Detection of the *JAK2* V617F Mutation by LightCycler PCR and Probe Dissociation Analysis

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A point mutation in the *JAK2* gene, a member of the tyrosine kinase family, was recently identified and shown to be associated with several myeloproliferative disorders. Several studies identified the same *JAK2* point mutation (1849G>T), resulting in the substitution of a valine to phenylalanine at codon 617 (V617F). We developed a simple and sensitive method to detect this mutation via polymerase chain reaction and probe dissociation analysis using the LightCycler platform, and we compared this method to existing restriction fragment-length polymorphism, direct sequencing, and amplification refractory mutation system methods. We found that the LightCycler method offered advantages of speed, reliability, and more straightforward interpretation over the restriction fragment-length polymorphism and sequencing approaches. (*J Mol Diagn* 2006, 8:330–334; DOI: 10.2353/jmoldx.2006.050130)

Studies of patients with myeloproliferative disorders have identified the presence of an activating point mutation (V617F, 1849G>T) in the Janus kinase 2 (*JAK2*) gene in a majority of patients with polycythemia vera (65 to 97%) and in a significant subset of patients with essential thrombocythemia (23 to 57%) and chronic idiopathic myelofibrosis (35 to 57%).^{1–3} The considerable variation in prevalence between studies may be related to patient selection criteria and the sensitivity of the assays used. Because the *JAK2* V617F mutation was only recently characterized, additional studies are expected to allow a more accurate estimate of its frequency in various disorders.

The point mutation constitutively activates *JAK2* and confers a survival and/or proliferative advantage to cells.³ *JAK* proteins function as intermediates between membrane receptors and signaling molecules. For example, *JAK2* is associated with receptors that bind hematopoietic growth

factors, including erythropoietin, thrombopoietin, interleukin-3, and granulocyte-macrophage colony-stimulating factor. The V617F mutation is in the pseudokinase (JH2) domain of *JAK2*, which is a negative regulatory region, and may interfere with inactivation of the kinase.^{4,5}

Because identification of the V617F mutation will be of potential use in the diagnosis, prognosis, and perhaps selection of treatment for myeloproliferative disorders, a quick and reliable assay to identify the mutation is needed.^{6–10} The most commonly used assays to detect the *JAK2* mutation are a restriction fragment-length polymorphism (RFLP) analysis for a restriction endonuclease site that is lost with the mutation, amplification refractory mutation system (ARMS), and direct sequencing. However, some of these methods have limitations in sensitivity, and in the case of RFLP, incomplete digestion can result in false-positive test results.

We describe a simple and sensitive method for detecting the V617F mutation in the *JAK2* gene via the polymerase chain reaction (PCR) and probe dissociation (melting curve) analysis to determine presence of the mutation. Comparison with a PCR-restriction digest method and DNA sequencing revealed key advantages of speed, reliability, and ease of interpretation. The test is easily automated with the LightCycler or other real-time PCR platforms.

Materials and Methods

Samples and DNA Extraction

DNA was extracted from blood samples collected in ethylenediamine tetraacetic acid anticoagulant with the QiaAmp DNA Blood Mini kit according to manufacturer's directions (Qiagen, Valencia, CA). Samples were obtained from patients seen in the hematology clinic at Stanford University Medical Center, and consent was obtained using a Stanford institutional review board-approved protocol for hematology research investigations. The set included patients with a clinical diagnosis of polycythemia vera ($n = 9$), essential thrombocythemia ($n = 6$), chronic idiopathic myelofibrosis ($n = 8$), atypical (*BCR-ABL*-negative) chronic myelogenous leukemia ($n = 2$), idiopathic hypereosino-

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Table 1. LightCycler and ARMS Assay Primer and Probe Sequences

LightCycler Primers	JAK2-for	5'-ACAACAGTCAAACAACAATTC-3'
	JAK2-rev	5'-ACACCTAGCTGTGATCC-3'
Probes	JAK2-wt	5'-LCred640-CGTCTCCACAGACACATACTC-C3blocker-3'
	JAK2-an	5'-AAAGGCATTAGAAAGCCTGTAGTTTACTTACTCT-Fluo-3'
ARMS Outer primers	Forward	5'-TCCTCAGAACGTTGATGGCAG-3'
	Reverse	5'-ATTGCTTTCCTTTTTCACAAGAT-3'
Specific primers	Wild type	5'-GCATTGGTTTAAATTATGGAGTATaTG-3'
	Mutant	5'-GTTTACTTACTCTCGTCTCCACAaAA-3'

nophilic syndrome and chronic eosinophilia ($n = 7$), other reactive thrombocytosis ($n = 2$), and overlap myelodysplastic syndrome/myeloproliferative disorder ($n = 3$).

