrently identify mutations in real-world patient samples when collected under the suspicion of MPN.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The application of the HRM assay can accelerate the laboratory approach to identifying MPN-associated mutations, allowing streamlined laboratory workflows, decreased cost and improved turnaround time for clinicians seeking evidence for the diagnosis of MPN.

INTRODUCTION

The identification of recurrent mutations in *JAK2*, *MPL* and *CALR* has become firmly established as a key component of the diagnostic criteria for Philadelphia-negative myeloproliferative neoplasms (MPNs) by both World Health Organization and International Consensus Classification guidelines.[1–3] In addition, current National Comprehensive Cancer Network guidelines recommend one of the following testing approaches for patients with clinical concern for MPN: (1) initial testing for *JAK2* exon 14 (p.V617F) mutations in all patients with reflex testing for *CALR* and *MPL* mutations in suspected essential thrombocythemia or primary myelofibrosis and reflex testing for *JAK2* exon 12 mutations in suspected polycythemia vera, or (2) simultaneous evaluation of all three genes.[4] The stepwise approach is rationalised by the fact that the majority of mutations in MPNs consist of the canonical *JAK2* p.V617F with fewer cases demonstrating *CALR* exon 9 mutations and even fewer carrying *MPL* exon 10 or *JAK2* exon 12 mutations.[5 6] However, this testing paradigm limits the ability to detect concurrent mutations, which may occur, although infrequently.[7–9] In addition, testing in many laboratories may include multiple testing modalities, send-out tests and staggered turnaround times (TATs), potentially delaying patient care. As a result, demand has emerged for an inexpensive, quick test to address all relevant targets with strong performance criteria across a broad variant allele frequency (VAF) range.[10]

High-resolution melting (HRM) analysis leverages the differential melting temperatures between unique sequence patterns, allowing the recognition of a variant sequence in a background of wild-type sequence. In HRM, the melt curve for each amplicon of the test sample of interest (eg, patient-derived) is compared with control samples of known sequence and VAF. Using the melting curve from a pure wild-type sample as a baseline, difference plots can be generated and visually inspected to detect the presence of a variant sequence.[11] HRM is particularly advantageous in a clinical laboratory setting due to

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Table 1 Primer sequences used for PCR by target

Target	Forward primer	Reverse primer
CALR exon 9	gcagagaaacaaatgaaggacaaac	AGGAAACAGCTATGACCATcttcctccttgtcctcctca
JAK2 exon 12	ccaacctcaccaacattacagag	AGGAAACAGCTATGACCATtccaatgtcacatgaatgtaaatcaa
JAK2 exon 13	AGGAAACAGCTATGACCATctcttgaagaatgaaagccttgg	gtttctgtgtgctttatccagaact
JAK2 exon 14	AGGAAACAGCTATGACCATtttctttgaagcagcaagtatg	agatgctctgagaaaggcatta
MPL exon 10	AGGAAACAGCTATGACCATtagcctggatctccttggtg	gcggtacctgtagtgtgcag

its low cost, relatively low complexity, high reliability, capacity for multiplex testing, ease of interpretation and quick overall TAT.[12–16] As the need for reliable front-line testing for multiple gene alterations increases, so too does the demand for a single assay to simultaneously assess all common mutations found in MPNs.[15] The current study evaluates the clinical utility of the HRM-based HemeScreen MPN assay as a genetic diagnostic approach for the evaluation of samples from patients with a clinical suspicion for MPN.

METHODS

Specimen origin and DNA extraction

A total of 1043 peripheral blood and bone marrow samples were collected over 11 months from patients with a clinical suspicion for an MPN. Samples were identified in both retrospective and prospective manners and were collected from patients at clinical sites around the USA with a goal of 1000 cases to ensure adequate coverage of all targets of interest. All specimens were initially sent to the Clinical Laboratory Improvement Amendments (CLIA)-certified Precipio laboratory in New Haven, CT, and 982 samples were ultimately found to be suitable for testing. DNA was extracted using a QIAamp DNA Blood Mini Kit on a QIAcube automated system (QIAGEN, Hilden, Germany, cat. 51104) according to the manufacturer's instructions and diluted with Tris-EDTA buffer to 10 ng/µL. The DNA samples were then divided evenly into two aliquots. The first aliquot was retained at the New Haven laboratory for HRM testing on a rolling basis, and the second aliquot was sent to the CLIA-certified Precipio laboratory in Omaha, NE for Sanger sequencing, which was performed in a batched manner. The teams at each site were blinded to both patient clinical information and results from the other laboratory.

