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A quantitative pyrosequencing-based test to detect *MPL* mutations

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ABSTRACT

Introduction Mutations in exon 10 of the myeloproliferative leukemia virus oncogene (*MPL*) are present in approximately 5% of patients with primary myelofibrosis (PMF) and 1% of patients with essential thrombocythemia (ET). *MPL* encodes a transmembrane receptor protein tyrosine kinase which acts as a receptor for thrombopoietin (TPO). The most common *MPL* mutations are the W515K and W515L mutations, which lead to augmented or constitutive activation of the JAK/STAT signaling pathway in the absence of TPO and contribute to the neoplastic phenotype. *MPL*-mutated patients typically demonstrate no evidence of *JAK2* mutations. Thus, *MPL* mutation testing represents an important component of the workup of patients suspected of having a myeloproliferative neoplasm. Here we describe a fast and easy quantitative pyrosequencing-based assay designed to detect *MPL* W515K and W515L mutations. **Materials and Methods** Wild-type, W515K, and W515L plasmids were synthesized using standard amplification and site-directed mutagenesis reactions. One W515K, three W515L mutant patient specimens, and twenty Jak2-mutated specimens were subjected to granulocyte enrichment followed by PCR amplification of *MPL* in duplicate. Amplicons were subjected to Pyrosequencing on the Pyromark Q24 instrument using a nucleotide dispensation order designed to detect and quantitate *MPL* codon 515. Sensitivity and reproducibility experiments were carried out using diluted plasmid DNA. **Results** The wild-type, W515K, and W515L plasmid specimens were amplified successfully and quantitative pyrosequencing indicated purity of >98%. One W515K and three W515L patient specimens yielded results similar to those from another reference laboratory, within 2%. Twenty *JAK2*-mutated specimens were determined to be wild-type for *MPL* using our assay. Plasmid dilution experiments demonstrated sensitivity to 5% mutant in a background of wild-type and reproducibility experiments demonstrated that the 5% sensitivity was 100% reproducible. **Conclusions** We have developed and validated a quantitative pyrosequencing-based assay designed to detect *MPL* mutations in PMF and ET. Our assay is robust, specific, sensitive, and reproducible and provides an important diagnostic aid for those patients who test negative for *JAK2* mutations.

BACKGROUND

The Philadelphia chromosome-negative (Ph-) myeloproliferative disorders (MPDs) polycythemia vera (PV), essential thombocythemia (ET), and primary myelofibrosis (PMF) are characterized by clonal proliferation of one or more myeloid lineages[1]. The *Jak2* V617F mutation is found in the vast majority of patients with PV, in 35-70% with ET, and 50% with PMF[2].

Mutations in exon 10 of the myeloproliferative leukemia virus oncogene (*MPL*) are present in approximately 5% of patients with PMF and in 1% with ET[2]. *MPL* encodes a transmembrane receptor tyrosine kinase which acts as a receptor for thrombopoietin (TPO), a glycoprotein cytokine responsible for thrombopoiesis[3]. The most common *MPL* mutations are the W515K and W515L mutations, which have been shown to activate the *Jak/STAT* signaling pathway in the absence of TPO and contribute to the oncogenic phenotype[4]. The vast majority of patients with *MPL* mutations test negative for the *Jak2* V617F mutation yet possess a phenotype consistent with a myeloproliferative neoplasm[5]. Detection and quantitation of *MPL* mutations can be used in the diagnosis and monitoring of myeloproliferative neoplasms and suggests either PMF or ET.

Here we describe a quantitative pyrosequencing-based assay designed to detect the W515K and W515L MPL mutations.

MATERIALS AND METHODS

Wild-type *MPL* was cloned into pDONR vector to generate p5E-WT *MPL*. WT *MPL* plasmid was subjected to site-directed mutagenesis reactions to generate W515K and W515L mutants. Mutant plasmids were subsequently diluted into WT plasmid for sensitivity experiments.

One W515K, three W515L, and twenty *Jak2*-mutated specimens were subjected to granulocyte enrichment followed by isolation of genomic DNA. Samples were subjected to PCR amplification of *MPL* exon 10 using primers that span exon 10 (see Figure 1). Amplicons were purified, mixed with a pyrosequencing primer, and analyzed for a W515 mutation using a Pyromark Q24 pyrosequencer (Qiagen, Valencia, CA).

