*The Journal of Molecular Diagnostics, Vol. 13, No. 5, September 2011*
*Copyright © 2011 American Society for Investigative Pathology and the Association for Molecular Pathology. Published by Elsevier Inc. All rights reserved.*
*DOI: 10.1016/j.jmoldx.2011.04.002*

# Sensitive Detection and Quantification of the *JAK2V617F* Allele by Real-Time PCR

## *Blocking Wild-Type Amplification by Using a Peptide Nucleic Acid Oligonucleotide*

Cornelis J.J. Huijsmans,* Jeroen Poodt,* Paul H.M. Savelkoul,[†] and Mirjam H.A. Hermans*

*From the Laboratory of Molecular Diagnostics,* Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands; and Medical Microbiology and Infection Control,[†] VU University Medical Center, Amsterdam, The Netherlands*

**A single G-to-T missense mutation in the gene for the JAK2 tyrosine kinase, leading to a V617F amino acid substitution, is commonly found in several myeloproliferative neoplasms. Reliable quantification of this mutant allele is of increasing clinical and therapeutic interest in predicting and diagnosing this group of neoplasms. Because *JAK2V617F* is somatically acquired and may be followed by loss of heterozygosity, the percentage of mutant versus wild-type DNA in blood can vary between 0% and almost 100%. Therefore, we developed a real-time PCR assay for detection and quantification of the low-to-high range of the *JAK2V617F* allele burden. To allow the assay to meet these criteria, amplification of the wild-type *JAK2* was blocked with a peptide nucleic acid oligonucleotide. *JAK2V617F* patient DNA diluted in *JAK2* wild-type DNA could be amplified linearly from 0.05% to 100%, with acceptable reproducibility of quantification. The sensitivity of the assay was 0.05% (*n* = 3 of 3). In 9 of 100 healthy blood donors, a weak positive/background signal was observed in DNA isolated from blood, corresponding to approximately 0.01% *JAK2V617F* allele. In one healthy individual, we observed this signal in duplicate. The clinical relevance of this finding is not clear. By inhibiting amplification of the wild-type allele, we developed a sensitive and linear real-time PCR assay to detect and quantify *JAK2V617F*. *(J Mol Diagn 2011, 13:558–564; DOI: 10.1016/j.jmoldx.2011.04.002)***

In 2005, the involvement of the *JAK2V617F* somatic point mutation in myeloproliferative neoplasms (MPNs) became clear. *JAK2V617F* is caused by a G-to-T transversion in the Janus kinase 2 *(JAK2)* gene resulting in a valine-to-phenylalanine amino acid substitution at codon 617.[1–4] JAK2 is a nonreceptor tyrosine kinase involved in the JAK-STAT signaling pathway.[5] The autoinhibitory pseudokinase JH2 domain is thought to be by the mutation altered in such a way that the JAK-STAT signaling pathway is constitutively activated, resulting in growth factor–independent cell proliferation.[6]

Since the discovery of *JAK2V617F*, other mutations in *JAK2* and other genes, such as Exon 12, *MPL*, and *TET2,* have been described. These mutations occur in a relatively small percentage of patients with MPN.[7–10] *JAK2V617F*, in contrast, is found in many patients: 65% to 97% of polycythemia vera (PV) cases, 23% to 57% of essential thrombocythemia cases, and 35% to 57% of chronic idiopathic myelofibrosis cases.[11,12] The diagnostic value of *JAK2* mutational analysis in MPN is now well established and endorsed in the classification of hematologic malignancies by the World Health Organization.[13]

Patients with PV often carry a *JAK2V617F* homozygous burden, in contrast to those with essential thrombocythemia, in whom homozygosity is rare.[14] This finding was also recapitulated in a large multicenter study: PV (*n* = 323: 67.8% heterozygous and 32.2% homozygous) and essential thrombocythemia (*n* = 639: 40.2% wild-type, 57.6% heterozygous, and 2.2% homozygous).[15] It is, therefore, more common for patients with PV to have a mutant allele burden >50%. Although the clinical value of the *JAK2V617F* allele burden is not yet fully understood, a correlation between disease phenotype and the proportion of mutant alleles has been postulated.[15,16] Splenomegaly, vascular events and pruritus in PV and splenomegaly, arterial thrombosis, and microvessel disease in essential thrombocythemia have been clinically correlated to mutant allele burdens.[17,18] Patients in the lowest quartile of mutant allele burden in idiopathic myelofibrosis had, overall, significantly shortened overall and leukemia-free survival compared with those with higher allele burdens and those negative for *JAK2V617F*.[19] In

Accepted for publication April 12, 2011.

Address reprint requests to Cornelis J.J. Huijsmans, MASc, Laboratory of Molecular Diagnostics, Jeroen Bosch Hospital, PO Box 90153, Nieuwstraat 34, 5211 NL, 's-Hertogenbosch, The Netherlands. E-mail: r.huysmans@jbz.nl.