HemeScreen HRM analysis

Polymerase chain reaction

JAK2 (exons 12, 13 and 14), *MPL* and *CALR* primer mixes were prepared containing 0.5 µM forward and reverse primer in 1× IDTE, pH 8.0 (Integrated DNA Technologies, Coralville, IA, USA). The primer sequences for each target are provided in table 1. A Master Mix for each target was then prepared containing 10 µL of 2× HRM Master Mix and 8 µL of the primer mix for each sample to be analysed alongside digitally verified wild-type and 5% mutant controls and a no template control (NTC). Eighteen microlitres of each target Master Mix was then aliquoted into a well of an Applied Biosystems MicroAmp EnduraPlate Optical 96-well Fast Plate (Thermo Fisher, Waltham, MA, USA, cat. 4425618) for all samples and controls. Two microlitres of sample DNA, wild-type control, 5% mutant control and NTC were added to the respective wells on the 96-well plate. Amplification and HRM were performed on a QuantStudio 3 Real Time PCR System (Thermo Fisher, cat. A28567). The amplification protocol involved 40 cycles of denaturation at 95°C for 15 s followed by annealing/extension at 60°C for 60 s.

High-resolution melting curve analysis

The HRM protocol was performed after amplification, consisting of a 10 s denaturation phase at 95°C for 15 s, a 60 s annealing phase at 65°C for 60 s and HRM data capture during a 0.025°C/second ramp up to 95°C. Initial data review was performed for QC metrics on the PCR instrument using QuantStudio Design and Analysis Desktop Software V.1.5.1 (Thermo Fisher), including positive control amplification and no amplification in the NTC. Since this software does not normalise the data for HRM analysis for mutation calling, melt curves were

