

light chain has undergone additional polymerization, a large change in conformation, or a combination of both (2, 3). The control Bence Jones protein had 2 molecular species that were consistent with monomer and dimer in the presence or absence of physiologic concentrations of salt.

Sedimentation equilibrium experiments were performed on the cryo κ -light chain protein that had been preequilibrated in the no-salt buffer and run at 6000 and 12 000 rpm at 20 °C (analysis could not be performed at 4 °C because of protein precipitation at higher concentrations). Sedimentation equilibrium centrifugation analysis with a self-association model revealed that the mean molecular mass of the cryo protein in the absence of salt at 20 °C was M_r 60 000. The sedimentation data were consistent with a mixture of 70% dimers and 30% tetramers.

Early studies investigating the mechanisms of temperature- and concentration-dependent cryoprecipitation have shown that low temperatures change the intramolecular environment of the protein, particularly with certain aromatic residues (4). It is possible that there are both salt- and temperature-dependent changes at 20 °C and that only the salt-dependent changes in polymerization are detected by either sedimentation equilibrium or by the apparent M_r measured by gel filtration at 4 °C. Low salt accurately reflects the presence of trimers and hexamers that would form a cryoprecipitate at the higher concentration of light chain protein found in undiluted urine stored at 4 °C. The sodium concentration in urine can vary considerably, and we did not ascertain the sodium in the urine of the cryo patient. It is noteworthy, however, that the control κ light chain did not show any salt-dependent changes in relative molecular mass, unlike the cryo protein.

It is likely that several physicochemical factors contributed to urine cryoglobulin formation in this patient, including the immunoglobulin light chain protein sequence, resulting from the inherent diversity in the variable regions of immunoglobu-

lins, and the concentration, because the protein had to be diluted substantially to prevent precipitation on the gel-filtration column at 4 °C. In addition, there was no evidence of clinical symptoms related to the urine cryo at physiologic temperature. The urine remained a clear liquid at room temperature and 37 °C and showed complete gel formation only at 4 °C. However, because we do not have data to confirm the role of these various factors, the extent to which these may have contributed to cryo formation in this patient remains speculative.

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Combined Locked Nucleic Acid and Molecular Beacon Technologies for Sensitive Detection of the *JAK2*^{V617F} Somatic Single-Base Sequence Variant

To the Editor:

We developed a quantitative and very sensitive method that combines molecular beacon (1) and locked nucleic acid (LNA) technologies (2) in a single sealed tube. We used the oncogenic somatic *JAK2*^{V617F} single-base sequence variant, observed in a broad range of Philadelphia chromosome-negative myeloproliferative diseases (MPDs) (3), to test this method of detection of somatic point variants. We used the molecular beacon to specifically detect the *JAK2*^{V617F} mutant allele and the LNA oligonucleotide to limit amplification of the wild-type *JAK2* sequences (see Fig. 1 in the Data Supplement that accompanies the online version of this letter at <http://www.clinchem.org/content/vol52/issue7>). With this method, we detected very small quantities of mutant alleles in tubes containing the homozygous *JAK2*^{V617F} mutant cell line (HEL cell line) mixed with various amounts of wild-type cells and in clinical specimens containing small amounts of *JAK2*^{V617F} mutated cells.

Primers and beacon probe were designed with the freeware MELT-CALC, Ver. 2.0 (<http://www.meltcalc.de/>). The melting temperature (T_m) of the molecular beacon stems was calculated by use of the Mfold algorithm (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi>), whereas the T_m

of the LNA was calculated via the Exiqon website (<http://lna-tm.com>).

We used unique PCR conditions (see Table 1 and experimental conditions in the online Data Supplement) that exploited the combined activities of the molecular beacon and LNA oligonucleotide to produce a fluorescent amplification signal highly specific to the *JAK2*^{V617F} DNA target (Fig. 1A). We tested different concentrations of LNA and found that a concentration of 1000 nM gave the most efficient blocking of the wild-type allele without significant alteration in the detection of the mu-

tated allele (Fig. 1A; also see Fig. 3 in the online Data Supplement). In addition, at this concentration, the low residual amount of wild-type allele present at the end of the PCR procedure was not recognized by the molecular beacon probe (Fig. 1A).

Dominguez and Kolodney (4) recommended the use of a mutated *Taq* polymerase, termed the Stoffel fragment, which lacks the 5'-3' exonuclease activity, on the basis of their hypothesis that this exonuclease activity would hydrolyze the LNA oligonucleotide and prevent it from blocking primer extension of the

wild-type target template. We found, however, that the conventional *Taq* polymerase included in the commercially available Supermix-UDG (Invitrogen) did not significantly alter the blocking activity of the LNA component.