**Table 1.** Overview and Some Characteristics of Currently Available *JAK2V617F* Detection (and Quantification) Techniques

| Technique | Sensitivity (%) | Advantages | Disadvantages | References |
|---|---|---|---|---|
| PCR-RFLP | 1–20 | No special equipment required, inexpensive | Risk of false positives due to incomplete digestion, labor intensive, post-PCR processing required | 4, 6, 21–26 |
| Direct sequencing | 5–20 | Simultaneous detection of other mutations | Labor intensive, sequencing equipment required, post-PCR processing required | 4, 6, 22, 23, 25, 27, 28 |
| Pyrosequencing | 2–10 | Simultaneous (real-time) detection of other mutations | Pyrosequencing equipment required, expensive | 6, 28, 29 |
| AS-PCR/ARMS | 0.01–5 | Highly sensitive | Post-PCR processing or real-time cycler required | 6, 21–25, 28, 30–33 |
| Real-time PCR | 0.01–5 | Highly sensitive, high-throughput, fast | Real-time cycler required | 6, 21, 24, 26, 27, 33–41 |
| HRM | 0.5–10 | Detection of other mutations | Real-time cycler required | 6, 21, 25, 27, 38, 42–45 |
| Allelic discrimination | 0.1 | Variable amplicon detection techniques possible | Real-time cycler required | 46, 47 |
| dHPLC | 1–2.5 | High-throughput, fast | Post-PCR processing required, technically challenging | 6, 23, 48 |
| PCR-MALDI-TOF | 0.01–1 | Highly sensitive, high-throughput, fast | MALDI-TOF equipment required, post-PCR processing required | 49, 50 |
| SSP-SMFD | 5 | High-throughput | Automated fluorescence cell sorter required, expensive | 50 |

AS-PCR/ARMS, allele-specific PCR/amplification refractory mutation system; dHPLC, denaturing high-pressure liquid chromatography; HRM, high-resolution melting curve analysis; PCR-MALDI-TOF, PCR -matrix-assisted laser desorption/ionization -time of flight; PCR-RFLP, PCR -restriction fragment length polymorphism; SSP-SMFD, sequence-specific primer–single molecule fluorescence detection.

addition, the amount of mutant allele burden in correlation to therapy may be of increasing interest in future diagnostic and predictive tests.[20]

With the discovery of *JAK2V617F*, the detection of this mutation became routine procedure in many laboratories. Since 2005, many assays to detect, and sometimes quantify, *JAK2V617F* have been described, evaluated, and compared.[4,6,21–51] Today, numerous techniques are used: PCR–restriction fragment length polymorphism (RFLP), direct sequencing, pyrosequencing, allele-specific PCR/amplification refractory mutation system, allelic discrimination, real-time PCR, high-resolution melting curve analysis, PCR, denaturing high-pressure liquid chromatography, PCR–matrix-assisted laser desorption/ionization–time of flight (PCR-MALDI-TOF) mass spectrometry, and sequence-specific primer–single molecule fluorescence detection. The techniques differ in a variety of characteristics, such as sensitivity (ranging from 0.01% to 20%, mutant allele diluted in wild-type allele), the potential for high-throughput diagnostics, and equipment required. An overview of currently available assays is presented in Table 1.

To increase the sensitivity of the *JAK2V617F* diagnostics, several techniques have been used in our laboratory during the past years. We started with an RFLP assay and subsequently used a semiquantitative real-time PCR and allele-specific minor groove–binding probes.[41] We then developed a quantitative real-time PCR using a locked nucleic acid (LNA) oligonucleotide to block amplification of the *JAK2* wild-type allele. We report herein replacement of the LNA moiety by peptide nucleic acid (PNA) for better blocking.

## Materials and Methods

### JAK2V617F-*Positive Blood Sample*

*JAK2V617F*-positive blood containing 97% mutant allele, estimated by RFLP, the absence of a wild-type peak in the sequence chromatogram, the presence of minimal wild-type signal in real-time PCR, and a hematologic analysis showing 3% lymphoid cells and 97% myeloid cells together with a high white blood cell count ($23.2 \times 10^9$/L)[41] was derived from a patient with PV and generated a mean ± SD $C_T$ value of 24.13 ± 0.02. A 100-fold dilution from DNA from this sample in *JAK2* wild-type DNA was used as a 1% *JAK2V617F*-containing positive control.

A second positive blood sample, used for serial dilutions in this study, was obtained from a 64-year-old man diagnosed as having possible PV, with a high number of erythrocytes [$7.64 \times 10^{12}$/L (reference range, 4.4 to $5.8 \times 10^{12}$/L)] and iron deficiency. *JAK2V617F* real-time PCR using the PNA oligonucleotide on genomic DNA derived from this sample generated a mean ± SD $C_T$ value of 23.88 ± 0.07. Thus, the sample used in this study was considered to contain $>$97% *JAK2V617F*.

### *Cohort Healthy Donors*

EDTA blood samples from 100 healthy blood donors—rendered anonymous—were used as a negative cohort. All the individuals were $<$30 years of age.

