Rapid Method for Detection of Mutations in the Nucleophosmin Gene in Acute Myeloid Leukemia

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Mutations in exon 12 of the nucleophosmin gene (*NPM1*) that cause the encoded protein to abnormally relocate to the cytoplasm are found at diagnosis in about 50% of karyotypically normal acute myeloid leukemias and are associated with a more favorable outcome. We have devised a PCR-based assay for *NPM1* exon 12 mutations using differential melting of an oligo probe labeled with a fluorescent dye. The nucleobase quenching (NBQ) phenomenon was used to detect probe hybridization, and an oligonucleotide containing locked nucleic acid (LNA) nucleotides was used as a PCR clamp to suppress amplification of the normal sequence and enhance the analytical sensitivity of the assay. After the NBQ assay, the specimens with a mutation were removed from the capillary and sequenced to identify the mutation. The use of the LNA clamp facilitates interpretation of the mutant sequence because of the lower intensity of the overlapping normal sequence. Analysis of a series of 70 patient specimens revealed 17 positive for an *NPM1* mutation and 53 negatives. All of the NBQ results (positives and negatives) were confirmed with other methods. The analytical sensitivity of the NBQ assay is variable depending on the concentration of the PCR clamp and other parameters. Using a 100 nmol/L concentration of the LNA clamp, *NPM1* mutations were detectable in a 10-fold excess of wild-type DNA. This assay may be valuable for screening disease specimens. (*J Mol Diagn* 2008, 10:338–345; DOI: 10.2353/jmoldx.2008.070175)

Nucleophosmin is a member of a family of chaperone proteins and is normally located in the nucleolus, where it appears to have a role in the maturation of ribosomes.¹ The nucleophosmin gene (official symbol: *NPM1*; also known as nucleolar phosphoprotein B23 and numatrin) has been found mutated in about 50% of newly diagnosed adult acute myeloid leukemia (AML) patients with

a normal karyotype.² It has also been shown to be a partner gene in several translocations including t(2;5)(p23;q35) in anaplastic large-cell non-Hodgkin's lymphoma,³ t(5;17)(q32;q12) in rare cases of acute promyelocytic leukemia,⁴ and t(3;5)(q25.1;q34) in AML and myelodysplastic syndrome.⁵ The mutations found in AML almost always involve a 4-bp insertion in a limited region of exon 12. The insertions cause a change in the reading frame, the destruction of the nucleolar localization signal, and the creation of a carboxy terminus that completes a nuclear export signal.⁶ As a result, the NPM1 protein relocates to the cytoplasm (NPMc⁺).^{6,7} Exceptions have been reported, including two AMLs with NPMc⁺ having an 8-nucleotide insertion in exon 11.^{8,9} The most common mutation, called type A, is a duplication of 4 nucleotides after position 863 in the cDNA (c.860_863dupTCTG) and accounts for about 75% of the mutations in adults, but a lower fraction of the pediatric cases.^{10,11} *NPM1* with AML-type mutations has been shown to gain the ability to transform primary mouse embryonic fibroblasts, probably due to inactivation of Arf.¹²

NPM1 mutations are associated with normal karyotype, internal tandem duplication in the juxtamembrane region of the *FLT3* gene (*FLT3* ITD), higher fraction of blasts in the bone marrow, higher white blood cell count in the peripheral blood, lower expression of the CD34 antigen, and female gender.^{2,13–16} The mutation is found at a lower frequency in pediatric AML.^{10,11,17–19} The mutations are entirely somatic, and almost always heterozygous.^{2,14} *NPM1* mutation has been seen in myeloid sarcoma^{20,21} and rarely in myelodysplasia patients.^{22,23}

In the absence of the *FLT3* ITD mutation, *NPM1* mutations are associated with improved outcome.^{2,13–16} Furthermore, there is evidence that adult AML patients with *NPM1* mutation and lacking *FLT3* ITD do well with standard chemotherapy and do not benefit from allogeneic hematopoietic stem cell transplant.^{15,24} The *NPM1* mutation status has been shown to be fairly stable. When patients with *NPM1* mutations relapse, the same mutation is frequently present.^{17,25} Thus, *NPM1* mutations provide

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a useful target for measurement of minimal residual disease.