Prior to validation, specimens were de-identified following University of Utah IRB#7275.

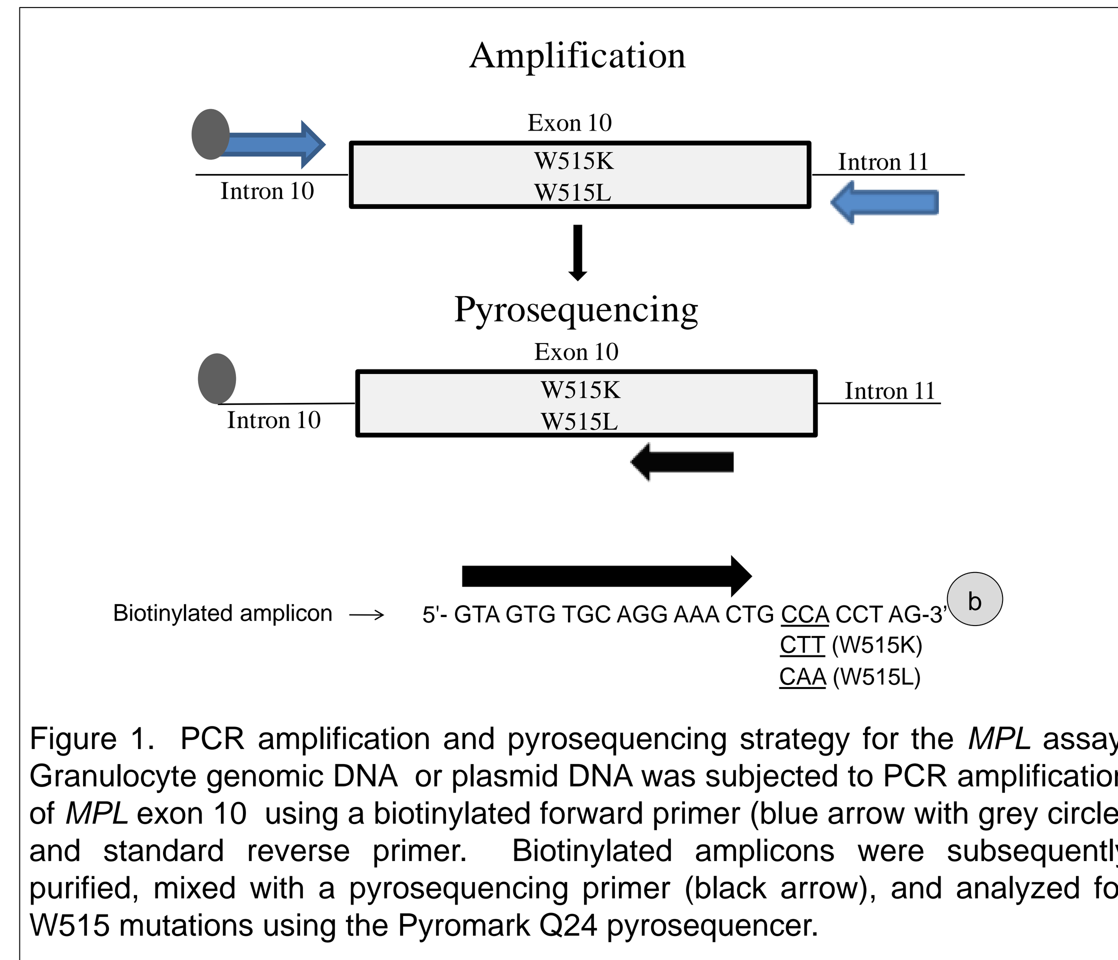

Figure 1. PCR amplification and pyrosequencing strategy for the *MPL* assay. Granulocyte genomic DNA or plasmid DNA was subjected to PCR amplification of *MPL* exon 10 using a biotinylated forward primer (blue arrow with grey circle) and standard reverse primer. Biotinylated amplicons were subsequently purified, mixed with a pyrosequencing primer (black arrow), and analyzed for W515 mutations using the Pyromark Q24 pyrosequencer.

Allele Quantification of T315I

Pyrosequencing reactions were loaded onto the Pyromark Q24 instrument (Qiagen) and subjected to a nucleotide dispensation order shown in Figure 2. Pyromark software calculated the peak heights for each incorporated nucleotide and reported % of C, T, and A nucleotides in codon 515 (see Figure 2, below).