**Table 2.** Sequences of Primers, Detection Probes, and PNA and LNA Blocking Oligonucleotides to Detect and Quantify *JAK2V617F* and to Detect Albumin by Real-Time PCR

| *JAK2 V617F*: primers and mutant probe[41] | Sequences |
|---|---|
| Forward primer | 5′-AAGCTTTCTCACAAGCATTTGGTTT-3′ |
| Reverse primer | 5′-AGAAAGGCATTAGAAAGCCTGTAGTT-3′ |
| Mutant probe | 5′-FAM-TCCACAGAAACATAC-MGB-BHQ-3′ |
| LNA wild-type blocking probe | 5′-ACAGACACATA-3′ |
| PNA wild-type blocking probe | 5′-Acetyl-CTCCACAGACACATAC-3′ |
| *Albumin*: primers and probe [53] | Sequences |
| Forward primer | 5′-TGAAACATACGTTCCCAAAGAGTTT-3′ |
| Reverse primer | 5′-CTCTCCTTCTCAGAAAGTGTGCATAT-3′ |
| Probe | 5′-FAM-TGCTGAAACATTCACCTTCCATGCAGA-TAMRA-3′ |

BHQ, black hole quencher; MGB, minor groove binder.

### Genomic DNA Isolation from Blood

Genomic DNA was isolated from 200 μL of blood that had been anticoagulated with EDTA using the Qiagen blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in DNase- and RNase-free water (Fisher Emergo B.V., Landsmeer, The Netherlands), quantified by photo spectrometer (Eppendorf BioPhotometer; Eppendorf, Hamburg, Germany), and diluted to 10 ng/μL in DNase- and RNase-free TE (10 mmol/L Tris-HCl, pH 8; 0.1 mmol/L EDTA).

### JAK2V617F *Real-Time PCR Assay with PNA and LNA Blocking*

Primers and probes were designed by Applied Biosystems (Foster City, CA) using TaqExpress software as previously described.[41] The wild-type probe was altered to function as a blocking oligonucleotide with PNA or LNA. Oligonucleotide sequences are listed in Table 2. The PCR (25 μL) contained 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 3 mmol/L $MgCl_2$ [prepared from 10× PCR buffer and 50 mmol/L $MgCl_2$ solution delivered with Platinum Taq polymerase (Invitrogen, Breda, The Netherlands)], 0.75 U of Platinum Taq polymerase, 4% glycerol (molecular biology grade; CalBiochem, VWR International, Amsterdam, The Netherlands), 200 μmol/L of each deoxyribonucleotide triphosphate (Invitrogen), 0.5 μL of ROX reference dye (Invitrogen), 900 nmol/L primers and 200 nmol/L probes (Applied Biosystems), 1 μmol/L PNA oligonucleotide (PanaGene, Daejeon, Korea; alternate source: Biosynthesis Inc., Lewisville, TX) or LNA oligonucleotide (Sigma-Proligo, The Woodlands, TX), and 50 ng of target DNA. The ABI Prism 7500 Fast sequence detection system (Applied Biosystems) was used for amplification and detection (10 minutes at 95°C, 45 cycles of 3 seconds at 95°C, and 30 seconds at 60°C and an infinite holding step at 25°C in Fast 7500 mode). The threshold was set at 0.008, and baseline was set from cycles 6 to 15 for both reporters. $C_T$ values were calculated using SDS version 1.3.1 software. A real-time PCR targeting the human albumin gene was used to test whether DNA extraction and dilution to 10 ng/μL were successful and to verify that the PCR was not inhibited. Amplification was performed as described previously herein with 900 nmol/L both primers and 200 nmol/L probe. Oligonucleotide sequences were described earlier and are denoted in Table 2.[52]

### Sensitivity and Linearity Testing of PNA versus LNA Blocking

To compare PNA and LNA blocking oligonucleotides, a *JAK2V617F* real-time PCR was performed on twofold (v/v) serial dilutions of genomic DNA from the patient sample containing >97% *JAK2V617F*. *JAK2V617F* DNA (10 ng/μL) was diluted in genomic DNA (10 ng/μL) of a *JAK2* wild-type individual. The lowest *JAK2V617F* concentration tested was 0.006% v/v. All the dilutions were tested in triplicate.

## Results

### Sensitivity and Linearity Testing of PNA versus LNA Blocking

LNA nucleotides are generally used as blocking oligonucleotides.[26,39,53] Because our data using the LNA oligonucleotide showed suboptimal sensitivity of 0.2%, we introduced a PNA blocker approach. To determine the sensitivity and linearity of the real-time PCR test using the PNA oligonucleotide versus the LNA oligonucleotide, twofold (v/v) serial dilutions of genomic DNA from a patient with PV containing 0.006% to >97% *JAK2V617F* were tested. The assay combined with the PNA oligonucleotide linearly detected *JAK2V617F* with a for quantification acceptable (mean $C_T$ value ± 1 $C_T$) reproducibility ranging to be approximately 97% to 0.05% ($n$ = 3 of 3). This range was determined for the real-time PCR with the LNA oligonucleotide to be 97% to 0.2% ($n$ = 3 of 3; Figure 1).