We tested the quantitative sensitivity of our method with serial dilutions of the HEL cell line DNA mixed with wild-type DNA, and the method was able to detect *JAK2*^{V617F} mutated alleles at proportions as low as 0.01% (Fig. 1B). The efficiency of this PCR was 91.9%, as demonstrated by the slope of the curve. Theoretically, the sensitivity could not be higher because, in a 100% homozygous mutant sample, a starting amount of 100 ng of genomic DNA would contain ~34 000 copies (based on a typical amount of 6 pg of DNA per diploid cell) of *JAK2*^{V617F} mutant allele, and a diluted sample containing 0.01% of this mutant gene would theoretically represent ~3 copies of the target sequence. We are thus very close to the limits of detection of any type of quantitative PCR assay. Of note, these results confirm that complete blocking of amplification of the wild-type allele is not an absolute prerequisite for very good quantitative sensitivity for the mutant allele.

We also compared the sensitivity of our tool with the Amplification Refractory Mutation System (ARMS)-PCR method for *JAK2*^{V617F} detection (5). We used ARMS-PCR to test DNAs from 23 patients carrying the *JAK2*^{V617F} sequence variant and observed that all results were perfectly concordant with our method. Moreover, 2 patients with clearly positive results by our method showed only a weak signal by ARMS-PCR (data not shown). The great sensitivity as well as the quantitative aspects of our method present 2 advantages: (a) in some cases of positive *JAK2*^{V617F} MPD, the variation is limited to small subclones; and (b) small molecules are being developed that specifically target the mutated protein (6). Our sensitive assay could be very useful in testing the efficacy of such molecules in the frame of therapeutic trials as well as in daily molecular follow-up.

In summary, we developed a very

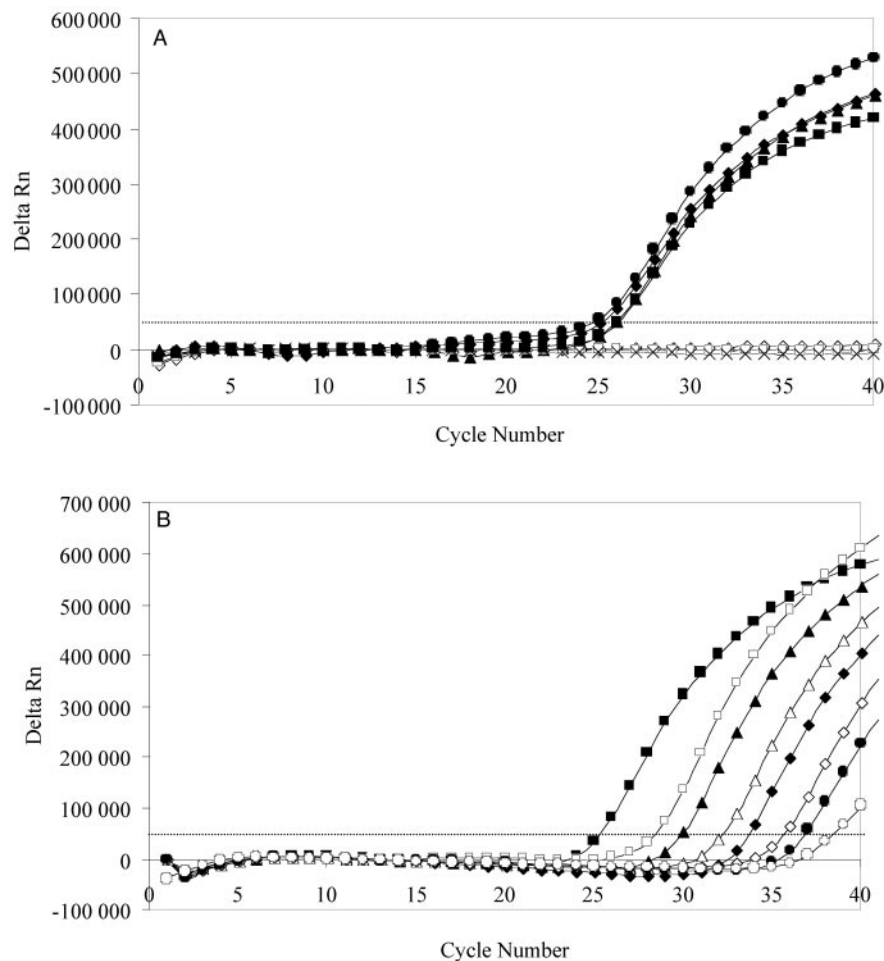


Fig. 1. Results generated with our method.