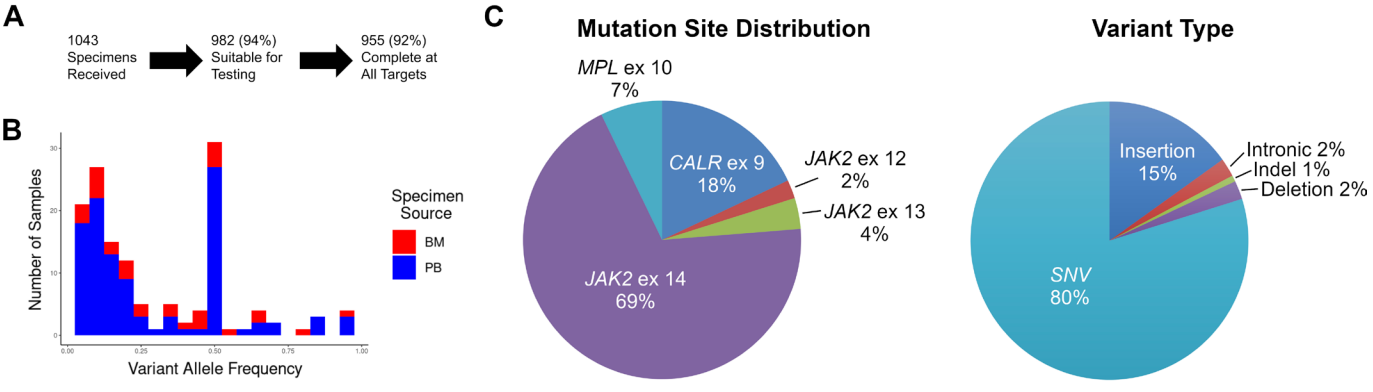

Figure 1 (A) Initially, 1043 specimens were received for testing on the basis of clinical concern for a myeloproliferative neoplasm (MPN), 982 (94%) of which were suitable for testing. While high-resolution melting (HRM) was successful in all tested specimens, an additional 26 specimens were excluded on the basis of failed Sanger sequencing, resulting in 955 (92%) cases with complete HRM and Sanger results at all targets. (B) Samples consisted of peripheral blood (90%) and bone marrow (10%) specimens and variants with allele frequencies ranging from <5% to 95%. (C) Among samples with mutations, the distribution was consistent with those reported in Philadelphia-negative MPNs with the majority consisting of *JAK2* exon 14 (p.V617F) mutations and fewer *MPL* and *CALR* mutations. Variants included 114 single nucleotide variants, 3 insertions (5 bp), 21 deletions (3–52 bp) and 1 deletion-insertion.

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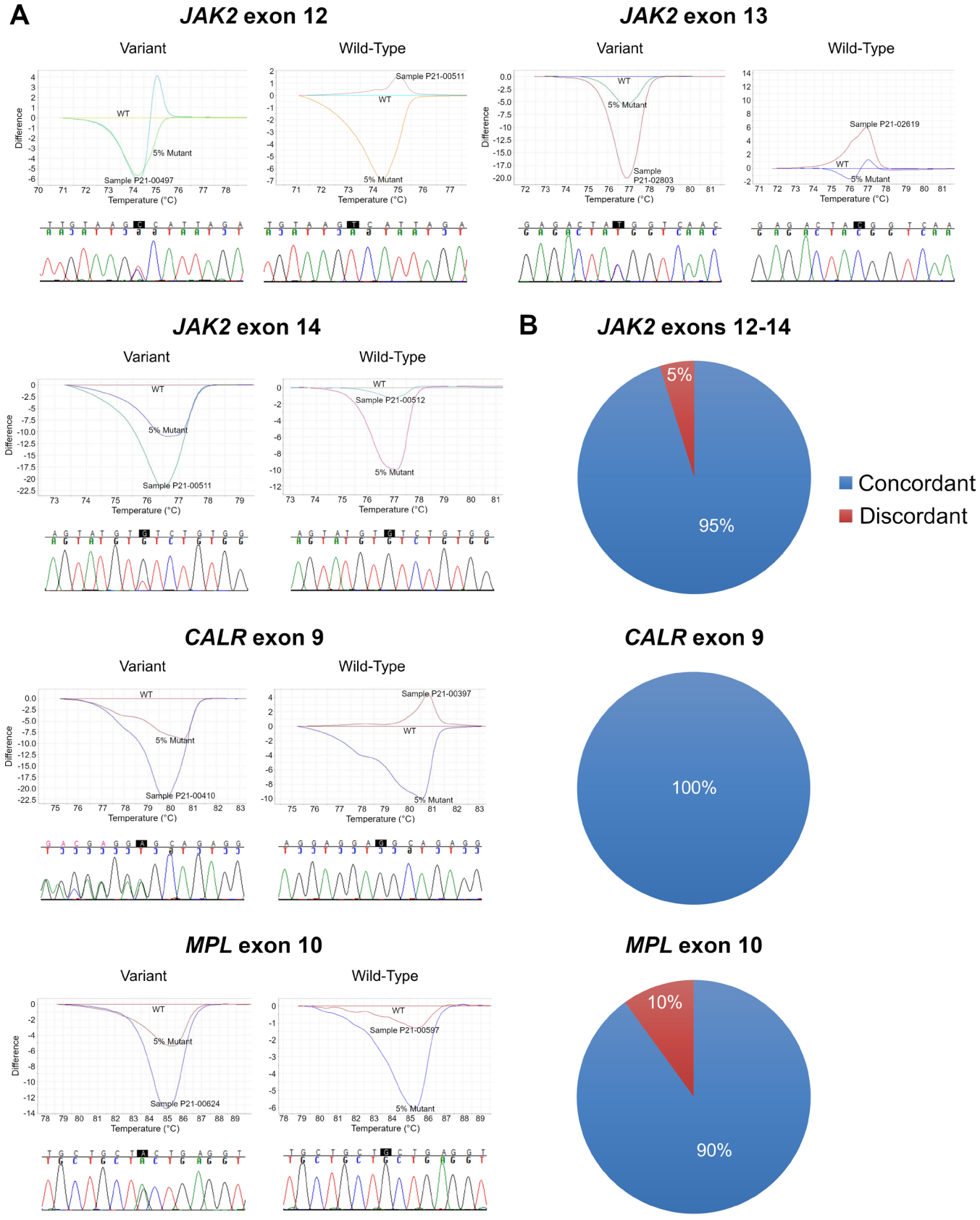