### Cohort Healthy Donors

One hundred healthy EDTA blood donors were tested in duplicate using the real-time PCR and the PNA blocking oligonucleotide. All individuals tested positive for the albumin housekeeping gene ($C_T$: mean ± SD, 23.16 ± 0.19; range, 22.60 to 23.88). All DNA isolations were considered successful with this value, and none of the

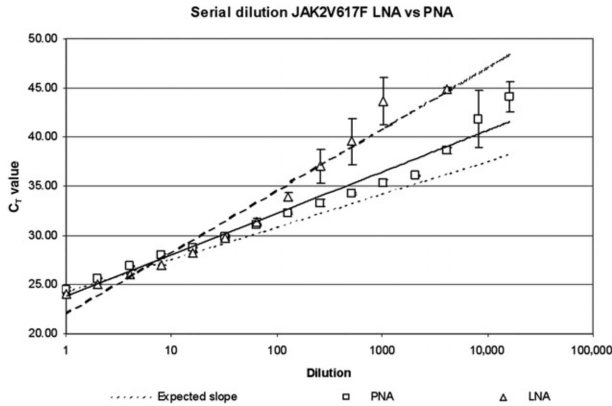


**Figure 1.** Standard curves (using twofold dilution steps) generated by *JAK2V617F* real-time PCR using PNA blocking (**squares**) and LNA blocking (**triangles**). Data are given as mean Ct ± SD.

samples showed PCR inhibition. Ninety-one patients (91%) were *JAK2V617F* negative. In nine patients (9%), a background signal corresponding to ±0.01% mutant allele was observed in one or both duplicates (eight patients and one patient, respectively). Two representative amplification plots are depicted in Figure 2. The results are summarized in Table 3.

## *Discussion*

We developed a highly sensitive *JAK2V617F* detection and quantification method by inhibiting amplification of the *JAK2* wild-type allele by means of a PNA wild-type blocking oligonucleotide. All $C_T$ values generated during sensitivity and linearity testing that were higher

**Table 3.** *JAK2V617F* Test Results from a Negative Cohort of 100 Healthy Individuals

| Result | *n* | Description |
|---|---|---|
| Total results | 200 | 100 healthy individuals tested in duplicate |
| Negative results | 190 | Negative for *JAK2V617F*/positive for albumin |
| Background $C_T \gtrsim 37.9$ | 10 | Mean background signal ± 3 SD (99.7% CI); eight patients single background signal; one patient in duplicate background signal |

The samples were amplified in duplicate ($n = 2 \times 100$). Signals were considered background when $C_T \gtrsim 37.9$.

than the cutoff $C_T$ value of 37.9 [determined with the cohort of healthy donors = mean $C_T$ value ±3 SD (99.7% CI)] were excluded. Compared with LNA blocking, PNA blocking resulted in a more sensitive and linear assay using the dilution series of *JAK2V617F* DNA in *JAK2* wild-type DNA: 0.2% to ±97% *JAK2V617F* DNA versus 0.05% to ±97% *JAK2V617F* DNA (Figure 1). More efficient/stable blocking by the PNA oligonucleotide probably causes the difference in sensitivity and linearity of the real-time PCR using the PNA blocker versus the LNA blocker. Unlike DNA or DNA analogues such as LNA, PNAs do not contain any phosphate groups, resulting in a noncharged backbone. As a result, PNA-DNA complexes lack charge repulsion and are, therefore, more stable than are DNA-DNA and DNA-LNA complexes.[54,55]

Although the clinical significance of a low *JAK2V617F* allelic burden in newly diagnosed patients is not