LightCycler Detection Method

Primers and probes were designed using GenBank sequence AL161450 and optimized using LightCycler Probe Design Software v1.0 (Idaho Technology, Salt Lake City, UT). The sequences are listed in Table 1. Analysis was performed in the LightCycler instrument (Roche, Indianapolis, IN). For the PCR reaction, 50 ng of genomic DNA was amplified with the LC FastStart DNA Master Hybridization Probe kit (Roche). Each 10- μ L reaction contained 1 $\times$ LightCycler-FastStart Reaction Mix Hybridization Probes, 1.5 mmol/L $MgCl_2$, 500 nmol/L each of forward and reverse primers, and 200 nmol/L of each hybridization probe. The amplification conditions consisted of one denaturation/activation cycle of 10 minutes at 95°C and 40 cycles of three-temperature amplification. Each cycle consisted of 95°C for 5 seconds, 55°C for 5 seconds, and 72°C for 10 seconds with a single fluorescent acquisition step at the 55°C hold. This was followed by a melting curve analysis of 95°C for 10 seconds, 45°C for 60 seconds, and a slow ramp (0.1°C/second) to 75°C with continuous fluorescent acquisition.

PCR-RFLP Detection Method

Analysis was performed using the *JAK2* Activating Mutation Assay for ABI Fluorescence Detection (InVivo-Scribe Technologies, San Diego, CA) according to the manufacturer's directions. Figure 1 illustrates the assay design. For PCR, 45 μ L of mastermix/AmpliTaq Gold (Applied Biosystems, Foster City, CA) was added to 100 ng of genomic DNA for a final volume of 50 μ L and cycled in an ABI 9700 with the following program: 95°C for 7 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 10 seconds; and 72°C for 10 minutes. After amplification, 25 μ L of the amplified reaction products are digested in a 30- μ L volume of 1 $\times$ NEB buffer 4 and 4 U of *Bsa*XI endonuclease (New England BioLabs, Newton, MA) at 37°C for at least 2 hours. Detection was performed by ABI Fluorescence Detection with ABI 3100 instrumentation.

ARMS Method

The allele-specific PCR testing for the *JAK2* V617F mutation was performed as described previously.⁵ This method uses two primer pairs to specifically amplify the normal and mutant sequences plus a positive control band in a single reaction. Outer primers flank the mutation region and create a 463-bp control product. A wild-type-specific forward primer pairs with the reverse control primer to form a 229-bp product; if *JAK2* V617F is present, a mutation-specific reverse primer forms a 279-bp product with the forward control primer. The discriminatory primers include mismatches, designated in lowercase in Table 1, to maximize discrimination of the two alleles. Products are resolved on a 2.5% agarose gel stained with ethidium bromide.

Sequencing Detection Method

A 50- μ L PCR reaction was performed on 50 ng of DNA in 1 $\times$ ABI BufferII, 1.5 mmol/L $MgCl_2$, 125 nmol/L of each dNTP, 0.8 μ mol/L each of forward and reverse primer (see Table 1 for sequence), and 5 U of AmpliTaq Gold. The amplification conditions on the ABI 9700 instrument consisted of one denaturation/activation cycle of 10 minutes at 95°C and 20 cycles of three-temperature touch-down amplification: 95°C for 40 seconds, 67 to 47°C for 30 seconds, 72°C for 30 seconds, an additional 18 cycles with the 47°C annealing temperature, and 72°C extension for 10 minutes. The amplicons were purified according to the manufacturer's directions using the Qiaquick PCR purification kit (Qiagen) and eluting in 50 μ L of AE Buffer. Five microliters of eluted amplicon was mixed with 1 μ L of 10 μ mol/L forward primer and 5 μ L of BigDye v.3 reaction ready mix and cycled according to standard ABI recommendations. Excess dye was precipitated with an equal volume of 2.2% sodium dodecyl sulfate, heated to 98°C for 5 minutes, and passed over a Centriprep column (Princeton Separations, Adelphia, NJ). After drying in a SpeedVac (Savant, Farmingdale, NY), samples were resuspended in formamide and sequenced on an ABI 3100 DNA Sequencer.

