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Minimal Residual Disease Monitoring of Acute Myeloid Leukemia by Massively Multiplex Digital PCR in Patients with *NPM1* Mutations

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The presence of minimal residual disease (MRD) is widely recognized as a powerful predictor of therapeutic outcome in acute myeloid leukemia (AML), but methods of measurement and quantification of MRD in AML are not yet standardized in clinical practice. There is an urgent, unmet need for robust and sensitive assays that can be readily adopted as real-time tools for disease monitoring. *NPM1* frameshift mutations are an established MRD marker present in half of patients with cytogenetically normal AML. However, detection is complicated by the existence of hundreds of potential frameshift insertions, clonal heterogeneity, and absence of sequence information when the *NPM1* mutation is identified using capillary electrophoresis. Thus, some patients are ineligible for *NPM1* MRD monitoring. Furthermore, a subset of patients with *NPM1*-mutated AML will have false-negative MRD results because of clonal evolution. To simplify and improve MRD testing for *NPM1*, we present a novel digital PCR technique composed of massively multiplex pools of insertion-specific primers that selectively detect mutated but not wild-type *NPM1*. By measuring reaction end points using digital PCR technology, the resulting single assay enables sensitive and specific quantification of most *NPM1* exon 12 mutations in a manner that is robust to clonal heterogeneity, does not require *NPM1* sequence information, and obviates the need for maintenance of hundreds of type-specific assays and associated plasmid standards. *(J Mol Diagn 2017, 19: 537—548; http://dx.doi.org/10.1016/j.jmoldx.2017.03.005)*

Acute myeloid leukemia (AML) is a fatal disease with dismal outcomes. Even after achieving initial remission, most patients relapse and ultimately succumb to their disease. The presence of minimal residual disease (MRD) using a variety of molecular and immunophenotypic criteria is predictive of outcome in patients with AML.[1–4] Flow cytometric methods of MRD detection are effective but require multiantibody panels and high levels of user expertise in the flow cytometric identification of rare cell populations.[5–7] Worldwide, there are no accepted standards for the identification and quantification of MRD in AML. PCR-based detection of MRD, in the form of fusion transcripts, can be accomplished in approximately 30% of patients with AML with abnormal cytogenetics, for example, inv(16), t(8;21), and t(15;17).[8] More than half of patients with AML have normal cytogenetics, and 40% to 50% of these patients, in turn, have *NPM1* mutations (*NPM1*muts).[9,10] Posttherapy monitoring of MRD in patients with *NPM1*mut AML using real-time quantitative PCR (qPCR) with allele-specific oligonucleotide primers has been evaluated in several clinical trials.[1,11] In all these studies, the levels of *NPM1*mut transcript were associated with patient

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outcome that pointed to *NPM1* as a stable marker for disease progression. The presence of the *NPM1*mut transcript was associated with high risk of relapse compared with patients with MRS and a lower survival rate, with detection after the second chemotherapy cycle the most discriminatory time point.[10] Conversely, a decrease in *NPM1*mut transcript copies correlated with hematologic remission, and half of the patients achieving complete remission presented <100 copies of the mutant transcript.[11] This value agrees with other studies in which *NPM1*mut levels >1% after treatment and >10% after allogeneic transplantation were associated with poor overall and disease-free survival.[12] Similarly, a threshold of 200 *NPM1*mut per 10^4 *ABL1* (*NPM1*mut/10^4 *ABL1*) copies was proposed for early detection of relapse after therapy by Krönke et al.[13]

Approximately 95% of *NPM1*muts consist of four nucleotide insertions in exon 12 at the 863 position, the most common of which is type A (c.860_863dupTCTG), found in approximately 75% of patients with *NPM1*mutAML and an additional 15% comprising both type B (c.863_ins864insCATG) and type D (c.863_864insCCTG) mutations.[3,9,14–17] The remaining patients often have rarer subtypes, with differing and sometimes highly patient-specific insertion sequences. More than 50 such frameshift insertion mutations have been reported,[18] and at least hundreds are theoretically possible. MRD testing in patients with *NPM1*mut AML currently requires prior DNA sequencing to identify the specific insertion sequence to match the patient to an appropriate allele-specific qPCR test. Thus, quantitative *NPM1* MRD testing requires maintenance of qPCR assays and plasmid standards for each mutation, with commercial plasmid standards being widely available for only the top three mutations. Digital PCR (dPCR) assays in which the standard type-specific qPCR assays for *NPM1*mut are directly adapted individually to dPCR format have been reported to circumvent the need for plasmid standards while demonstrating excellent agreement with qPCR for the detection of rare *NPM1*muts on clinical validation.[18] Despite this concordance, custom tests are still required for every new *NPM1*mut that is encountered.

The need to develop patient-specific MRD tests currently poses regulatory challenges. In the United States, for instance, each patient-specific MRD test for rare *NPM1*mut would comprise a separate laboratory developed test and, as such, would require submission of a new or amended application with subsequent approval by regulatory agencies before the laboratory developed test is clinically reportable. In addition, a practical problem encountered by oncologists is that *NPM1*mut diagnosis is sometimes made without sequencing using capillary electrophoresis[19] with no widely used, clinically actionable sequencing technology sensitive enough to sequence *NPM1* during remission. In such cases, reporting of MRD as mutated *NPM1* transcripts is precluded using any current approach. Therefore, a substantial number of patients with *NPM1*mut AML would be ineligible for MRD assessment because they lack *NPM1* sequence information and/or the availability of an appropriate test. Finally, although *NPM1*muts are generally stable over time,[20–22] there have been reports of intrapatient heterogeneity[23] and type switching,[24] both of which could result in false-negative determinations of MRD status in individual cases. Next-generation sequencing–based approaches can circumvent these challenges because they do not require prior sequence information for detection of *NPM1*muts in the context of MRD and have sensitivities that exceed flow cytometry.[23,25] However, this technology currently does not express MRD in *NPM1*mut/10^4 *ABL1* copies used by much of the concurrent and historical work. Thus, MRD testing for *NPM1* in AML would be significantly enhanced by a more robust assay that is usable on more patients, simplifies the testing process for practitioners, and better addresses the possibility of intrapatient heterogeneity or clonal evolution. We present a new single dPCR-based test that detects 95% of *NPM1*mut types in AML using a single multiplex assay pool that enables quantitative assessments in a manner that is robust to *NPM1* evolution, mixture, and subtype even when sequence information is missing.