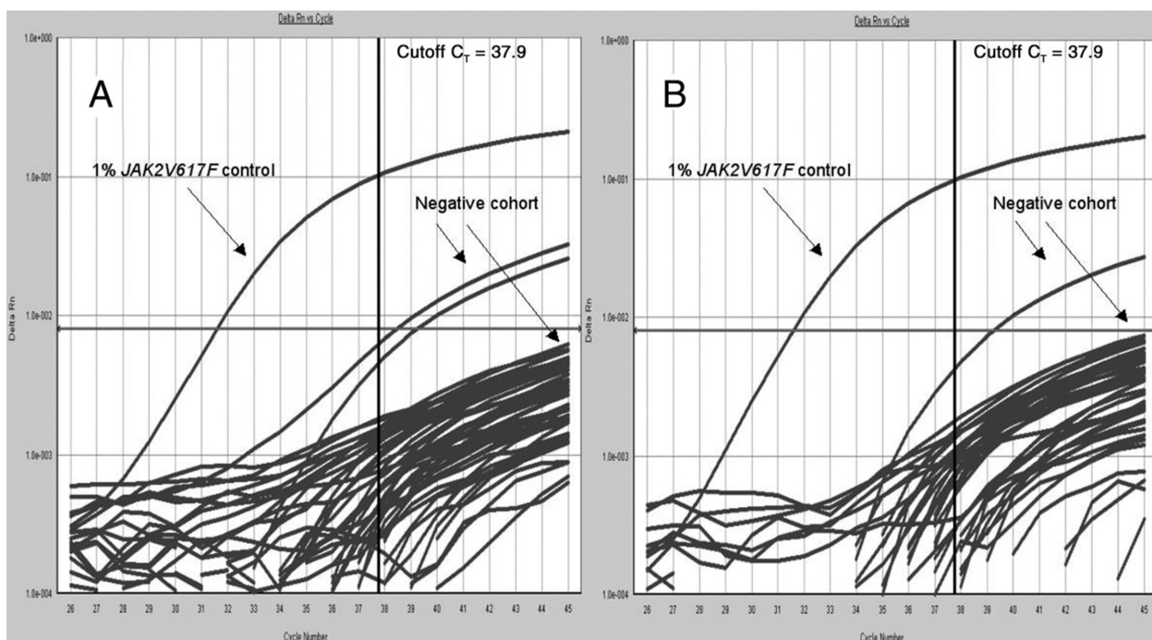


**Figure 2.** Amplification plots of *JAK2V617F* real-time PCR of 25 (**A**) and 22 (**B**) healthy individuals in duplicate (*JAK2V617F* negative cohort) and one 1% *JAK2V617F* positive control. The amplification plots shown are representative of the entire cohort. Gray horizontal line (at Delta Rn 0.8e-002) indicates threshold; black vertical line, cutoff $C_T$ value of 37.9.

clear,[38,40,52] highly sensitive mutant allele detection is currently used for minimal residual disease detection after allogeneic bone marrow transplantation.[56] In addition, quantification of *JAK2V617F* will help in monitoring new therapeutic strategies. A prerequisite for reliable quantification is the linearity of the assays from high to low allele burdens.[57]

In the cohort of 100 healthy individuals (Table 3), 9% generated *JAK2V617F* background signals (Figure 2). A weak positive/background *JAK2V617F* signal was observed in duplicate in one healthy individual. First, these background signals could be due to a technical issue, such as incomplete blocking of wild-type amplification. Second, *JAK2V617F* could be present in these patients in an extremely small number of circulating cells. Although this phenomenon has been described for other *JAK2V617F* assays, its significance is not clear.[38,40,52] To prevent false positives in the clinical setting, a cutoff $C_T$ value was determined. The mean $C_T$ value ($C_T$ values >40 are generally considered unreliable and were, therefore, excluded) ± 3 SD of the negative cohort was determined, and signals with a generated value of $C_T \geq 37.9$ (CI = 99.7%) were considered to be background. One patient generated duplicate *JAK2V617F* background signals (Table 3). Although both $C_T$ values were ≥37.9 and should be considered background, further analysis of this material may be of interest. Cloning and subsequent sequencing of these amplicons could be of value to determine whether the signals were nonspecific or that *JAKV617F* was, in fact, present. In addition, an assessment of the presence of erythropoietin-independent erythroid colonies might be useful in this and similar cases.[58] In addition, such a patient could undergo testing for the presence of the *JAK2V617F* predisposing haplotype.[59–61] In the routine diagnostic setting, a *JAK2V617F* allele burden of 0.1% to 1.0% is considered weak positive, and a remark stating that the clinical relevance of this finding is unclear is added to the test report. In addition, we have requested a follow-up sample after 6 months.

Although sequencing is the gold standard for mutation screening, it is less suitable for the detection of *JAK2V617F* because the mutation can be present at low levels relative to the wild-type sequence. PCR-RFLP, pyro sequencing, sequence-specific primer–single molecule fluorescence detection, high-resolution melting curve analysis, and denaturing high-pressure liquid chromatography are all less sensitive than is the real-time PCR described herein (Table 1), which has a sensitivity of 0.05% and, thereby, is comparable with techniques such as allele-specific PCR/amplification refractory mutation system, allelic discrimination, and PCR-MALDI-TOF (Table 1).[4,21–51] In addition, interpretation of PCR-RFLP results is complicated by the risk of incomplete digestion, resulting in *JAK2V617F* false positives (Table 1).

Besides the higher or equal sensitivity, the real-time PCR using the PNA oligonucleotide has some additional advantages compared with other assays: i) it is fast (<2 hours for DNA extraction and real-time PCR); ii) it eliminates the need of post-PCR processing, a key step in techniques such as allele-specific PCR/amplification refractory mutation system, sequencing, PCR-RFLP, PCR, and PCR-MALDI-TOF, reducing the risk of contamination; and iii) it has a reduced hands-on time of approximately 30 minutes.

In 2006, Sidon et al[39,40] described a similar real-time PCR as the assay described in this study using an LNA blocking oligonucleotide and claimed a sensitivity of their assay of 0.01% *JAK2V617F* diluted in wild-type allele. The sensitivity of this test, however, was determined using a HEL cell line, which is known to have more than two mutant alleles per cell, instead of patient DNA.[29]

Prospective evaluation of the real-time PCR with PNA oligonucleotide in a diagnostic setting for 1 year now included the analysis of 293 patients suspected of having MPN. Fourteen patients (4.8%) showed a *JAK2V617F* allele burden >50%, 16 (5.5%) between 1% and 50%, and 2 (0.7%) between 0.1% and 1%. The results generated during the performance of routine *JAK2* diagnostics indicate a robust and rapid test with excellent reproducibility: the overall CV with different batches of controls, reagents, and various real-time PCR cyclers was 1.5% for the 1% control ($n = 27$) and 1.4% for the 50% control ($n = 24$). Reproducibility is essential for a reliable quantitative assay[57]; therefore, these observations further confirm that the developed assay is well suited for the application.