Figure 2 (A) For each target, variant (left) and wild-type (WT, right) patterns on high-resolution melting (HRM), represented by difference plots (top row), and Sanger sequencing (bottom row) were identified among the submitted patient samples. For each test, a 5% mutant control was run as a point of comparison, and a sample was only called positive if the sample melting curve varied from the wild-type curve to a degree greater than the mutant control curve when visualised on a difference plot. This process allowed for accurate identification of variants, occasionally even at levels below 5% variant allele frequency. (B). Overall, mutations were identified in each of the five targeted domains, the majority of which were detected by both HRM and Sanger sequencing.

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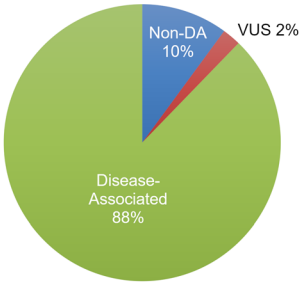
Variant Categorization



Overall Clinical Performance

	JAK2 ex 12-14	*CALR* ex 9	*MPL* ex 10	Overall
Sensitivity	97.0%	100.0%	88.9%	96.8%
Specificity	99.9%	99.2%	99.9%	98.8%
PPV	97.0%	68.0%	88.9%	92.3%
NPV	99.9%	100.0%	99.9%	99.5%

Summary of Variants Identified

Target	Variant	Detection Rate	Type	Effect
JAK2 exon 14	c.1849G>T; p.V617F	94/96 (98%)	SNV	DA
CALR exon 9	c.1099_1150del; p.L367fs*46	11/11 (100%)	Del (52 bp)	DA
MPL exon 10	c.1544G>T/C; p.W515L/S	9/10 (90%)	SNV	DA
CALR exon 9	c.1191_1199del; p.E398_D400del	6/6 (100%)	Del (9 bp)	Non-DA
CALR exon 9	c.1154_1155insTTGTC; p.K385fs*47	3/3 (100%)	Ins (5 bp)	DA
JAK2 exon 12	c.1641+6T>C; p.?	1/3 (33%)	Intronic SNV	Non-DA
JAK2 exon 13	c.1711G>A; p.G571S	1/2 (50%)	SNV	VUS
CALR exon 9	c.1090_1123del; p.E364Nfs*55	1/1 (100%)	Del (33 bp)	DA
CALR exon 9	c.1101_1152del; p.K368Rfs*45	1/1 (100%)	Del (52 bp)	DA
CALR exon 9	c.1102_1104del; p.K368del	1/1 (100%)	Del (3 bp)	Non-DA
CALR exon 9	c.1122_1126delinsTTCTTGTCTT; p.K372Ffs	1/1 (100%)	Del/Ins	DA
CALR exon 9	c.1142_1144del; p.E381del	1/1 (100%)	Del (3 bp)	Non-DA
JAK2 exon 13	c.1686C>T; p.G562=	1/1 (100%)	SNV	Non-DA
JAK2 exon 13	c.1710C>T; p.Y570=	1/1 (100%)	SNV	Non-DA
JAK2 exon 13	c.1691G>T; p.R564L	1/1 (100%)	SNV	VUS
MPL exon 10	c.1536G>A; p.L512=	1/1 (100%)	SNV	Non-DA