(A), amplification curves of both mutant and wild-type *JAK2* sequences, with and without the use of LNA oligonucleotide. As observed, the variations in *JAK2*^{V617F} threshold cycle remain very narrow with the different concentrations of LNA blocking sequence used. Moreover, the wild-type sequence is not detected by the molecular beacon. Shown are curves for mutant DNA plus no (◆), 10 nM (▲), 100 nM (●), and 1000 nM LNA (■); wild-type DNA plus no (◇) and 1000 nM LNA (□); and a control containing water and 1000 nM LNA (✱). (B), amplification curves obtained from serial dilutions of homozygous *JAK2*^{V617F} mutant allele diluted in wild-type DNA. As observed, a detection limit of 0.01% *JAK2*^{V617F} could be reached. The dotted line indicates the fluorescent detection threshold. Shown are curves for 100% *JAK2*^{V617F} (■); 10% *JAK2*^{V617F} (□); 5% *JAK2*^{V617F} (▲); 1% *JAK2*^{V617F} (△); 0.5% *JAK2*^{V617F} (◆); 0.1% *JAK2*^{V617F} (◇); 0.05% *JAK2*^{V617F} (●); and 0.01% *JAK2*^{V617F} (○). Results of regression analysis: $y = -3.538x + 31.255$ ($R^2 = 0.9957$).

sensitive and rapid method that combines the advantages of LNA and molecular beacon technologies. Our tool, which can be used to detect any type of single-base variation, is a simple PCR performed in <40 min, under universal conditions and in sealed tubes, hence limiting the risk of false positivity and contributing to the high sensitivity offered by our PCR assay.

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How Accurate Is the Antiprimer Quenching-Based Real-Time PCR for Detection of Her2/neu in Clinical Cancer Samples?

To the Editor:

I read with great interest the recent article by Li et al. (1), in which they presented a novel, cost-effective quantitative PCR technology for the analysis of clinical cancer samples. They developed an antiprimer quenching-based real-time PCR (aQRT-PCR) that uses fluorescently labeled PCR primers in combination with a universal quenching antiprimer, reducing the cost of labeling. In agreement with their in-house fluorescence in situ hybridization (FISH) and immunohistochemistry results, their multiplex aQRT-PCR approach detected chromosomal Her2/neu amplification in microdissected breast cancer samples; in formalin-fixed, paraffin-embedded specimens; and in plasma circulating DNA. Hence, Li et al. (1) concluded that aQRT-PCR could be used as a simple, versatile, reliable, and low-cost alternative for Her2/neu detection in clinical cancer samples.

Her2/neu overexpression is considered a strong prognostic and predictive marker for breast cancer. Overexpression of Her2/neu is seen in ~30% of invasive human breast carcinomas. Because of their low cost and ease of performance, immunohistochemical methods are commonly used to measure Her2/neu protein. In ~90% of breast carcinomas, Her2/neu protein overexpression is attributable to gene amplification. Although FISH measurement of Her2/neu gene amplification is considered the gold standard for clinical detection of Her2/neu, FISH analysis is more time-consuming and expensive than immunohistochemical methods and is therefore not preferred for primary screening of Her2/neu status.

Quantitative real-time PCR using paraffin-embedded tissue is a suggested alternative standardized approach to Her2/neu detection (2–4), but I think that the multiplex aQRT-PCR for Her2/neu detection, as presented by Li et al. (1), should be used

very cautiously in clinical cancer samples. Her2/neu overexpression has been linked with polysomy of chromosome 17. Polysomy 17 has been reported in breast carcinoma cases with borderline or low Her2/neu protein concentrations in the absence of gene amplification (5, 6). Thus, for reliable quantification of Her2/neu gene amplification, an internal control for chromosome 17 should be used. The use of a probe directed against the centromere of chromosome 17, as in dual-color FISH, can differentiate between true Her2/neu gene amplification and polysomy of chromosome 17. Because Li et al. (1) used glyceraldehyde-3-phosphate dehydrogenase (GAPDH), located on chromosome 12, as an internal control, centromere involvement could not be distinguished from true gene amplification. Moreover, because no details on the FISH procedure were reported, the comparison of the aQRT-PCR with their in-house FISH results might be questioned. From the data in the article, it is not clear whether a single-color (Her2/neu) or dual-color (Her2/neu probe–centromere 17 probe) FISH protocol was used.

I understand that the main point of the report by Li et al. (1) was the development and validation of the accuracy of the aQRT-PCR methodology, and I think that this method has great potential for analysis of clinical cancer samples. However, it requires revision for Her2/neu detection. I hope to see optimization of the aQRT-PCR method for Her2/neu detection in clinical cancer samples in future work by Li et al., as this would be of great value for clinical cancer research.

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