In summary, blocking of wild-type *JAK2* with the PNA oligonucleotide resulted in a more sensitive real-time PCR assay to detect and quantify *JAK2V617F* with a longer range of linearity compared with the LNA oligonucleotide approach. Nine percent of healthy individuals in this negative cohort generated background *JAK2V617F* signals. Therefore, a cutoff $C_T$ value was introduced to prevent false-positive test results in a clinical setting.

## Acknowledgments

We thank Kathelijn Geraats-Peters for critical reading of the manuscript and Colin Ingham for valuable comments.

## References

1. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC: A gain-of-function mutation of *JAK2* in myeloproliferative disorders. N Engl J Med 2005, 352:1779–1790
2. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau1 F, Lacout C, Garçon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval J, Constantinescu SN, Casadevall N, Vainchenker W: A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005, 434:1144–1148
3. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Fröhling S, Döhner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG: Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005, 7:387–397
4. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR: Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005, 365:1054–1061

5. Schindler C, Darnell JE Jr: Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 1995, 64: 621–651
6. Steensma DP: JAK2 V617F in myeloid disorders: molecular diagnostic techniques and their clinical utility: a paper from the 2005 William Beaumont Hospital Symposium on Molecular Pathology. J Mol Diagn 2006, 8:397–411
7. Tefferi A: Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia 2010, 24:1128–1138
8. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, Warren AJ, Gilliland DG, Lodish HF, Green AR: JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 2007, 356:459–468
9. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, Cuker A, Wernig G, Moore S, Galinsky I, DeAngelo DJ, Clark JJ, Lee SJ, Golub TR, Wadleigh M, Gilliland DG, Levine RL: MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med 2006, 3:e270
10. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL: TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 2009, 23:905–911
11. Nelson ME, Steensma DP: JAK2 V617F in myeloid disorders: what do we know now, and where are we headed? Leuk Lymphoma 2006, 47:177–194
12. Tefferi A, Gilliland DG: The JAK2V617F tyrosine kinase mutation in myeloproliferative disorders: status report and immediate implications for disease classification and diagnosis. Mayo Clin Proc 2005, 80:947–958
13. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009, 11:937–951
14. Scott LM, Scott MA, Campbell PJ, Green AR: Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera, but not essential thrombocythemia. Blood 2006, 108:2435–2437
15. Vannucchi AM, Antonioli E, Guglielmelli P, Rambaldi A, Barosi G, Marchioli R, Marfisi RM, Finazzi G, Guerini V, Fabris F, Randi ML, De Stefano V, Caberlon S, Tafuri A, Ruggeri M, Specchia G, Liso V, Rossi E, Pogliani E, Gugliotta L, Bosi A, Barbui T: Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. Blood 2007, 110:840–846
16. Passamonti F, Rumi E: Clinical relevance of JAK2 (V617F) mutant allele burden. Haematologica 2009, 94:7–10
17. Vannucchi AM, Antonioli E, Guglielmelli P, Longo G, Pancrazzi A, Ponziani V, Bogani C, Ferrini PR, Rambaldi A, Guerini V, Bosi A, Barbui T; MPD Research Consortium: Prospective identification of high-risk polycythemia vera patients based on JAK2(V617F) allele burden. Leukemia 2007, 21:1952–1959
18. Antonioli E, Guglielmelli P, Poli G, Bogani C, Pancrazzi A, Longo G, Ponziani V, Tozzi L, Pieri L, Santini V, Bosi A, Vannucchi AM; Myeloproliferative Disorders Research Consortium (MPD-RC): Influence of JAK2V617F allele burden on phenotype in essential thrombocythemia. Haematologica 2008, 93:41–48
19. Tefferi A, Lasho TL, Schwager SM, Strand JS, Elliott M, Mesa R, Li CY, Wadleigh M, Lee SJ, Gilliland DG: The clinical phenotype of wild-type, heterozygous, and homozygous JAK2V617F in polycythemia vera. Cancer 2006, 106:631–635
20. Tefferi A: Mutational analysis in BCR-ABL-negative classic myeloproliferative neoplasms: impact on prognosis and therapeutic choices. Leuk Lymphoma 2010, 51:576–582
21. Cankovic M, Whiteley L, Hawley RC, Zarbo RJ, Chitale D: Clinical performance of JAK2 V617F mutation detection assays in a molecular diagnostics laboratory: evaluation of screening and quantitation methods. Am J Clin Pathol 2009, 132:713–721