Figure 3 (A) Overall, the vast majority of variants detected were disease-associated (DA) with few non-DA and variants of unclear clinical significance (VUS) observed. (B) The high-resolution melting (HRM)-based assay identified known DA variants (89%), variants of undetermined significance (2%) and non-DA variants (9%) with a positive predictive value (PPV) of 92.3% and negative predictive value (NPV) of 99.5%. (C) Mutation distribution reflected what is reported in the literature with the majority of variants consisting of the canonical *JAK2* p.V617F mutation, of which 94/96 (98%) were detected by the HRM assay. Non-DA variants were predominantly benign 3 or 9 bp *CALR* deletions.

analysed on a separate computer with HRM V.3.1 and/or HRM V.3.2 software (Thermo Fisher), through which difference plots and derivative melt curve plots were generated and visually inspected. A sample was considered positive if it demonstrated a unique melting profile compared with the wild-type control, which was specified to mean a variance from wild-type to a degree equal to or greater than the mutant control curve(s) as visualised on the generated difference plots. The assay was determined to have a limit of detection of 2% for *JAK2* p.V617F and 5% for all other targets.

Sanger sequencing

Sanger sequencing was performed in the Precipio laboratory in Omaha, NE on the amplicons produced by the HRM process described previously. For samples that failed Sanger sequencing on the original amplicon, the second DNA aliquot was used, and PCR amplification for each target was performed on a C1000 Touch PCR System (Bio-Rad Laboratories, Hercules, CA, USA, cat. 1851196) with the same reagents, primers and samples/controls described in the HRM protocol. All PCR products were then subjected to gel electrophoresis using E-Gel Precast 2% Agarose Gels on a Mother and Daughter E-Base (Invitrogen, Waltham, MA, USA, cat. A39560) to confirm the presence of the target amplicon as well as appropriate control performance. PCR products were then purified with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA, cat. A63882) per the manufacturer's instructions. The cycle sequencing reactions were carried out using standard Sanger sequencing methods with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, cat. 4337455) on the C1000 Touch PCR System. Excess dye-terminators in the reactions were removed with Agencourt CleanSEQ paramagnetic beads (Beckman Coulter, cat. A29161). Unidirectional sequencing was performed on an ABI 3730XL DNA Analyzer (Thermo Fisher, cat. 3730XL) and analysed with Sequencher V.5.4.6 software (Gene Codes Corp, Ann Arbor, MI, USA).

A sample was considered positive if a variant was present at 5% VAF or greater; otherwise, the sample was considered negative for the variant in question. VAF was estimated by visual estimation and confirmed by using a pixelated ruler to determine the actual peak height of the mutant peak to the calculated total peak height of the wild-type and mutant peaks. Samples with poor Sanger sequencing quality were re-sequenced if sufficient PCR product was present based on gel electrophoresis. Samples with insufficient PCR product for resequencing or with persistently poor sequencing quality were considered to have failed Sanger sequencing (27 samples). Samples that did not survive transport, failed to produce a PCR product or had inadequate DNA material for testing were excluded from analysis.

Droplet digital PCR

Samples appearing to have a *JAK2* p.V617F VAF <5% underwent orthogonal variant confirmation with the *JAK2* p.V617F c.1849G>T ddPCR Mutation Assay (Bio-Rad Laboratories, cat. 10049550, assay ID: dHsaMDV27944642), which was performed on a QX200 ddPCR system (Bio-Rad Laboratories, cat. 1864001) according to the manufacturer's instructions. A comparative study of Sanger sequencing to ddPCR using clinical and control samples was performed to validate the identification of low-level *JAK2* p.V617F mutations (online supplemental figure 1).

Statistical analysis

Concordance was calculated using Sanger sequencing and supporting ddPCR results as the gold standard, from which overall analytical performance was determined. Variants were categorised as disease-associated (DA), variants of uncertain significance (VUS) or non-DA according to established protocols for variant classification. Considering DA and VUS to be clinically true positive results, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated on individual component and complete assay bases.