Results

In this study, we developed an assay to detect the V617F mutation in the *JAK2* gene using the LightCycler platform

Figure 1. Principle of PCR-RFLP assay for detection of the V617F mutation in *JAK2*. Samples are amplified with fluorescently labeled primers, followed by restriction digestion with *BsaXI* (5'...₉(N)AC(N)₅CTCC(N)₁₀... 3') and subsequent sizing by capillary electrophoresis with ABI fluorescence detection. A wild-type sample will have a green peak at 97 bp and blue peaks at 140 and 170 bp. The *JAK2* V617F mutation results in the loss of a restriction site and will appear as a blue/green peak at 267 bp.

and compared it with detection by direct DNA sequencing and a commercially available PCR-RFLP method. DNA samples from 37 patients seen in the hematology clinic for myeloproliferative disorders were tested for the *JAK2* V617F mutation by both the LightCycler and PCR-RFLP methods. Eight of the samples, three of which were positive, three negative, and two discrepant, were assayed by direct sequencing and sent to an outside laboratory for confirmatory ARMS testing.

Analytical Sensitivity

Currently, the relevance of zygosity status of *JAK2* V617F-associated diseases and clinical phenotype is being evaluated.⁷⁻⁹ The presence of a clonal population of somatically mutated cells (heterozygous or homozygous) in a background of wild-type cells makes it difficult to distinguish a "true" heterozygous state at the single-cell level. For this reason, none of the assays performed would be appropriate for reliably establishing true heterozygosity or homozygosity for the mutation.

Each assay method was evaluated for sensitivity by running a serial dilution panel of homozygous V617F TIB180 cell line DNA (Coriell Institute, Camden, NJ) in wild-type DNA at concentrations of 20, 10, 5, 3, and 1%. The LightCycler method had melting curves with robustly reproducible discernible differences (within-run and be-

tween runs) from negative (wild type) down to the 5% level (Figure 2). Our laboratory defined a melting peak between ~3 and 10% as a weak positive result, although the threshold for a weak positive with this assay may vary between laboratories. The PCR-RFLP and ARMS method had similar discernible differences between the negative and positive dilutions down to ~5%. In contrast, a positive result by sequencing was difficult to discern below the 20% level.

Test Performance

Testing by the LightCycler method yielded 21 positives and 16 negatives. The PCR-RFLP method yielded 17 positives, 19 negatives, and 1 indeterminate result. There was result agreement between 32 of the 37 samples. Of the discordant results, four samples were negative or indeterminate by the Invivoscribe (RFLP) method and positive at a weak level by the LightCycler method. As shown in Figure 2, populations as low as 5% can be easily detected by this method, whereas with the RFLP method, the same samples are more difficult to discern from negative. In fact, reinspection of the postdigestion electropherograms of the discordant samples did show a possible increase in the size of the small 267-bp peak that always remained undigested (for assay design, see Figure 1). There was one sample that was positive by

Figure 2. Results of a dilution series of homozygous positive cell line TIB180 diluted in wild-type DNA at 20% (1), 10% (2), 5% (3), 3% (4), and 1% (5). **A:** LightCycler assay in which the *JAK2* wild-type allele appears as a melting peak at 61°C and the V617F mutant allele appears as a melting peak at 54°C. **B:** ARMS assay in which the *JAK2* wild-type allele appears as a band at 279 bp and the V617F mutant allele at 229 bp. An amplification control band at 463 bp also amplifies.

RFLP and negative by the LightCycler method. Inspection of the RFLP electropherogram indicated resistance to digestion in the sample by an increase in size of the typically present 170-bp initial digestion fragment, consistent with a false-positive result due to incomplete digestion. Results of ARMS testing in an independent laboratory corroborated the LightCycler results. Using DNA sequencing, it was possible to detect three samples that were strongly positive by other techniques, but two samples that were deemed weakly positive appeared negative due to the reduced sensitivity of the sequencing method.

The performance and interpretation time for each assay starting from extracted gDNA varied widely from 1.75 hours for the LightCycler method to 7.25 hours for the sequencing method (Figure 3). Also, the LightCycler

method offers the advantage of a single-tube format, reducing the potential for sample manipulation errors.