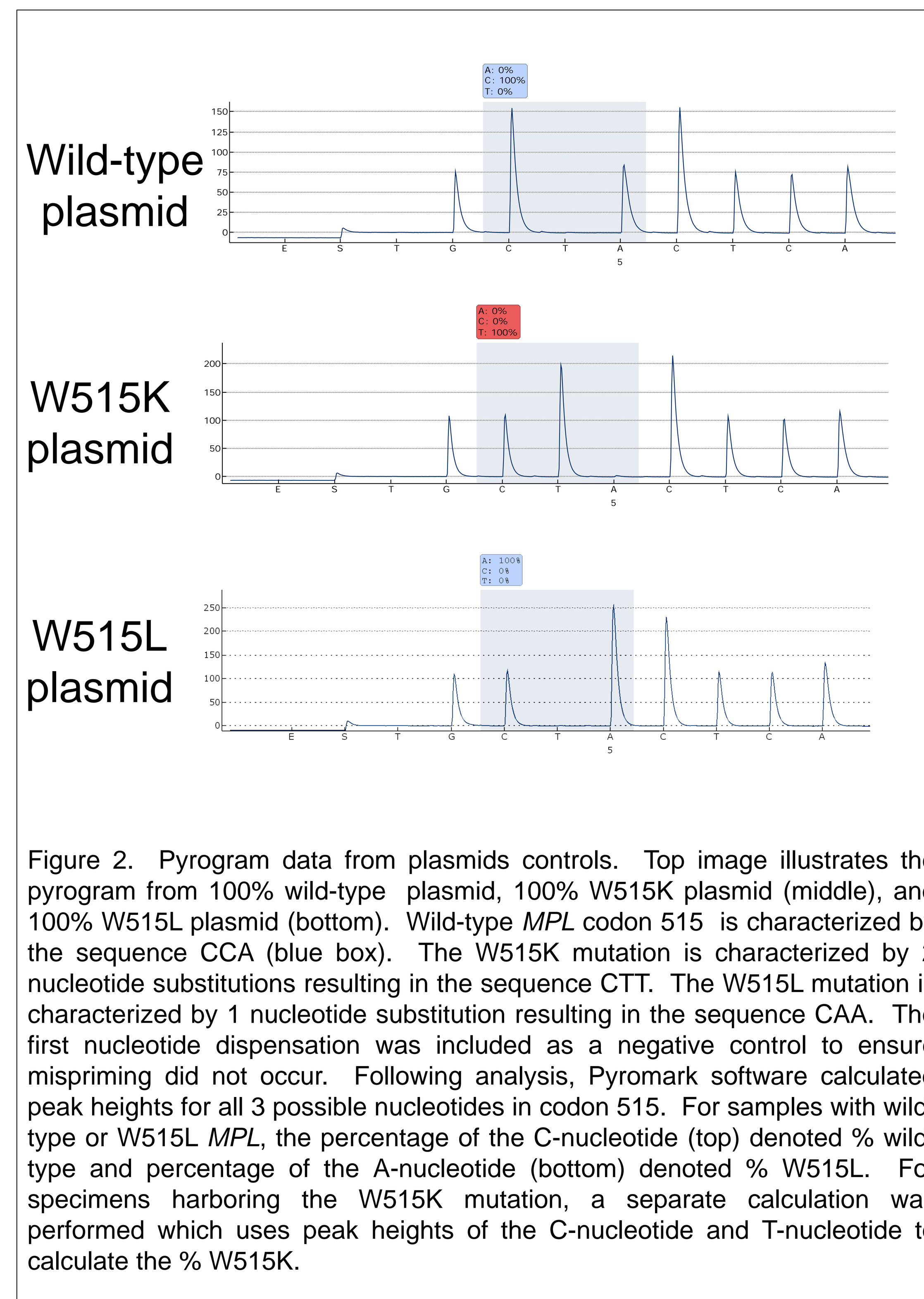

Figure 2. Pyrogram data from plasmids controls. Top image illustrates the pyrogram from 100% wild-type plasmid, 100% W515K plasmid (middle), and 100% W515L plasmid (bottom). Wild-type *MPL* codon 515 is characterized by the sequence CCA (blue box). The W515K mutation is characterized by 2 nucleotide substitutions resulting in the sequence CTT. The W515L mutation is characterized by 1 nucleotide substitution resulting in the sequence CAA. The first nucleotide dispensation was included as a negative control to ensure mispriming did not occur. Following analysis, Pyromark software calculated peak heights for all 3 possible nucleotides in codon 515. For samples with wild-type or W515L *MPL*, the percentage of the C-nucleotide (top) denoted % wild-type and percentage of the A-nucleotide (bottom) denoted % W515L. For specimens harboring the W515K mutation, a separate calculation was performed which uses peak heights of the C-nucleotide and T-nucleotide to calculate the % W515K.

RESULTS

Analysis of Quantitative MPL W515 Pyrosequencing Accuracy & Specificity

One W515K and three W515L patient specimens shown to harbor *MPL* mutations by pyrosequencing at M.D. Anderson Cancer Center were subjected to quantitative pyrosequencing analysis using our assay and we observed 100% concordance with M.D. Anderson results (data not shown).

We also analyzed 20 *Jak2*-mutated patient specimens and all 20 harbor wild-type *MPL* (data not shown).

Quantitative *MPL* W515 Pyrosequencing Sensitivity

Mutant plasmids (W515K or W515L) were diluted into wild-type *MPL* plasmid to 50%, 25% 10%, 5%, and 1% mutant. Each plasmid dilution was subjected to PCR amplification in duplicate and pyrosequencing analysis in singlet. Figure 3 (below) illustrates resulting pyrograms for W515K dilutions (left column) and W515L dilutions (right column). We achieved quantitation reproducibly in the 5% plasmid dilutions for W515K and W515L.