RESULTS

Of 1043 samples originally received, 982 were adequate for paired HRM and Sanger sequencing, and 955 (97%) had successful Sanger sequencing at all targets. Successful samples consisted of peripheral blood (90%) and bone marrow aspirate (10%) specimens. In the evaluable study cohort, 136/982 (14%)

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specimens carried 139 variants, which included 114 single nucleotide variants, 3 insertions (5 bp), 21 deletions (3–52 bp) and 1 insertion/deletion with VAF ranging from <5% to 95% (figure 1, online supplemental table 1). Overall, HRM and Sanger sequencing had an analytical concordance of 99.4% by case. Among the 139 variants, the HRM assay detected 133 (96%), including 9/10 *MPL* variants, 25/25 *CALR* variants, 1/3 *JAK2* exon 12 variants, 4/5 *JAK2* exon 13 variants and 94/96 *JAK2* exon 14 variants (figure 2). Variants undetected by HRM included 1 *MPL* variant (c.1544G>T p.W515L, VAF 5%), 2 *JAK2* exon 12 variants (both c.1641+6T>C, VAF 50%), 1 *JAK2* exon 13 variant (c.1711G>A p.G571S, VAF 50%) and 2 *JAK2* exon 14 variants (both p.V617F, VAF 50% and 10%). In 3/139 (2%) variant-carrying cases, concurrent variants in both *JAK2* exon 14 and a second target were present. The dual mutations included *JAK2* p.V617F and *CALR* p.E398_D400del (1/139; 0.7%), *JAK2* p.V617F and *CALR* p.E381del (1/139; 0.7%) and *JAK2* p.V617F and *JAK2* p.R564L (1/139; 0.7%), all of which were detected by the HRM assay (online supplemental table 1).

Considering only DA or VUS to be clinically positive cases and using Sanger sequencing results as the gold standard, the HRM assay overall had a sensitivity of 96.7% and a specificity of 98.7% with a PPV of 92.3% and a NPV of 99.5% (figure 3). Regarding the most common mutation, *JAK2* p.V617F, 94/96 (97.9%) cases were positively identified. Both undetected cases were from peripheral blood samples of good quality with p.V617F VAF of 10% and 50%, and no clear cause for the lack of detection by HRM was identified. Interestingly, a number of variants that are not known to be associated with clinical disease were identified using HRM and confirmed by Sanger sequencing. These were most numerous in *CALR* exon 9, where benign 3 or 9 bp deletions accounted for 8/25 (32%) cases, yielding a 68% PPV for that target despite a 100% NPV. Additional cases with non-DA variants were seen with *MPL* (1), *JAK2* exon 12 (2) and *JAK2* exon 13 (2) (figure 3). Each of these cases yielding variant profiles demonstrated a corresponding 'non-reported' variant. All cases positive by HRM were confirmed by Sanger sequencing to contain a genetic variant.

DISCUSSION

Molecular tests in the clinical laboratory interrogating *JAK2*, *MPL* and *CALR* are important for the complete evaluation and accurate diagnosis of MPN. Here, data from patient samples demonstrate the evaluation and clinical utility of the HemeScreen MPN assay, involving simultaneous HRM analysis for the assessment of samples from patients with clinical suspicion for an MPN. The assay demonstrated a 99.5% NPV in a large number of real-world patient samples. Combined with this clinical performance, the panel's greatest strength lies in its ability to interrogate all major MPN-associated targets simultaneously without the need to perform second-line studies to rule out relevant variants, thereby avoiding associated delays in patient care.

The necessity for delivery of genetic testing results in a clinically relevant timescale is increasingly important for the timely management of patients. From a laboratory standpoint, this assay offers benefits in cost-effectiveness by requiring relatively inexpensive instrumentation and reagents, requiring less technologist and pathologist time, and avoiding the need for up-front single-gene and next-generation sequencing panel testing. Notably, the panel does not report a specific variant, and secondary sequencing studies would be necessary in a subset of positive cases to confirm both the variant identity and clinical implications. However, given the low frequency of positive results in clinical practice as well as the opportunity to perform concurrent testing at all targets, the number of secondary tests would be minimal and could allow for improved cost and TAT benefits overall.