This paper describes procedures for detecting mutations in the region of exon 12 of the *NPM1* gene in which the vast majority of mutations have been found, and the determination of the sequence of the mutation. The purpose of this test is to assist in the assignment of newly diagnosed AML patients into prognostic categories. It is likely that this information, together with the results from other tests, will be used in selecting the best therapeutic modality for each patient. The information derived from this analysis will also be useful in the development of strategies for measurement of minimal residual disease during the therapy of these patients.²⁵⁻²⁷

Materials and Methods

DNA Preparation

DNA was extracted from EDTA-anticoagulated peripheral blood or bone marrow using the QIAamp Blood DNA extraction kit (QIAGEN Inc., Valencia, CA). The concentration of DNA was determined by spectrophotometry using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). DNA mixtures to test sensitivity of the assays were prepared based on these concentrations using diagnostic AML specimens with an *NPM1* mutation and specimens that tested negative for *NPM1* mutations. The stated sensitivities were based on the naïve assumption that the diagnostic specimens with a mutation contained the mutation in every cell. Thus, the actual sensitivity of the assays was greater than the stated sensitivity.

NPM1 Exon 12 Mutation Assay

The PCR and acquisition of fluorescence data were done in the Roche LightCycler (Roche, Basel, Switzerland). The PCRs were done in LightCycler capillary tubes in a volume of 20 μ l with 0.25 μ mol/L upstream primer NPM-7, 1 μ mol/L downstream primer NPM-3, 0.2 μ mol/L probe (NPM-QP1), $MgCl_2$ to achieve a final concentration of 2.5 mmol/L, 50 to 100 ng of genomic DNA, and 2 μ l of Roche FastStart DNA Master Hybridization Probes 10X reaction mix, which contains the buffer, deoxynucleoside-5'-triphosphates (dNTPs), and TaqDNA polymerase. The sequences of the primers and probe are described in Table 1, and were purchased from Integrated DNA Tech-

nologies (Coralville, IA). The sequence of the locked nucleic acid (LNA) clamp (NPM-CLMP) is also described in Table 1, and was purchased from Sigma-Proligo (Woodlands, TX). The clamp was used at a concentration of 0.1 μ mol/L for the standard assay and 0.25 μ mol/L for increased sensitivity. All of the thermal cycles started with 10 minutes at 95°C to activate the hot-start TaqDNA polymerase. The standard assay was then cycled 70 times between 95°C for 2 seconds, 62°C for 5 seconds, and 72°C for 5 seconds. The increased sensitivity assay was cycled 100 times between 95°C for 2 seconds, 76°C for 10 seconds, 62°C for 5 seconds, and 72°C for 5 seconds. The temperature transitions were all done at the maximal rate of 20°C/second, except for the transition from the annealing to extension temperature that was done at 1°C/second. After PCR, the melting curve thermal profile was 95°C for 10 seconds, 40°C for 1 minute, then 72°C with a 0.1°C/second temperature transition rate while continuously collecting fluorescence data.

DNA Sequencing

Amplicons from the LightCycler assay with a suspected mutation and controls were removed from the LightCycler capillaries by removing the cap, inverting the capillaries in 1.5-ml microcentrifuge tubes, and centrifuging at full speed for about 20 seconds. The amplicons were treated with ExoSap (Amersham Biosciences, Piscataway, NJ) to remove the primers and dNTPs, then 1 μ l was sequenced using the NPM-3 downstream PCR primer as sequencing primer and Applied Biosystems (ABI, Foster City, CA) BigDye Terminator v.3.1 chemistry. The sequencing reactions were purified using the CleanSeq system (Agencourt Bioscience, Beverly, MA) and then resolved by capillary electrophoresis on the ABI 3100 Prism Genetic Analyzer. The mutations were determined by comparison with the normal *NPM1* sequence (GenBank accession number NM_002520) and a normal control that was included in each run.