Materials and Methods

Primary Sample Isolation and Cell Culture

Mononuclear cell isolation was performed using Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. GM12878 cell line (Coriell, Camden, NJ) was cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum and 2 mmol/L L-glutamine (ThermoFisher, Pittsburgh, PA). OCI-AML3 cells were cultured in α–minimal essential medium supplemented with 20% heat-inactivated fetal bovine serum (ThermoFisher). Patient samples and healthy donor samples were acquired with patient consent in accordance with the Declaration of Helsinki and following Weill Cornell Medicine Institutional Review Board–approved regulations and protocols.

RNA Extraction

Cells were washed with Dulbecco's phosphate-buffered saline, and total RNA was isolated using the RNeasy extraction kit (Qiagen, Valencia, CA) as per the manufacturer's specifications.

Primers, Probes, Synthetic Targets, and Plasmid Standards

*NPM1*mut detection was performed using primers and probes described by Gorello et al,[11] with modifications when performing dPCR. The *ABL1* gene was used as endogenous control.[26] Multiplex dPCR reactions for *NPM1* detection consisted of a common forward primer (5′-GAAGAATTGCTTCCGGATGACT-3′) and probe (5′FAM-ACCAAGAGGCTATTCAA-MGB-3′) as previously

described[11] combined with a degenerate reverse primer (5′-CTTCCTCCACTGCNNNNCAGA-3′) adapted from the study by Gorello et al[11] to generate a multiplex reaction pool (where N is a mixture of A, C, G, and T). A VIC (4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein)—labeled *ABL1* probe was used for dPCR.[27] Additional primers are indicated in Table 1. Probes were synthesized by ThermoFisher and primers by ThermoFisher or Integrated DNA Technologies (Coralville, IA). Synthetic targets for rare *NPM1* subtypes were generated as gBlocks (Integrated DNA Technologies). A synthetic *NPM1*mut target pool and wild-type *NPM1* (GeneArt fragments) were obtained from ThermoFisher. Sequences for all synthetic DNA targets are listed in Supplemental Table S1. Plasmid reference standards were used for quantification by qPCR (Qiagen).

Reverse Transcription

cDNA was synthesized from 500 ng of total RNA with SuperScript VILO cDNA Synthesis (Life Technologies, Carlsbad, CA). Reactions were incubated at 25°C for 10 minutes, 42°C for 60 minutes, and at 85°C for 5 minutes.

qPCR

qPCR reactions were performed on a QuantStudio 5K platform (Applied Biosystems, Carlsbad, CA) as described in Gorello et al.[11] Annealing temperature varied according to target; individual values are specified for each assay in Table 1.

Droplet dPCR

Droplet dPCR was performed using the RainDrop platform (RainDance Technologies, Billerica, MA) or QX-200 platform (BioRad, Hercules, CA). For the RainDrop platform, reaction mixtures consisted of 1× Taqman Genotyping Master Mix (Life Technologies), 1× Droplet Stabilizer (RainDance Technologies), 500 nmol/L of each forward and reverse primer for *NPM1* and *ABL1,* and 250 nmol/L of each probe. After generation of droplet partitions, 10 minutes of polymerase activation (95°C) were followed by 45 cycles of denaturation (95°C) and annealing and extension (58°C for multiplex assay) using BioMetra TAdvanced Thermocycler (Göttingen, Germany). Other temperatures are indicated in Table 1. Reactions were terminated with a 98°C incubation and held at 10°C until analysis. For the QX-200 platform, reactions were performed using the droplet dPCR Supermix for Probes (no dUTP) (BioRad) and processed according to the manufacturer's protocols on a C1000 Touch Thermal Cycler (BioRad) using the amplification protocol and primer and probe concentrations as above. Droplet positivity was quantified using the manufacturer's software: RainDrop Analyst II version 1.1 (RainDance Technologies) or QuantaSoft version 1.0 (BioRad).

Limit of Detection

Limit of detection was determined as the mean *NPM1*mut/10^4 *ABL1* ratio for 12 blank samples ± 3 SDs according to International Union of Pure and Applied Chemistry recommendations.[28,29]

Table 1 Primers and Probes Used in This Study

Target	Reverse primer sequence	Annealing temperature, °C
NPM1 multiplex	5′-CTTCCTCCACTGCNNNNCAGA-3′	58
NPM1 deoxyinosine (dI)	5′-CTTCCTCCACTGC(dI)$_4$CAGA-3′	58
NPM1 5-nitroindole (5NI)	5′-CTTCCTCCACTGC(5NI)$_4$CAGA-3′	58
NPM1 universal	5′-GCCAGATATCAACTGTTACAGAAATG-3′	58
c.860_863dupTCTG (type A)	5′-CTTCCTCCACTGCCAGACAGA-3′	62
c.863_864insCATG (type B)	5′-CCTCCACTGCCATGCAGAG-3′	60
c.863_864insCCTG (type D)	5′-CCTCCACTGCCAGGCAGA-3′	61
c.863_864insCTTG (type DD1)	5′-CCTCCACTGCCAAGCAGAG-3′	62
c.863_864insTATG	5′-CTTCCTCCACTGCCATACAGA-3′	60
c.863_864insTCGG	5′-CTCCACTGCCCGACAGAGA-3′	60
c.863_864insTAAG	5′-CTTCCTCCACTGCCTTACAGAGA-3′	63
c.863_864insCGTG	5′-CCTCCACTGCCACGCAG-3′	60
c.863_864insTTTG	5′-TCCTCCACTGCCAAACAGA-3′	60
c.863_864insCAAA	5′-TCCTCCACTGCTTTGCAGA-3′	60
c.863_864insTAGG	5′-CTTCCTCCACTGCCCTACAGAG-3′	60
c.863_864insCTCG	5′-CCTCCACTGCCGAGCAGA-3′	60
c.864_864delinsCCGTT	5′-TCCTCCACTGAACGGCAGA-3′	60
c.865_866insCAGC	5′-CTTCCTCCACTGCCTGGCAGA-3′	60
c.863_864insGCCG	5′-CTTCCTCCACTGCCCGGCAGA-3′	61

Sequences for reverse primers are listed for all *NPM1* mutation (*NPM1*mut) subtypes tested and massively multiplex *NPM1*mut and NPM1 universal assays, which amplifies both wild-type and mutant *NPM1*. Annealing temperature is indicated for universal, multiplex, *NPM1* c.863_864insCTTG, *NPM1* c.865_866insCAGC, and *NPM1* c.863_864insGCCG assays.