Accordingly, follow-up testing could be performed in several ways depending on the clinical history and variant identified by HRM. Positive HRM findings in *JAK2* exon 14 are essentially all pathogenic V617F mutations, requiring no further verification to support a diagnosis of *JAK2*-positive MPN, although reflex to a broader sequencing panel may be useful for potential prognostic data. In contrast, positive *CALR* results by HRM bear a relatively higher possibility of a false-positive result due to benign 3 bp or 9 bp deletions and may yield better results through confirmation of the specific *CALR* variant. Similarly, positive *JAK2* exons 12/13 and *MPL* by HRM warrant either single-gene or broader panel sequencing to confirm the mutation and diagnosis. The decision to pursue either single gene or sequencing panel may be best guided by clinical suspicion, anticipated TAT of secondary testing and/or cost of testing, and is a decision best reached in collaboration with the laboratory's referring clinicians.

In this study, a PCR and HRM-based assay for the identification of recurrent alterations in MPN was established and assessed. The sequence alterations varied from single base substitutions resulting in no change in length of the amplicon to insertion-deletions resulting in length affecting alterations ranging from 3 to 52 bp. Each variant was detected as a reproducible distortion in the shape of the fluorescence versus temperature curves of the samples containing the genetic variant when compared with the curves of the samples with the wild-type sequence. As observed in previous studies,[13 17] the distortion in the curve shape can be attributed to heteroduplex formation between the variant sequences, contributed by the abnormal alleles in the tumour DNA, and wild-type sequence, contributed by a normal allele in the tumour DNA or from DNA from contaminating normal cells. The ability to readily detect a range of alterations with high accuracy is thus an advantage of this assay format. Although disease-associated *JAK2* exon 12 mutations were absent from this cohort, a benign *JAK2* exon 12 variant present in this cohort was detected using this assay format. This cohort also exhibited a relative paucity of disease-associated *JAK2* exon 13 variants. Finally, the lack of available clinical data for these patients precludes the ability to correlate the relevance of positive variants with the patients' clinical context and final diagnosis.

In summary, this is the first report of clinical performance data for the HemeScreen MPN assay, a simultaneous, HRM-based platform for the identification of recurrent somatic variants in genes that are known to play a role in the pathogenesis of MPN. The data demonstrate the assay's capacity as a screening tool with a high negative predictive value across all five panel targets. Combined with its analytical performance characteristics, the panel's low cost and potential for quick TAT leave it well positioned to complement a laboratory's testing paradigm, which emphasises simple workflows for disease-group-based panel testing. Overall, the ease of use and versatility of the platform facilitate implementation and accessibility across a wide resource spectrum and in a large variety of clinical laboratory settings.

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Contributors AM conceptualised and designed the study and is responsible for the overall content as guarantor. AM and BLL developed the HRM assay. DP performed HRM setup and testing. SLF performed Sanger sequencing setup. AM and DP performed HRM data analysis. BLL and SLF performed Sanger sequencing data

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analysis. AM, KG and CMS performed data review and statistical analyses. AM, CMS, GY and KSJE-J performed data analysis and interpretation. CMS and KSJE-J prepared the manuscript with input from all authors.