Alternate Assays

Two alternate assays for the *NPM1* exon 12 mutations were done for validation of the LightCycler assay. The first assay was PCR with a fluorescently labeled primer followed by separation using capillary electrophoresis. The PCR was done in a volume of 25 μ l with primers NPM-11 and NPM-3F (sequence of primers in Table 1) at

Table 1. Sequences of Oligodeoxyribonucleotides

Name (NPM-)	Position	Sequence (5'-3')*	Position in NT_023133
3	Exon 12	GGACAGCCAGATATCAACTGTTACAG	15647229-15647204
7	Intron 11	TATGAAGTGTGTGGTTCTTAAC	15647067-15647090
QP1	Exon 12	F-TATTCAAGATCTCTGGCAGTGG-C3	15647118-15647139
CLMP	Exon 12	AsAs+G+ATCT+CT+G+GCA+GT+GG-C3	15647123-15647139
11	Intron 11/exon 12	TTTTCCAGGCTATTCAAGATC	15647108-15647128
3F	Exon 12	F-GGACAGCCAGATATCAACTGTTACAG	15647229-15647204

*Other than the bases A, T, C, and G, the oligos are described by a 5' fluorescein dye (F-) in the QP1 probe and 3F primer, the positions of the LNA nucleotides (+ before the LNA) in the CLMP PCR LNA clamp, the phosphorothioate linkages in the clamp (s), and the C3 spacer (-C3) on the 3' terminus of the probe (QP1) and clamp (CLMP).

a final concentration of 1 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ of each dNTP, 2 units of HotStar TaqDNA polymerase, and 2.5 μl of the 10X buffer provided by the enzyme manufacturer (QIAGEN). Template was added in a volume of 1 μl containing 50 to 100 ng of DNA. The reaction was cycled 27 times between 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 40 seconds, preceded by 15 minutes at 95°C, and followed by 5 minutes at 72°C. After PCR, the products were diluted 1:10 in water, then 1 μl was mixed with ABI Hi-Di Formamide and size markers (ABI GeneScan 350 ROX) and resolved by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer. The results were evaluated and printed using the computer program GeneMapper v4.0 (ABI).

The second assay was heteroduplex analysis. The PCR was done in a volume of 25 μl with primers NPM-7 and NPM-3 each at a final concentration of 1 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ of each dNTP, 2 units of QIAGEN HotStar TaqDNA polymerase, and 2.5 μl of the 10X buffer provided by the enzyme manufacturer. Template was added in a volume of 1 μl containing 50 to 100 ng of DNA. The reaction was cycled 35 times between 95°C for 15 seconds, 64°C for 15 seconds, and 72°C for 40 seconds, preceded by 10 minutes at 95°C, and followed by 5 minutes at 72°C. After PCR, 5 μl of each reaction was resolved by electrophoresis on a preformed 10% polyacrylamide mini-gel in Tris-borate-EDTA buffer (Invitrogen, Carlsbad, CA) together with molecular weight markers (MspI digest of pUC18 plasmid DNA; Sigma, St. Louis, MO), stained with SYBR Gold (Invitrogen) and photographed.

Results

The purpose of this work was to develop and validate a method for detection of the *NPM1* mutations found frequently in AML with a normal karyotype. We attempted to design an assay using differential melting of an oligodeoxynucleotide probe to distinguish the alleles, and nucleobase quenching (NBQ) of a fluorescent dye linked to the probe to generate the signal, because we have found this method useful for low-volume genotyping in several situations.^{28–30} In contrast to our previous efforts directed toward detection of single defined germline mutations, the *NPM1* alterations found in AML are a collection of more than 30 different mutations, all of which cause relocation of the encoded protein to the cytoplasm.^{6,31} In addition, the mutations are somatic and likely confined to the AML clone. For these reasons, the assay must be able to detect a variety of different mutations in a small region, and have sufficient analytical sensitivity to detect the mutation in a smaller fraction of the total DNA than an assay for a heterozygous germline mutation.