Discussion

Comparison of four different assay techniques for detection of the *JAK2* V617F mutation revealed clear advantages of a LightCycler-based method over PCR-RFLP, direct DNA sequencing, and ARMS. Foremost is the ease of interpretation. With the sensitivity and probe dissociation curves of the LightCycler, it is easier to detect samples with low levels of *JAK2* V617F-positive cells (Figure 2) than it is with PCR-RFLP or sequencing. Sequencing was unable to detect levels below an approximately 20% clonal load. The PCR/RFLP assay was almost as sensitive

Figure 3. Time and motion comparison. **White bars** indicate hands-on time; **gray bars** indicate instrument time (not requiring technologist's labor). Inter., interpretation; Set dig., set-up of restriction digestion; Set seq. rxn, set-up of DNA sequencing reaction.

but had the possibility of incomplete digestion causing a false-positive result. Assay results often demonstrated a low level of undigested product remaining in the negative control. Because a weakly positive sample would also have a low level of undigested product, it is not possible in such instances to unequivocally discern the true negatives with that method alone. The ARMS and LightCycler-based methods allow for more straightforward interpretation of weakly positive samples. Those individuals with a low clonal copy number can be flagged as probable positives and retested further in the clinical course. Alternatively, the sample can be treated with techniques to enrich the clonal cell population in the DNA extraction step.

The LightCycler methodology allows for considerable time efficiency over the other three assays. The time savings occur in the removal of postamplification steps such as restriction digest, electrophoresis, amplicon clean-up, and cycle sequencing. The direct labor savings is at least 2.5 hours, with technologist hands-on-time of 0.75 hours, compared with approximately 2 hours for the other methods. The time savings can reduce turnaround times and free up laboratory instrumentation and personnel for other applications. Further labor savings can easily be realized by incorporating automation with the Magnapure/LightCycler or another robotic platform.

The new melting probe-based method incorporates amplification and detection in a single tube with no additional manipulations after the PCR set-up. This increases the reliability of the assay by reducing the possibility for sample mix-up and erroneous result reporting. In contrast, the other three assay methods have at least two postamplification steps where sample must be transferred between tubes and additional reagents added.

The ultimate role of the JAK2 V617F mutation in the clinical management of patients with myeloproliferative disorders remains to be fully established. The LightCycler-based assay that we have introduced, however, can be a valuable tool in the clinical molecular laboratory for quick and accurate identification of the subset of patients who carry this mutation. In addition, it ultimately may be useful for monitoring response to therapy.

References

1. 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
2. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villevall JL, Constantinescu SN, Casadevall N, Vainchenker W: A unique clonal JAK2 mutation leading to constitutive signalling causes polycythemia vera. *Nature* 2005, 434:1144–1148
3. 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
4. Levine RL, Loriaux M, Huntly BJ, Loh M, Beran M, Stoffregen E, Berger R, Clark JJ, Willis SG, Nguyen K, Flores N, Estey E, Gattermann N, Armstrong S, Look AT, Griffin JD, Bernard OA, Gilliland DG, Druker BJ, Deininger MW: The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood* 2005, 106:3377–3379
5. 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 Sothier, Mercher T, D'Andrea A, Frohling S, Dohner 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
6. Tefferi A, Lasho TL, Gilliland G: JAK2 mutations in myeloproliferative disorders. *N Engl J Med* 2005, 353:1416–1417
7. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase A, Grand FH, White H, Zoi C, Loukopoulou D, Terpos E, Vervessou E, Schultheis B, Emig M, Ernst T, Lengfelder E, Hehlmann R, Hochhaus A, Oscier D, Silver RT, Reiter A, Cross NCP: Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood* 2005, 106:2162–2168
8. Tefferi A, Sirhan S, Lasho TL, Schwager SM, Li CY, Dingli D, Wolanskyj AP, Steensma DP, Mesa R, Gilliland DG: Concomitant neutrophil JAK2 mutation screening and PRV-1 expression analysis in myeloproliferative disorders and secondary polycythemia. *Br J Haematol* 2005, 131:166–171
9. Wolanskyj AP, Lasho TL, Schwager SM, McClure RF, Wadleigh M, Lee SJ, Gilliland DG, Tefferi A: JAK2 mutation in essential thrombocythemia: clinical associations and long-term prognostic relevance. *Br J Haematol* 2005, 131:208–213
10. Tefferi A, Gilliland DG: The JAK2 (V617F) tyrosine kinase mutation in myeloproliferative disorders: status report and immediate implications for disease classification and diagnosis. *Mayo Clin Proc* 2005, 80:947–958