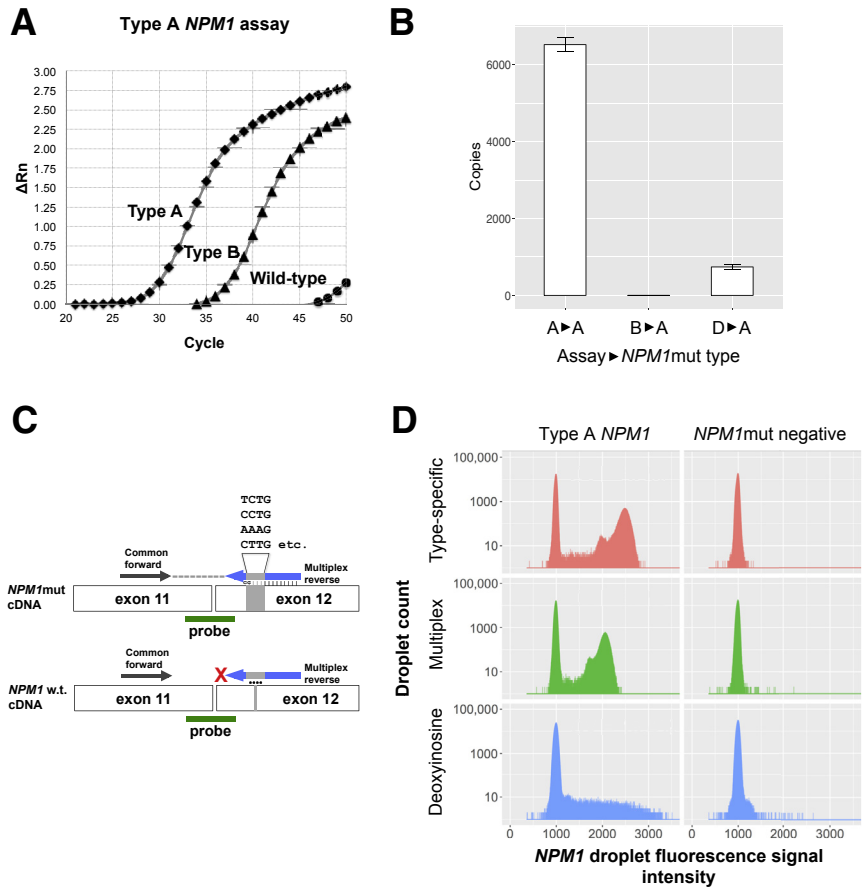

Figure 1 Cross-detection between *NPM1* assays motivates design of a digital PCR-based multiplex *NPM1* assay. **A:** Real-time quantitative PCR (qPCR) amplification curves indicating normalized reporter value (ΔRn) as a function of the PCR cycle. The type A *NPM1* assay is able to amplify 2000 copies of Ipsogen reference plasmids for type A and type B *NPM1* mutations (*NPM1*muts) but not wild-type *NPM1*. **B:** *NPM1*mut copies detected by qPCR with type A, type B, or type D assays when type A plasmid standard was used as template. **C:** Schematic of massively multiplex assay indicating relative positions of the common forward primer, multiplex reverse primer, and common probe. Productive amplification occurs with *NPM1*mut templates (**top row**) but not wild-type *NPM1* templates (**bottom row**). **D:** Histograms indicate the distribution of positive droplets of type-specific, multiplex, and deoxyinosine-based *NPM1* mutation assays in type A *NPM1* patient samples (*NPM1*mut acute myeloid leukemia; type A) alongside *NPM1*-negative mononuclear cells (negative). The vertical axis indicates $\log_{10}$ (absolute counts) with the horizontal axis indicating droplet signal intensity. The CV of positive droplets is indicated. Data are expressed as means ± SEM (**B**). $n = 3$ (**B**).

RNA Sequencing

Total RNA was used to generate sequencing libraries using the Kapa Biosystems Stranded mRNA Sequencing Kit with an mRNA capture beads protocol according to the manufacturer instructions (Kapa Biosystems, Wilmington, MA). Targeted enrichment for ultradeep RNA sequencing was performed using Integrated DNA Technologies Lockdown probes. Hybridization and washing were performed as per the company's specifications. Paired end 150-bp sequencing data were generated on an Illumina HiSeq 2500. Reads were aligned to the human reference genome (GRCh38) using the STAR aligner version 2.5.2a.[30] Postalignment processing included duplicate marking using SamBlaster version 0.1.21[31] and splice junction splitting using splitNreads commit a84339a (splitNreads, *https://github.com/mjafin/splitNreads*, last accessed August 31, 2015). Variant calling was performed using FreeBayes version 0.9.21.[32]

Targeted DNA Sequencing

Genomic DNA was extracted from frozen pellets using the Qiagen QiaAmp DNA Micro Kit. Libraries for DNA sequencing were prepared using 250 ng of material with the Kapa Biosystems HyperPrep Kit following the manufacturer's instructions. Libraries were hybridized for targeted enrichment with a custom-designed panel specific for hematologic malignant neoplasms. Capture probes were synthesized by Nimblegen (Roche Diagnostics, Indianapolis, IN); hybridization, washing, and purification steps were performed following their protocol. Paired-end sequencing (150 bp) was performed on an Illumina Hiseq 2500. Reads were aligned to the human reference genome (hg37d5) using BWA-MEM version 0.7.10 (*http://bio-bwa.sourceforge.net*).[33] Variant calling was performed using VarDict version 1.4.6 (*https://github.com/AstraZeneca-NGS/VarDictJava*).[34]