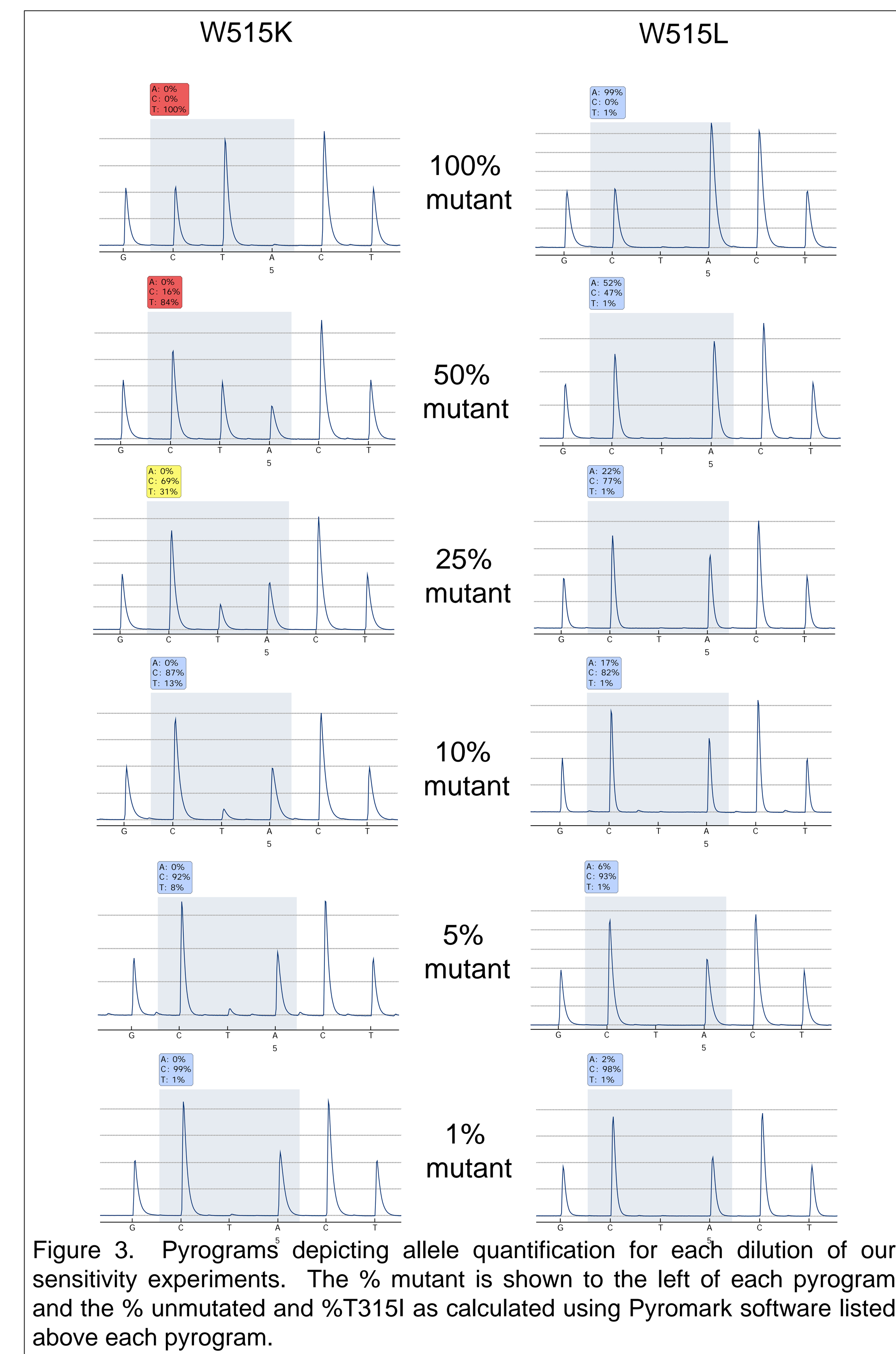

Figure 3. Pyrograms depicting allele quantification for each dilution of our sensitivity experiments. The % mutant is shown to the left of each pyrogram and the % unmutated and %T315I as calculated using Pyromark software listed above each pyrogram.

Quantitative *MPL* Pyrosequencing Reproducibility

We subjected the 5% W515K and W515L plasmid dilutions to PCR and pyrosequencing in triplicate on one day (within-run reproducibility) and in triplicate on three consecutive days (between-run reproducibility). Using the 5% W515K mutant plasmid dilution, we observed 5.8% mutant (between-run) and 5.4% mutant (within-run). Using the 5% W515L mutant plasmid dilution, we observed 6.0% mutant (between-run) and 6.4% mutant (within-run) (see Table 1).

Within-run reproducibility

	W515K % Wild-type	W515K % Mutant		W515L % Wild-type	W515L % Mutant
Replicate 1	94.6%	5.4%	Replicate 1	93.0%	7.0%
Replicate 2	94.4%	5.6%	Replicate 2	95.0%	5.0%
Replicate 3	93.7%	6.3%	Replicate 3	93.0%	6.0%
Mean	94.2%	5.8%	Mean	93.7%	6.0%
Std. dev.	0.47%	0.47%	Std. dev.	1.2%	1.0%

Between-run reproducibility