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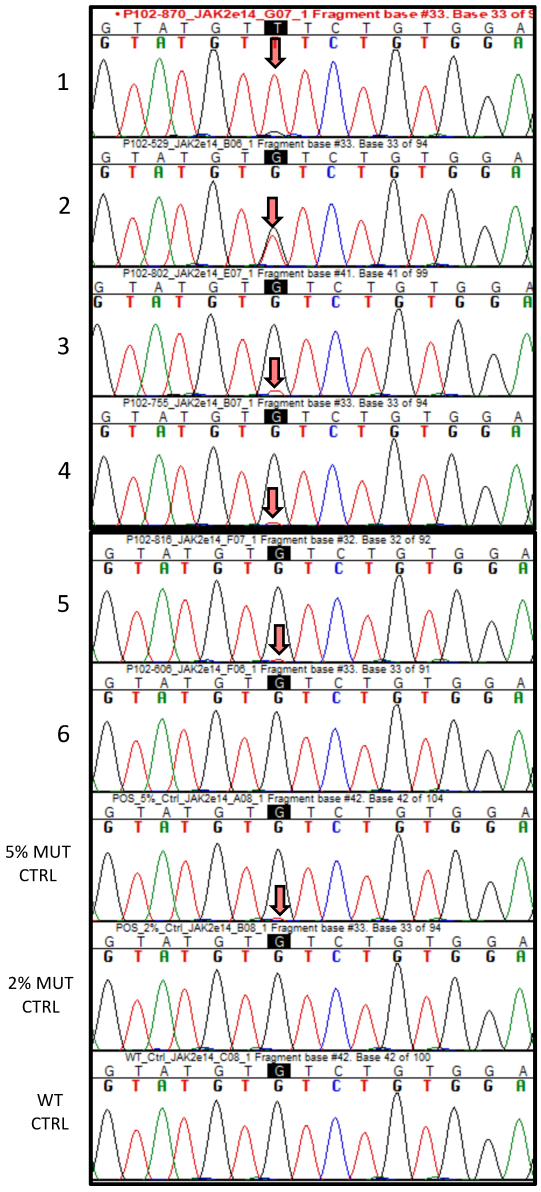
Sample



Sample	Original Sequencing Results with Estimated VAF[a]	Retested Sequencing Results with Pixelated Ruler VAF[b]	ddPCR Results (Fractional Abundance)
1 (P102-870)	c.1849G>T; p.V617F, 95%	c.1849G>T; p.V617F, 91%	93%
2 (P102-529)	c.1849G>T; p.V617F, 45%	c.1849G>T; p.V617F, 44%	50%
3 (P102-802)	c.1849G>T; p.V617F, 5%	c.1849G>T; p.V617F, 10%	10%
4 (P102-755)	c.1849G>T; p.V617F, <5%	c.1849G>T; p.V617F, 5%	7%
5 (P102-816)	c.1849G>T; p.V617F, 5%	c.1849G>T; p.V617F, 4%	6%
6 (P102-606)	NVD	NVD	NVD
7 (P102-478)	c.1849G>T; p.V617F, 10%	-	14%
8 (P102-558)	NVD	-	NVD
9 (P102-596)	c.1849G>T; p.V617F, 5%	-	9%
10 (P102-601)	c.1849G>T; p.V617F, 5%	-	10%
11 (P102-606)	NVD	-	NVD
12 (P102-638)	c.1849G>T; p.V617F, 80%	-	86%
13 (P102-718)	NVD	-	NVD
14 (P102-756)	c.1849G>T; p.V617F, 5%	-	8%
15 (P102-757)	NVD	-	NVD
16 (P102-993)	c.1849G>T; p.V617F, 10%	-	9%
5% MUT CTRL	c.1849G>T; p.V617F, 5%	c.1849G>T; p.V617F, 4%	6%
2% MUT CTRL	N/A	NVD	2%
WT CTRL	NVD	NVD	NVD

Sixteen samples of wild-type and varying mutant variant allele frequency were retested using fresh aliquots of stored DNA using the HRM/Sanger Sequencing protocol and droplet digital PCR for *JAK2* Exon 14. Amplification for the HRM/Sanger protocol was performed as originally described. Droplet digital PCR was performed using manufacturer's recommended conditions.

As seen in the representative electropherograms, the mutant peaks are evident even at low VAF (red arrows). In this study, only the sequence at the mutation area of interest was interrogated relative to baseline and controls. Mutations were called only if they were peak-under-peak centered. The 2% mutant control was not tested in the original cohort.

[a] The VAF was determined by visually comparing the peak height of the mutant peak to the estimated total peak height of the wild-type peak and mutant peaks.

[b] The VAF was determined by using a pixelated ruler to determine the actual peak height of the mutant peak to the calculated total peak height of the wild-type and mutant peaks.