Results

Cross-Detection between Existing qPCR-Based *NPM1* Allele-Specific Assays

NPM1 allele-specific assays are able to cross-detect other *NPM1* subtypes but not wild-type *NPM1*. Representative qPCR amplification curves (Figure 1A) reveal the ability of the established type A *NPM1* assay[11] to detect 2000 copies of reference plasmid for type A or type B *NPM1* insertions (Ipsogen) alongside wild-type *NPM1*. The type A assay effectively discriminated between the type A *NPM1*mut and

the wild-type *NPM1*, reproducing the known specificity of this assay for *NPM1*mut. However, the same type A–specific assay was notably less selective between type A and type B *NPM1*muts, where type B mutations had delayed reaction kinetics and approached a reaction plateau above 45 cycles that was not achieved by wild-type *NPM1*. Next, we determined the extent to which the established *NPM1* type-specific assays for the most common mutations (types A, B, and D) could cross-detect the type A *NPM1*mut by testing each of the three assays against the same amount of type A plasmid (Figure 1B). When the type A–specific assay was correctly matched to the type A *NPM1*mut, means ± SEM of 6524 ± 176 *NPM1*mut copies were detected. However, when the type B–specific and type D–specific assays were used against the same amount of type A target, means ± SEM of only 0.3 ± 0.3 and 740 ± 60 copies were detected, respectively. The resulting underestimation of *NPM1*mut copies arising from the mismatch of assays to mutations was significant ($P < 0.001$; one-way analysis of variance). Despite these observations, the apparent ability to achieve a reaction plateau in the case of *NPM1*mut but not wild-type *NPM1* irrespective of mismatch between assay and mutation suggested the possibility that an end point–sensitive technology, such as dPCR, could demonstrate quantitative characteristics in these scenarios. We hypothesized that the observed specificity for *NPM1*muts versus wild-type was likely because *NPM1*mut primers need to loop out their insertion sequence in an energetically unfavorable manner to successfully amplify wild-type *NPM1*, with the insertion sequence itself being a significant but lesser contributor to specificity. We further hypothesized that most *NPM1*mut AMLs would be detectable with a single assay if it were possible to develop a multiplex pool of assays against all known and theoretical insertion *NPM1*muts with subsequent quantification of reaction end points by dPCR.

Development of Massively Multiplex dPCR Assay for *NPM1*muts

Multiplex insertion-specific assays covering all known and theoretical four nucleotide insertions were synthesized for the most common insertion site (position 863) (Table 1). The relative position of primers and probes is indicated schematically in Figure 1C. In parallel, we synthesized assays in which the common insertion site was spanned by universal bases deoxyinosine[35] and 5-nitroindole,[36] which have relaxed base-pairing properties relative to the four standard nucleotides, thus permitting base pairing across a range of insertion sequences. As a baseline, the established qPCR–based assay for type A *NPM1*muts[11] was adapted to dPCR. The performance of these assays was initially compared using cDNA from a patient with AML who tested positive for a type A *NPM1*mut by clinical sequencing (Genoptix Medical Laboratory, Carlsbad, CA). Both the type-specific assay and the multiplex assay produced distinct positive droplet populations indicative of successful amplification of *NPM1*mut with CV values of 5.5% and 5.1%, respectively, among positive droplets (Figure 1D). In contrast, dPCR reactions conducted with deoxyinosine-containing primers produced a nondistinct smear of positive droplets (CV = 19.4%) (Figure 1D), rendering it less useful for MRD detection. 5-Nitroindole–containing primers failed to produce amplified product. Given that the multiplex assay was both the most successful and cost-effective option tested, we continued to evaluate the multiplex assay on other *NPM1*mut and in serial AML cases.

Agreement with Established Assays

To test whether the *NPM1* multiplex assay agrees with established subtype-specific qPCR and dPCR assays already used in MRD assessment, we generated a spike-in dilution series of the cell line OCI-AML3 (type A *NPM1*) into GM12878 (wild-type *NPM1*), ranging from 1:1000 cells to 1:50,000 cells (OCI-AML3:GM12878). *NPM1*mut subtype and variant allele fraction were verified by next-generation sequencing. For each dilution, we quantified *NPM1*mut/10^4 *ABL1* transcript copies according to methods and reporting convention established by Gorello et al.[11] The established type A allele-specific assays in qPCR were then compared with type-specific and multiplex assays in dPCR. For each comparison, we computed Lin's concordance correlation coefficient (ρ_c),[37] which evaluates the extent to which pairwise comparisons agree (ie, fit to a diagonal line with a slope of 1 and *y*-intercept of 0). Previous studies noted general concordance between MRD measurements obtained by qPCR and dPCR using allele-specific primers across a range of common and rare *NPM1*muts.[18] Indeed, we similarly noted excellent concordance between qPCR and dPCR across the range of cell dilutions tested ($\rho_c = 0.998$; 95% CI, 0.994–0.999; qPCR versus dPCR) (Figure 2A). *NPM1*mut/10^4 *ABL1* ratios were further concordant when comparing the new *NPM1* multiplex assay to the type-specific assay in dPCR ($\rho_c = 0.999$; 95% CI, 0.997–0.999; multiplex versus type specific) (Figure 2B), producing essentially identical *NPM1*mut/10^4 *ABL1* ratios (Figure 2C). The 1:50,000 dilution demonstrated *NPM1*mut/10^4 *ABL1* ratios close to double the background seen in negative control GM12878. However, increasing cDNA input 10-fold improved the signal/noise ratio for 1:50,000 cell dilution (Supplemental Figure S1C). OCI-AML3 serial dilutions in MV411, a myelomonocytic cell line, produced similar results (data not shown). This concordance was preserved when using an alternative dPCR technology (BioRad QX200) (Supplemental Figure S1, A and B), indicating that results across platforms are generally comparable. To transition from cell lines to primary samples, we further tested the *NPM1* multiplex assay against type-specific assays in primary AML samples identified as positive for common, type A *NPM1* (Figure 2D), or rare naturally occurring mutation types in patient samples, such as DD1 (c.863_864insCTTG) (Figure 2E), c.863_864insTATG, c.865_866insCAGC, and