22. Frantz C, Sekora DM, Henley DC, Huang CK, Pan Q, Quigley NB, Gorman E, Hubbard RA, Mirza I: Comparative evaluation of three JAK2V617F mutation detection methods. Am J Clin Pathol 2007, 128:865–874
23. Kannim S, Thongnoppakhun W, Auewarakul CU: Two-round allele specific-polymerase chain reaction: a simple and highly sensitive method for JAK2V617F mutation detection. Clin Chim Acta 2009, 401:148–151
24. Kremer M, Horn T, Koch I, Dechow T, Gattenloehner S, Pfeiffer W, Quintanilla-Martínez L, Fend F: Quantitation of the JAK2V617F mutation in microdissected bone marrow trephines: equal mutational load in myeloid lineages and rare involvement of lymphoid cells. Am J Surg Pathol 2008, 32:928–935
25. Lay M, Mariappan R, Gotlib J, Dietz L, Sebastian S, Schrijver I, Zehnder JL: Detection of the JAK2 V617F mutation by LightCycler PCR and probe dissociation analysis. J Mol Diagn 2006, 8:330–334
26. Shammaa D, Bazarbachi A, Halas H, Greige L, Mahfouz R: JAK2 V617F mutation detection: laboratory comparison of two kits using RFLP and qPCR. Genet Test Mol Biomarkers 2010, 14:13–15
27. James C, Delhommeau F, Marzac C, Teyssandier I, Couédic JP, Giraudier S, Roy L, Saulnier P, Lacroix L, Maury S, Tulliez M, Vainchenker W, Ugo V, Casadevall N: Detection of JAK2 V617F as a first intention diagnostic test for erythrocytosis. Leukemia 2006, 20: 350–353
28. Lippert E, Girodon F, Hammond E, Jelinek J, Reading NS, Fehse B, Hanlon K, Hermans M, Richard C, Swierczek S, Ugo V, Carillo S, Harrivel V, Marzac C, Pietra D, Sobas M, Mounier M, Migeon M, Ellard S, Kröger N, Herrmann R, Prchal JT, Skoda RC, Hermouet S: Concordance of assays designed for the quantification of JAK2V617F: a multicenter study. Haematologica 2009, 94:38–45
29. Jelinek J, Oki Y, Gharibyan V, Bueso-Ramos C, Prchal JT, Verstovsek S, Beran M, Estey E, Kantarjian HM, Issa JP: JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia Blood 2005, 106:3370–3373
30. Chen Q, Lu P, Jones AV, Cross NC, Silver RT, Wang YL: Amplification refractory mutation system, a highly sensitive and simple polymerase chain reaction assay, for the detection of JAK2 V617F mutation in chronic myeloproliferative disorders. J Mol Diagn 2007, 9:272–276
31. Tan AY, Westerman DA, Dobrovic A: A simple, rapid, and sensitive method for the detection of the JAK2 V617F mutation. Am J Clin Pathol 2007, 127:977–981
32. Vannucchi AM, Pancrazzi A, Bogani C, Antonioli E, Guglielmelli P: A quantitative assay for JAK2(V617F) mutation in myeloproliferative disorders by ARMS-PCR and capillary electrophoresis. Leukemia 2006, 20:1055–1060
33. Wolstencroft EC, Hanlon K, Harries LW, Standen GR, Sternberg A, Ellard S: Development of a quantitative real-time polymerase chain reaction assay for the detection of the JAK2 V617F mutation. J Mol Diagn 2007, 9:42–46
34. Bousquet M, Le Guellec S, Quelen C, Rigal-Huguet F, Delsol G, Brousset P: Frequent detection of the JAK2 V617F mutation in bone marrow core biopsy specimens from chronic myeloproliferative disorders using the TaqMan polymerase chain reaction single nucleotide polymorphism genotyping assay: a retrospective study with pathologic correlations. Hum Pathol 2006, 37:1458–1464
35. Hammond E, Shaw K, Carnley B, P'ng S, James I, Herrmann R: Quantitative determination of JAK2 V617F by TaqMan: an absolute measure of averaged copies per cell that may be associated with the different types of myeloproliferative disorders. J Mol Diagn 2007, 9:242–248
36. Kröger N, Badbaran A, Holler E, Hahn J, Kobbe G, Bornhäuser M, Reiter A, Zabelina T, Zander AR, Fehse B: Monitoring of the JAK2-V617F mutation by highly sensitive quantitative real-time PCR after allogeneic stem cell transplantation in patients with myelofibrosis. Blood 2007, 109:1316–1321
37. Merker JD, Jones CD, Oh ST, Schrijver I, Gotlib J, Zehnder JL: Design and evaluation of a real-time PCR assay for quantification of JAK2 V617F and wild-type JAK2 transcript levels in the clinical laboratory. J Mol Diagn 2010, 12:58–64
38. Rapado I, Albizua E, Ayala R, Hernández JA, Garcia-Alonso L, Grande S, Gallardo M, Gilsanz F, Martinez-Lopez J: Validity test study of JAK2 V617F and allele burden quantification in the diagnosis of myeloproliferative diseases. Ann Hematol 2008, 87:741–749
39. Sidon P, Heimann P, Lambert F, Dessars B, Robin V, El Housni H: Combined locked nucleic acid and molecular beacon technologies for sensitive detection of the JAK2V617F somatic single-base sequence variant. Clin Chem 2006, 52:1436–1438