	W515K % Wild-type	W515K % Mutant		W515L % Wild-type	W515L % Mutant
Replicate 1	94.6%	5.4%	Replicate 1	94.0%	6.0%
Replicate 2	94.4%	5.6%	Replicate 2	95.0%	5.0%
Replicate 3	93.7%	6.3%	Replicate 3	93.0%	7.0%
Replicate 4	94.9%	5.1%	Replicate 4	93.0%	7.0%
Replicate 5	94.9%	5.1%	Replicate 5	93.0%	7.0%
Replicate 6	94.8%	5.2%	Replicate 6	93.0%	7.0%
Replicate 7	94.4%	5.6%	Replicate 7	95.0%	5.0%
Replicate 8	95.3%	4.7%	Replicate 8	93.0%	7.0%
Replicate 9	94.8%	5.2%	Replicate 9	93.0%	7.0%
Mean	94.6%	5.4%	Mean	93.6%	6.4%
Std. dev.	0.45%	0.45%	Std. dev.	0.88%	0.88%

Table 1 illustrates within-run (top) and between-run (bottom) reproducibility results of the *MPL* assay using a 5% W515K mutant plasmid dilution (left) and 5% W515L mutant plasmid dilution (right).

CONCLUSIONS

- We developed a quantitative pyrosequencing MPL assay designed to detect the W515K and W515L mutations found in a subset of *Jak2*-unmutated MPD patients.
- Our assay shows 100% specificity, accuracy, and reproducibility with sensitivity of 5% and is easily integrated into a clinical laboratory setting.

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