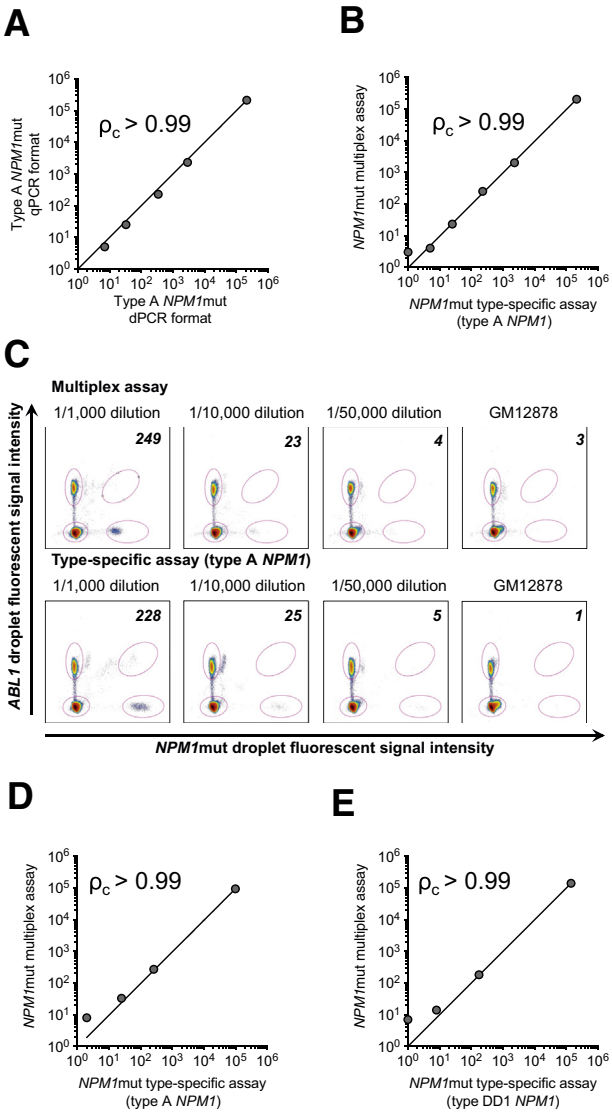

Figure 2 Performance of multiplex *NPM1* minimal residual disease assay on spike-in dilution series. **A:** Correlation of real-time quantitative PCR (qPCR)—based type A—specific assay (vertical axis) to digital PCR (dPCR)—based adaptation of the type A—specific assay (horizontal axis) when using cDNA derived from undiluted OCI-AML3 or OCI-AML3 spiked into GM12878 at 1:1000, 1:10,000, or 1:50,000 cells or GM12878 alone. Axes indicate *NPM1* mutation (*NPM1*mut)/10^4 *ABL1*. **B:** Correlation of the massively multiplex *NPM1* assay (vertical axis) to dPCR-based adaptation of the type A—specific assay (horizontal axis) on the same dilution series as **A**. Axes indicate *NPM1*mut/10^4 *ABL1*. **C:** Dot plot of dPCR data for the multiplex assay (**top row**) and type A—specific assay adapted to dPCR (**bottom row**) on the same spike-in dilution series as **A** and **B**. Dilutions are indicated. The vertical axis indicates the signal intensity of droplets in the *ABL1* channel (VIC), and the horizontal axis indicates positive droplets in the *NPM1* mutant channel (FAM). *NPM1*mut/10^4 *ABL1* copies are indicated in the upper right hand corner of each scatterplot. **D:** Correlation of the massively multiplex *NPM1* assay (vertical axis) to dPCR-based adaptation of the type A—specific assay (horizontal axis) on cDNA from *NPM1*mut acute myeloid leukemia (AML; type A) undiluted or diluted into cDNA from healthy cord blood at 1:1000 or 1:10,000 (w/w) alongside cord blood cDNA alone. Axes indicate *NPM1*mut/10^4 *ABL1*. **E:** Same as **D** except using a type DD1—specific dPCR assay on cDNA from a rare *NPM1*mut AML (type DD1). Lin's concordance correlation coefficient is indicated (ρ_c).

c.863_864insGCCG (Supplemental Figure S2). The cDNA from primary samples was diluted into healthy cord blood cDNA at 1:1000 and 1:10,000 (w/w). Concordance was noted between massively multiplex assay and type-specific assays for all mutation subtypes tested ($\rho_c = 0.995$ to 1.000).

To determine the lower limit of detection of multiplex assay, background signal was measured in a panel of 12 *NPM1*mut-negative samples. The panel included primary AML samples (bearing rearrangements rarely found to co-occur with *NPM1,* such as CBFβ-MYH11 or AML1/ETO),[15] bone marrow from healthy donor, cord blood, and the GM12878 and MV411 cell lines. Total *NPM1* transcripts were also quantified in the same set of samples using an *NPM1*-universal primer with binding site downstream of the 863 position in exon 12 of *NPM1* transcript. *NPM1*-universal primer nonselectively amplifies all *NPM1* transcripts irrespective of the presence or absence of a 4-nucleotide insertion (ie, the number of *NPM1* copies counted consist of wild-type and, if present, mutant transcripts). Background levels in 12 *NPM1*mut wild-type normal primary samples and cell lines tested ranged from 0 to 7 *NPM1*mut/10^4 *ABL1* (median, 1 *NPM1*mut/10^4 *ABL1*) for the multiplex assay compared with 0 to 1 *NPM1*mut (median, 0 *NPM1*mut/10^4 *ABL1*) for the type-specific assay (*NPM1*mut type A) (Supplemental Figure S3). Thus, the limit of detection for the multiplex and type A—specific assays under dPCR conditions are estimated at 2.4 and 1.0, respectively, for normal primary blood samples, well below reported decision cutoffs of 100 to 1000 *NPM1*mut/10^4 *ABL1* copies.[38] Both allele-specific and massively multiplex assays produced a single amplicon as determined by gel electrophoresis thus further corroborating specificity (Supplemental Figure S4).