Our overall strategy for genotyping using melting curve analysis is to first generate an amplicon using asymmetric PCR so that there will be an excess of the DNA strand that hybridizes to the probe. This increases the signal by avoiding competition between probe hybridization and renaturation of the amplicon.³² The probe was designed so that the fluorophore is located in proximity to a deox-

yguanylate residue on the opposite strand when the probe is base-paired to the amplicon. This causes a decrease in the fluorescent signal due to the quenching effect of the guanine base.³³ After hybridization, the temperature is raised and fluorescence is monitored continuously to detect the temperature at which the probe dissociates from the complementary strand. When the probe/amplicon hybrid dissociates (melts), the fluorescent signal increases due to elimination of the quenching effect of the guanine base. A mismatch between the probe and template causes a reduction in the melting temperature (T_m) that is characteristic for each different mutation. The probe is matched to the wild-type sequence, so that a mutation will produce a reduction in the T_m . A NBQ assay designed initially without a PCR clamp was optimized for MgCl_2 concentration, annealing temperature, primer concentrations, and cycle number. The analytical sensitivity of this assay was sufficient to detect *NPM1* mutations in 20 to 25% of the cells, which is barely sufficient for screening diagnostic AML specimens (data not shown).

We desired to increase the analytical sensitivity of the assay to make it usable on specimens with a lower fraction of cells with a mutation. We enhanced the assay with the use of a PCR clamp, similar to the strategy used by Thiede et al³⁴ to increase the sensitivity of other assays for *NPM1* mutations. The clamp is a modified oligonucleotide that has the same sequence as the normal (Table 1), is blocked on the 3' end by a C3-spacer to prevent extension, and has phosphorothioates for the two 5'-most linkages to inhibit degradation by the 5'→3' exonuclease activity of Taq DNA polymerase. It uses LNA nucleotides at several positions to increase the avidity of the clamp for the normal sequence. The T_m of the clamp was estimated using a free program available at the Exiqon web site (<http://lna-tm.com/>). The strategy is to inhibit amplification of the normal sequence so that there will be preferential amplification of template DNA with a mutation. Figure 1 shows that the use of the PCR clamp increased the analytical sensitivity so that a specimen with 10% of the cells having an *NPM1* mutation (type B) was detected. Without the clamp, the melting curves of the normal and mutant DNAs were indistinguishable. With the clamp, the mutation was revealed by the lower T_m trough (Figure 1). Figure 2 shows the results of a clamped NBQ assay for several different *NPM1* exon 12 mutations. Different mutations produce different shifts in the T_m of the probe amplicon hybrid. However, the T_m difference is not sufficient to identify the actual mutation.

We employ nucleotide sequencing to confirm the presence of, and to identify, the mutation. As described in Materials and Methods, we remove the contents of the LightCycler capillary and sequence the DNA using the downstream PCR primer. Sequencing electropherograms are shown in Figure 3 for a normal amplicon (top panel), the most common *NPM1* mutation, c.860_863dupTCTG (type A mutation, middle panel), and c.864_865delGCinsCTGGCG (University of Rochester Medical Center [URMC] mutation, lower panel), which, to our knowledge, has not been previously reported. The two mutation panels in Figure 3

Figure 1. Graph of $-d(F1)/dT$ versus temperature showing that the use of a LNA PCR clamp enhances the sensitivity of the NBQ assay. The sensitivity control was prepared by mixing DNA from a diagnostic AML patient specimen with a type B mutation and normal DNA in a 1:10 ratio. The 10% mutant and normal templates were amplified with or without the LNA PCR clamp as indicated. Both no-template controls are also shown. The direction of change in fluorescence intensity when the quenched probe dissociates from the amplicon (increase) is the opposite of that seen when using a dual probe fluorescence resonance energy transfer system to generate the fluorescent signal. For this reason we see a trough, or inverted peak, when using the LightCycler software. The higher T_m trough is from the normal sequence that is fully base-paired with the probe, and the lower T_m trough is due to the mismatched mutant sequence.

show results for amplicons generated with and without the use of the LNA clamp during PCR. Without the clamp, the sequence shows overlapping normal and mutant sequence. With the clamp, the signal from the

Figure 2. Melting troughs ($-d(F1)/dT$ versus temperature) from the types A, B, D and URMC mutations in exon 12 of the *NPM1* gene. The mutant melting troughs are labeled in the figure, as well as the normal and no DNA template controls.