40. Sidon P, El Housni H, Dessars B, Heimann P: The JAK2V617F mutation is detectable at very low level in peripheral blood of healthy donors. Leukemia 2006, 20:1622
41. Poodt J, Fijnheer R, Walsh IB, Hermans MH: A sensitive and reliable semi-quantitative real-time PCR assay to detect JAK2 V617F in blood. Hematol Oncol 2006, 24:227–233
42. Er TK, Lin SF, Chang JG, Hsieh LL, Lin SK, Wang LH, Lin CW, Chang CS, Liu TC: Detection of the JAK2 V617F missense mutation by high resolution melting analysis and its validation. Clin Chim Acta 2009, 408:39–44
43. Olsen RJ, Tang Z, Farkas DH, Bernard DW, Zu Y, Chang CC: Detection of the JAK2(V617F) mutation in myeloproliferative disorders by melting curve analysis using the LightCycler system. Arch Pathol Lab Med 2006, 130:997–1003
44. Sutton BC, Allen RA, Zhao ZJ, Dunn ST: Detection of the JAK2V617F mutation by asymmetric PCR and melt curve analysis. Cancer Biomark 2007, 3:315–324
45. Tanaka R, Kuroda J, Stevenson W, Ashihara E, Ishikawa T, Taki T, Kobayashi Y, Kamitsuji Y, Kawata E, Takeuchi M, Murotani Y, Yokota A, Hirai M, Majima S, Taniwaki M, Maekawa T, Kimura S: Fully automated and super-rapid system for the detection of JAK2V617F mutation. Leuk Res 2008, 32:1462–1467
46. Nussenzveig RH, Swierczek SI, Jelinek J, Gaikwad A, Liu E, Verstovsek S, Prchal JF, Prchal JT: Polycythemia vera is not initiated by JAK2V617F mutation. Exp Hematol 2007, 35:32–38
47. Passamonti F, Rumi E, Pietra D, Della Porta MG, Boveri E, Pascutto C, Vanelli L, Arcaini L, Burcheri S, Malcovati L, Lazzarino M, Cazzola M: Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood 2006, 107:3676–3682
48. Sattler M, Walz C, Crowley BJ, Lengfelder E, Jänne PA, Rogers AM, Kuang Y, Distel RJ, Reiter A, Griffin JD: A sensitive high-throughput method to detect activating mutations of Jak2 in peripheral-blood samples. Blood 2006, 107:1237–1238
49. Fu JF, Shi JY, Zhao WL, Li G, Pan Q, Li JM, Hu J, Shen ZX, Jin J, Chen FY, Chen SJ: MassARRAY assay: a more accurate method for JAK2V617F mutation detection in Chinese patients with myeloproliferative disorders. Leukemia 2008, 22:660–663
50. Koren-Michowitz M, Shimoni A, Vivante A, Trakhtenbrot L, Rechavi G, Amariglio N, Loewenthal R, Nagler A, Cohen Y: A new MALDI-TOF-based assay for monitoring JAK2 V617F mutation level in patients undergoing allogeneic stem cell transplantation (allo SCT) for classic myeloproliferative disorders (MPD). Leuk Res 2008, 32:421–427
51. Ohyashiki K, Hori K, Makino T, Ohyashiki JH: Automated JAK2V617F quantification using a magnetic filtration system and sequence-specific primer-single molecule fluorescence detection. Cancer Genet Cytogenet 2007, 179:19–24
52. Xu X, Zhang Q, Luo J, Xing S, Li Q, Krantz SB, Fu X, Zhao ZJ: JAK2(V617F): Prevalence in a large Chinese hospital population. Blood 2007, 109:339–342
53. Rijnders RJ, Christiaens GC, Bossers B, van der Smagt JJ, van der Schoot CE, de Haas M: Clinical applications of cell-free fetal DNA from maternal plasma. Obstet Gynecol 2004, 103:157–164
54. Denys B, El Housni H, Nollet F, Verhasselt B, Philippé J: A real-time polymerase chain reaction assay for rapid, sensitive, and specific quantification of the JAK2V617F mutation using a locked nucleic acid-modified oligonucleotide. J Mol Diagn 2010, 12:512–519
55. Karkare S, Bhatnagar K: Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. Appl Microbiol Biotechnol 2006, 71:575–586
56. Steckel NK, Koldehoff M, Ditschkowski M, Beelen DW, Elmaagacli AH: Use of the activating gene mutation of the tyrosine kinase (VAL617Phe) JAK2 as a minimal residual disease marker in patients with myelofibrosis and myeloid metaplasia after allogeneic stem cell transplantation. Transplantation 2007, 15:1518–1520
57. Bernard PS, Wittwer CT: Real-time PCR technology for cancer diagnostics. Clin Chem 2002, 48:1178–1185
58. Prchal JF, Axelrad AA: Bone-marrow responses in polycythemia vera. N Engl J Med 1974, 290:1382
59. Olcaydu D, Harutyunyan A, Jäger R, Berg T, Gisslinger B, Pabinger I, Gisslinger H, Kralovics R: A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. Nat Genet 2009, 41: 450–454
60. Jones AV, Chase A, Silver RT, Oscier D, Zoi K, Wang YL, Cario H, Pahl HL, Collins A, Reiter A, Grand F, Cross NC: JAK2 haplotype is a major risk factor for the development of myeloproliferative neoplasms. Nat Genet 2009, 41:446–449
61. Kilpivaara O, Mukherjee S, Schram AM, Wadleigh M, Mullally A, Ebert BL, Bass A, Marubayashi S, Heguy A, Garcia-Manero G, Kantarjian H, Offit K, Stone RM, Gilliland DG, Klein RJ, Levine RL: A germline JAK2 SNP is associated with predisposition to the development of JAK2(V617F)-positive myeloproliferative neoplasms. Nat Genet 2009, 41:455–459