The multiplex dPCR assay for detection of *NPM1*mut demonstrated overall agreement with established type-specific assays in the spike-in dilution series and the primary AML cells for common and rare *NPM1* subtypes. Moreover, *NPM1*mut/10^4 *ABL1* ratios produced by the multiplex assay agreed with established qPCR—based assays. We thus proceeded to determine its robustness against a variety of rare *NPM1*muts and its ability to monitor MRD in actual patients.

Other Rare *NPM1*mut Types

To further assess the general robustness of the multiplex assay and its ability to detect rare *NPM1*muts, we generated a synthetic *NPM1*mut target pool that contained all possible four nucleotide insertions at the c.863 position of exon 12 such that 100% of DNA fragments contain *NPM1* insertions. The sequence of the synthetic target was verified by Sanger sequencing (Table 2). The synthetic fragment and primers are indicated schematically in Figure 3A. *NPM1* copies were quantified using a multiplex assay or *NPM1* universal assay. If the multiplex *NPM1*mut assay can detect all *NPM1* c.863 insertions, the expectation is

Table 2 Sequence of Synthetic *NPM1* Mutant Transcripts

Insertion	Synthetic sequence
NPM1-c863del864G_insCCGTT	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGCCGTTCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insCAAA	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGCAAAGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insCGTG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGCGTGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insCTCG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGCTCGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insTAGG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGTAGGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insTATG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGTATGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insTCGG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGTCGGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863_864insTTTG	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGTTTGGCAGTGGAGGAAGTCTCTTTAAG-3′
NPM1-c863ins4N	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGNNNNGCAGTGGAGGAAGTCTCTTTAAGAAAATAG-TTTAAACAATTTGTTAAAAAATTTTCCGTCTTATTTCATTTCTGTAACAGTTGATATCTG-GCTGTCCTTTTTATAATGCAGAGTGAGAACTTTCCCTACCGTGTTTGATAAATGTTGTCC-AGGTTCTATTGCCAAGAATGTGTTGT-3′
NPM1-wt	5′-GACCTAGTTCTGTAGAAGACATTAAAGCAAAAATGCAAGCAAGTATAGAAAAAGGTGGT-TCTCTTCCCAAAGTGGAAGCCAAATTCATCAATTATGTGAAGAATTGCTTCCGGATGACT-GACCAAGAGGCTATTCAAGATCTCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAGTTTA-AACAATTTGTTAAAAAATTTTCCGTCTTATTTCATTTCTGTAACAGTTGATATCTGGCTG-TCCTTTTTATAATGCAGAGTGAGAACTTTCCCTACCGTGTTTGATA-AATGTTGTCCAGG-TTCTATTGCCAAGAATGTGTTGT-3′

Sequences for mutant and wild-type *NPM1* synthetic cDNA fragments.

that the number of *NPM1*-positive partitions will not differ statistically if the synthetic target is amplified by *NPM1* multiplex (mutant specific) or the *NPM1* universal primer set. Indeed, we found that the universal and mutant-specific multiplex primer sets resulted in 0.093% positive partitions, indicating that counting was accurate irrespective of subtype (odds ratio, 1.002; 95% CI, 0.956−1.049; $P = 0.934$) (Figure 3B). Given the reported instances of clonal heterogeneity[23] and type switching[24] in selected AML cases and success with the multiplex target pool in Figure 3B, we next sought to determine the ability of the massively multiplex assay to quantify *NPM1*muts in the setting of clonal heterogeneity (Figure 3C). To this end, plasmids harboring type A and/or type B *NPM1*muts were spiked into *NPM1* wild-type GM12878 cDNA alone or combined as a 50:50 mixture of type A and type B (A:B) to target a total of approximately 350 *NPM1*mut/10^4 *ABL1* copies in all cases. Each of the targets was tested with allele-specific assays for type A or type B *NPM1*muts alongside the massively multiplex assay. As expected, the type-specific assays produced accurate measurements, within approximately 5% of the targeted 350 *NPM1*mut/10^4 *ABL1* ratios ranging from 331 to 335 *NPM1*mut/10^4 *ABL1* copies when each assay was correctly matched to the correct *NPM1*mut. Notably, the massively multiplex assay demonstrated similar accuracy against all targets, including the mixture detecting 326, 362, and 360 *NPM1*mut/10^4 *ABL1* copies for the type A, type B, and A:B (50:50) mixture, respectively. However, in sharp contrast, the type-specific assays variably underestimated the *NPM1*mut/10^4 *ABL1* ratio when either used with the wrong *NPM1*mut or challenged with the A:B mixture, producing unreliable measurements ranging from 11 to 278 *NPM1*mut/10^4 *ABL1* copies.