Figure 3. Sequence electropherograms showing the most common *NPM1* mutation (type A, c.860_863dupTCTG) and the mutation discovered at the URMC (c.864_865delGCTGCTGGCG), as labeled in the figure. The **upper** panel is a normal sequence for comparison. The sequencing reactions were done using the downstream PCR primer. The reverse complements are shown. The panel for each mutation shows the sequence after the PCR was done in the presence (+clamp) or absence (-clamp) of a LNA PCR clamp, as labeled on the **left** side of the figure. The mutated nucleotides are underlined in the sequence interpretation between the +clamp and -clamp electropherograms. In the absence of the clamp, the sequence of the mutation can be determined from the overlapping normal and mutant signals by subtracting the normal base at each position. The clamp is used to inhibit amplification of the normal. Even though a bit of the normal sequence can still be seen in the +clamp electropherograms, the sequence of the mutation can be deciphered more easily because the normal sequence is much less intense.

normal sequence is diminished, easing the interpretation of the mutation.

The NBQ assay was validated by comparison of results with two other assays for *NPM1* exon 12 mutations. The other two assays were fragment analysis with fluorescence detection after separation by capillary electrophoresis (Figure 4), and heteroduplex analysis on a polyacrylamide gel (Figure 5). The fragment analysis method is one of the most commonly used for detection of *NPM1* gene mutations because the mutations almost always involve a net insertion of four nucleotides.^{24,34,35} In the heteroduplex method, after amplification using the PCR,

Figure 4. Example of the fragment analysis method used for validation of the NBQ assay. The figure shows a result from a specimen positive for an *NPM1* mutation (Mutant, right panel) and a specimen with a normal sequence in this region (Normal, left panel). The double peaks are likely due to variable non-templated A addition. The y axis is fluorescence intensity in arbitrary units, and the x axis is the size of the fragment in bp.

the products are resolved by electrophoresis on a 10% polyacrylamide gel. Heteroduplexes that form between normal and mutant strands electrophorese slower and form a band at a higher apparent molecular weight than the wild-type band. This method depends on the mutation being heterozygous so that there is normal as well as mutant DNA to form the heteroduplexes. This method is not commonly used for analysis of *NPM1* mutations, but one report did use a very similar methodology.¹⁸ Fragment analysis was used for validation of 23 specimens, heteroduplex analysis for 29 specimens, and 18 specimens were validated with both methods. Seventy patient specimens were studied in all, which were mostly from AML patients. There was 100% concordance between the NBQ assay and the alternate methods for 17 specimens positive for an *NPM1* mutation and 53 negatives. The mutations included 12 type A (c.860_863dupTCTG), two type B (c.863insCATG), two type D (c.863insCCTG), and one newly discovered mutation (c.864_865delGCinsCTGGCG).

Although most of the *NPM1* mutations in AML patients are 4-bp insertions after position 863 in the coding sequence, several mutations have been noted that have breakpoints a few bases downstream after position 869.³¹ Although these downstream mutations still have a mismatch between the amplicon and the NBQ probe, and are likely to be detectable in our assay, we did not

Figure 5. Example of the heteroduplex method used for validation of the NBQ assay. The figure is a photograph of a stained polyacrylamide gel showing results from a series of specimens, one of which is positive for an *NPM1* mutation, indicated by a + above the lane. The white arrowhead on the gel photograph points toward the heteroduplex band. The specimens with no evidence for mutation are indicated by a - above the lane. The M lane contains the molecular weight markers (MspI digest of pUC18 plasmid DNA). The 163-bp amplicon produced by the PCR is indicated in the figure. The approximately 480-bp band in all of the lanes is of unknown origin.