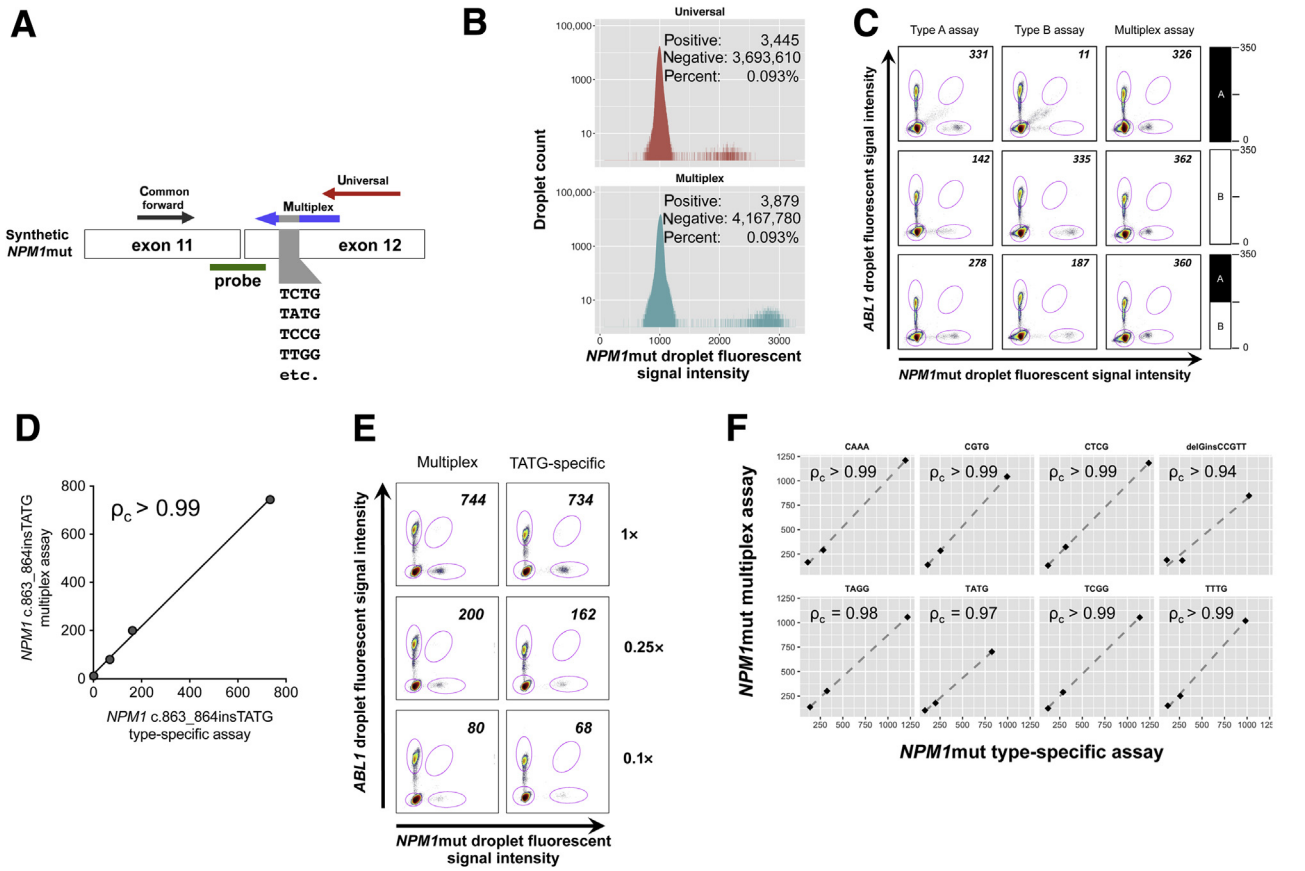

Figure 3 Multiplex *NPM1* assay accurately detects rare *NPM1* mutation (*NPM1*mut) types. **A:** Schematic representation of synthetic *NPM1*mut target consisting of a pool of degenerate *NPM1*mut insertion sequences. Amplification is performed using common forward primer with multiplex reverse primer pool (mutant specific) or the universal reverse primer (amplifies both mutant and wild-type). Common probe is used for all reactions. **B:** Histogram indicating counts and distribution of fluorescence signal intensity of *NPM1*-positive and -negative droplets. **C:** Plasmids harboring type A (**top row**), type B (**middle row**), or a 50:50 mixture of type A and B (**bottom row**) *NPM1* mutations were spiked into GM12878 background cDNA. A target of 350 *NPM1*mut/10^4 *ABL1* copies was used in all cases and the expected content indicated graphically by the bar plot on each row. Detection was attempted by digital PCR (dPCR) using the specific assays for the type A or type B mutation alongside the massively multiplex assay. The vertical axis of each dot plot indicates the signal intensity of *ABL1*-positive droplets, and the horizontal axis indicates *NPM1*mut-positive droplets. *NPM1*mut/10^4 *ABL1* ratios are indicated in the upper right corner. **D:** Synthetic template for *NPM1* c.863_864insTATG is spiked into GM12878 cDNA targeting approximately 1000 *NPM1*mut/10^4 *ABL1* copies and left undiluted (1×) or diluted fourfold (0.25×) or 10-fold (0.1×) alongside negative control cell line GM12878. The axes indicate *NPM1*mut/10^4 *ABL1* as determined by the *NPM1* multiplex assay (vertical axis) versus type-specific assay (horizontal axis). **E:** Scatterplot of dPCR data for the multiplex assay and c.863_864insTATG-specific assay adapted to dPCR for the dilution series from **A** with dilutions 1×, 0.25×, and 0.1× shown. **F:** Correlation between *NPM1*mut/10^4 *ABL1* ratios for a different synthetic rare *NPM1* insertion mutations spiked into GM12878 cDNA targeting an approximately 1000 *NPM1*mut/10^4 *ABL1* starting ratio. Undiluted spike-in mixture (1×) is then compared with fourfold (0.25×) and 10-fold (0.1×). The position 863 insertion sequence is indicated over each plot except for delGinsCCGTT, which is *NPM1* c.864_865delGinsCCGTT. The axes indicate *NPM1*mut/10^4 *ABL1* ratios for the multiplex assay (vertical axis) versus type-specific assays (horizontal axis). Lin's concordance correlation coefficient is indicated (ρ_c).

Next, to further explore the ability of multiplex assay to detect *NPM1*mut transcripts independently of the subtype sequence, we proceeded to test our *NPM1*mut-specific multiplex assay on individual rare insertion sequences reported recently by Ivey et al[10] along with the published type-specific assay for those mutations (Table 1). *NPM1* copies were determined using both the *NPM1* multiplex assay and type-specific assay, testing a subset of rare *NPM1* insertions on dual dPCR platforms. First, *NPM1* c.863_864insTATG synthetic targets in a dilution series were spiked into GM12878 alongside nonspiked GM12878 controls. *NPM1*mut/10^4 *ABL1* ratios obtained by type-specific and multiplex assay were concordant (ρ_c = 0.998; 95% CI, 0.981−1.000) (Figure 3D), producing similar values and distinct clusters of positive dPCR partitions/droplets (Figure 3E). Measurements of *NPM1*mut copies were also consistent across dPCR platforms using the *NPM1* multiplex assay (ρ_c = 0.985; 95% CI, 0.861−0.999) (Supplemental Figure S5 and Supplemental Table S1). We additionally generated synthetic *NPM1*mut spike-in dilution series for eight rare *NPM1*muts and compared *NPM1*mut/10^4 *ABL1* ratios derived from multiplex and type-specific assays (Figure 3F). All the *NPM1*muts demonstrated substantial concordance between type-specific and multiplex assays (ρ_c = 0.97 to 0.99), with the notable exception of *NPM1* c.864_865delGinsCCGTT, which contains an extra

mismatch, likely leading to reduced PCR priming efficiency. The multiplex assay detected *NPM1* insertions in all cases tested.