Figure 6. Analysis of *NPM1* mutation c.869_873delGGAGGinsTGTTTTCTC. **A:** Melting trough ($-d(F1)/dT$ versus temperature) from c.869_873delGGAGGinsTGTTTTCTC compared with a type A mutation and a normal control. The mutant melting peaks are labeled in the figure, as well as the normal and no DNA template controls. The melting trough for the c.869_873delGGAGGinsTGTTTTCTC mutation is labeled with an asterisk. **B:** The mutant amplicons were removed from the LightCycler capillaries and sequenced using the downstream PCR primer. The reverse complement sequence trace is shown for the c.869_873delGGAGGinsTGTTTTCTC mutation with the overlapping normal sequence at a lower intensity due to the PCR clamp. The sequence interpretation of the mutant and normal DNA is above the trace, in upper and lower case, respectively, as labeled in the figure. The nucleotides inserted in the mutant and deleted from the normal are underlined. The sequence of the 3' part of the probe is above the mutant sequence in a shaded box to show the single mismatched base between the probe and mutant amplicon.

find any of these mutations in our initial validation. For this reason we obtained an AML specimen with the *NPM1* mutation c.869_873delGGAGGinsTGTTTTCTC from the Johns Hopkins leukemia cell bank to confirm the ability of the NBQ assay to detect the downstream insertions. This mutation was detected in the NBQ assay with a T_m about 3°C lower than that of the normal sequence compared with an approximately 8°C ΔT_m for the type A mutation (Figure 6A). A comparison of the mutation sequence with the probe sequence shows that the mutant amplicon has only a single base mismatch with the probe at the penultimate 3' position (Figure 6B).

The increased analytical sensitivity obtained with the PCR clamp tempted us to further optimize the assay to see how much analytical sensitivity we could achieve with this format. The use of a higher concentration of the clamp increased the relative signal from the mutant compared with the normal, but also lowered the overall signal obtained from the assay (data not shown). Additional

PCR cycles were used to increase the overall signal. We also found an enhancement using an additional step (10 seconds at 76°C) in the cycle before annealing to give the clamp a head-start over the primers. Using a concentration of 250 nmol/L for the clamp and 100 cycles, we achieved an analytical sensitivity for the type A mutation of greater than 1% (data not shown).

Discussion

We have designed and validated a homogeneous PCR assay to detect *NPM1* mutations in exon 12 that are found in about half of AMLs with a normal karyotype. The assay uses nucleobase quenching to detect probe hybridization as described by Crocket and Wittwer.³³ The probe has a normal sequence. Mutations in the amplicon in the target region of the probe lower the thermal stability of the probe/amplicon hybrid that we detect as a lower T_m .

To enhance the analytical sensitivity of the method, we have used a PCR clamp to inhibit amplification of the normal sequence. The effect of the clamp on improving the analytical sensitivity of the test is shown in Figure 1. The clamp may enhance the assay in two ways. Besides blocking amplification of the normal sequence, it may also compete with the probe for binding to the normal sequence before determining the T_m . The sequencing results in Figure 3 show that the clamp does inhibit the amplification of the normal sequence. This effect also facilitates interpretation of the sequence electropherograms by removing enough of the normal signal so that the mutation can be read directly without subtraction of the overlapping normal sequence.

The use of a clamp to suppress amplification of a normal sequence and enhance detection of a mutation has been done previously, usually using a peptide nucleic acid oligo.³⁶ The use of a PCR clamp containing LNA nucleotides is more recent, with the advantages of lower cost and greater versatility due to the ability to mix LNA and standard deoxyribonucleotides in the oligo.^{34,37} In an LNA nucleotide, the ribose ring is constrained by a methylene bridge between the 2'-O and 4'-C atoms, which locks it into a conformation that increases the thermal stability of base-paired hybrids and increases the decrement in T_m due to mismatches.³⁸

The clamp in the work reported here (CLMP in Table 1) was designed using phosphorothioate linkages for the first two internucleotide bonds with the intention of limiting hydrolysis by the 5'→3' exonuclease activity of the Taq DNA polymerase. We have not tested whether this alteration is necessary by trying a clamp lacking the phosphorothioate linkages. We found the clamp had no effect on the amplification of the same PCR product when using QIAGEN's HotStar Taq DNA polymerase in a standard PCR, even with the clamp at a 3 μmol/L concentration, compared with a 0.1 μmol/L concentration that was effective in the LightCycler assay (data not shown). We also tried Clontech's (Mountain View, CA) Titanium TaqDNA polymerase, which lacks the 5'→3' exonuclease activity, and similarly found the clamp ineffective under these conditions (data not shown). Other workers have achieved suc-

cess using PCR clamps containing LNA using Applied Biosystem's AmpliTaq Gold or Stoffel fragment, and Invitrogen's Platinum Taq polymerase.^{34,37,39} Further work is needed to increase our understanding of PCR clamping using LNA oligos to aid in the design of this type of assay.

We have validated the clamped NBQ assay in a survey of 70 specimens, revealing 100% concordance with other assays for 17 positives and 53 negatives. The assay detected the three most common *NPM1* exon 12 mutations that account for more than 90% of the total mutation load,³¹ and also detected a previously unpublished mutation. The newly discovered mutation, c.864_865delGCinsCTGGCG, is similar to those previously reported with respect to destruction of the nucleolar localization signal and creation of a nuclear export signal. We also demonstrated that the assay can detect a mutation with a more downstream breakpoint, c.869_873delGGAGGinsTGTTTTCTC, which has only a one-nucleotide difference with the probe at the penultimate 3' position (Figure 6). Thus, the homogeneous PCR assay presented here should be able to detect all of the *NPM1* exon 12 mutations published to date,³¹ except for two mutations that were reported during the development of this test.¹⁸ Our assay should also be able to detect the one *NPM1* exon 12 mutation that has been reported that does not have a four-nucleotide insertion, because three of the five single base changes in this mutant gene lie in the target sequence of our NBQ probe.¹⁸ This mutation would be missed by methods that use the increased size of a PCR product with a 4-bp insertion to detect *NPM1* mutations. Several mutations have been reported outside of the exon 12 hot spot that would be undetectable by the NBQ assay presented here, as well as all of the proposed assays that focus on exon 12.^{8,9,40} Immunostaining for cytoplasmic nucleophosmin may be the most clinically sensitive assay for the detection of *NPM1* gene alterations in AML.⁶

The NBQ assay we developed and validated has the usual advantages of homogeneous systems for mutation detection, in terms of speed, lower hands-on time, and less potential for carryover contamination due to the avoidance of post-PCR processing, at least for specimens that are not subjected to nucleotide sequencing. The use of a PCR clamp to increase the analytical sensitivity does have the disadvantage of making the assay less able to quantify the relative proportion of mutant DNA. Detection of *NPM1* mutations by fragment analysis with fluorescence detection after separation by capillary electrophoresis, although less efficient, preserves the ability to quantify the mutation, and has an analytic sensitivity similar to that of the clamped NBQ assay (data not shown). Our initial work using a heteroduplex assay (Figure 5) revealed that it is less efficient and has less analytical sensitivity than the two other assays. However, it may be possible to improve the analytical sensitivity of the heteroduplex assay with further optimization. Overall, we prefer the clamped NBQ assay and use it as our standard test for detection and characterization of mutations in exon 12 of the *NPM1* gene in AML.

We have also optimized an even more sensitive assay that can detect mutations in a 1:100 dilution of a diagnostic positive in normal DNA by increasing the concentration of the PCR clamp and the number of cycles. It is

likely that further optimization of the assay, including optimization of the sequence of the LNA clamp, would allow greater analytical sensitivity, but we have not pursued this aspect. For detection of minimal residual disease, a test that is more sensitive and also capable of quantifying the mutant DNA is preferable. Several such assays, using a real-time PCR strategy, have been described.^{25–27} The starting point for such assays is the determination of the sequence of the *NPM1* mutation. The test we describe is valuable for screening diagnostic specimens and, together with sequence analysis, provides the information needed for choosing an assay to quantify disease in subsequent specimens.

Studies to detect and measure mutations and alterations in the expression of particular genes will open a door toward optimizing the choice of therapeutic modalities for each patient. This individualized approach has great potential to improve the outcome for AML patients.⁴¹

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