Serial Monitoring of MRD in Patients with AML

To ascertain the potential utility of the multiplex assay in patient care, we determined molecular MRD levels sequentially in three patients with AML undergoing treatment at the Leukemia Program of Weill Cornell Medicine and New York Presbyterian Hospital. Patients had confirmation of *NPM1*mut by an independent diagnostic laboratory, and all clinical decision making was based on the results from the Clinical Laboratory Improvement Amendments–certified laboratory. Bone marrow aspirates (BMAs) and/or peripheral blood (PB) were collected periodically from patients with AML seen during their care. The patients signed informed consent documents approved by the institutional review board and agreed to participate in a research protocol for serial sampling of PB and bone marrow. One of these patients presented to our clinic in remission without prior sequencing having been diagnosed for *NPM1*mut AML only by capillary electrophoresis.

Patient 1 was confirmed positive for type B *NPM1*mut (c.863_864insCATG) by routine clinical sequencing performed as standard care (Genoptix). *NPM1* MRD levels were assessed during a period of 801 days (Figure 4A). PB and BMAs demonstrated significant decreased levels of *NPM1*mut ($<$100 *NPM1*mut/10^4 *ABL1*) after therapy measured at day 297 as determined by the type-specific assay (type B) and *NPM1* multiplex assay, with both assays demonstrating overall agreement and trending upward over time. *NPM1*mut levels are expected to be higher in BMA versus PB based on previous reports.[10,15] However, the present case demonstrated higher PB mutant *NPM1* percentages compared with BMA at day 514 and 731. The patient eventually evolved to relapsed disease after 836 days.

Patient 2 is a 62-year-old woman with a history of breast cancer treated with multiagent chemotherapy and radiation who presented for care at our center after having been diagnosed with AML with normal cytogenetics and treated elsewhere. She received a standard cytarabine and anthracycline-based induction, followed by four cycles of high-dose cytarabine consolidation. The patient had an *NPM1*mut identified at the time of diagnosis using capillary electrophoresis by a clinical diagnostic laboratory. However, a diagnostic specimen was not available to determine the patient's *NPM1* sequence. We thus followed up *NPM1*mut MRD levels for 329 days (Figure 4B) using the *NPM1* multiplex assay. A surge in *NPM1*mut transcripts was noted in BM from day 28, reaching 1012 *NPM1*mut/10^4 *ABL1* copies. Leftover RNA initially isolated for MRD assessment was submitted for ultradeep targeted mRNA-sequencing hybrid capture sequencing to determine the *NPM1* subtype. This analysis revealed the *NPM1* subtype as type D (c.863_864insCCTG) supported by 48 (0.34%) of 14,271 reads (Figure 4D) and incidentally identified an *IDH1* (p.Arg132His; R132H) unknown in this patient supported by 32 (0.9%) of 3679 reads (Figure 4E). The type-specific and multiplex assay closely tracked each other, with levels of *NPM1*mut gradually increasing in the PB and BMA during MRD monitoring through this patient's eventual clinical and morphologic relapse 6 months later. On relapse, clinical DNA sequencing (Genoptix) identified the *IDH1* R132H mutation at 6% variant allele fraction but not the type D *NPM1*mut. Targeted deep DNA sequencing then confirmed the presence of the type D *NPM1*mut at 3% variant allele fraction. The patient is currently undergoing treatment with an investigational isocitrate dehydrogenase (IDH1) inhibitor, which was associated with an observed reduction in *NPM1*mut transcripts during the resistant disease stage.

Patient 3 presented with a rare *NPM1*mut subtype (c.865_866insCAGC) confirmed by routine clinical sequencing. A type-specific assay was designed to retrospectively monitor *NPM1* status alongside multiplex assay during a period of 660 days (Figure 4C). *NPM1*mut copies drastically decreased after standard induction therapy after day 27, consistent with treatment response and progression into remission status. The patient received a stem cell transplant and remained in remission for $>$1 year, after which the patient relapsed as indicated by the increase in *NPM1*mut copies with levels comparable to those observed at baseline. Reemergence of an *IDH1* mutation present at baseline was observed by next-generation sequencing, and at this point treatment was started with an IDH1 inhibitor under clinical trial. Finally, the patient evolved to resistant disease and secondary remission, with *NPM1*mut levels trending downward. The patient is currently in second remission. Type-specific and multiplex assays showed close agreement at all times in a case that is not otherwise tractable for clinically reportable molecular MRD evaluation by the most common qPCR tests.

Discussion

Historically, the most important prognostic criteria in AML have been age and cytogenetics. Recently, molecular genetics are also emerging as important prognostic criteria and, in some cases, a means to identify potential targets for treatment. Despite these advances, outcomes remain poor, especially for older patients with AML and for those of any age with unfavorable cytogenetics. Recent studies suggest that assessment of *NPM1*mut transcript copies as a marker of MRD improves risk stratification over established cytogenetic and molecular criteria.[2–4,10,39] Indeed, a recent prospective study of 223 patients revealed subgroups of patients with *NPM1*muts with improved survival when the PCR results were *NPM1* negative, despite having prognostically adverse co-occurring mutations, such as *FLT3*-ITD and/or *DNMT3A*.[10] In addition, several studies have found that patients with AML who are treated with allogeneic stem cell transplantation in first remission have distinctly more

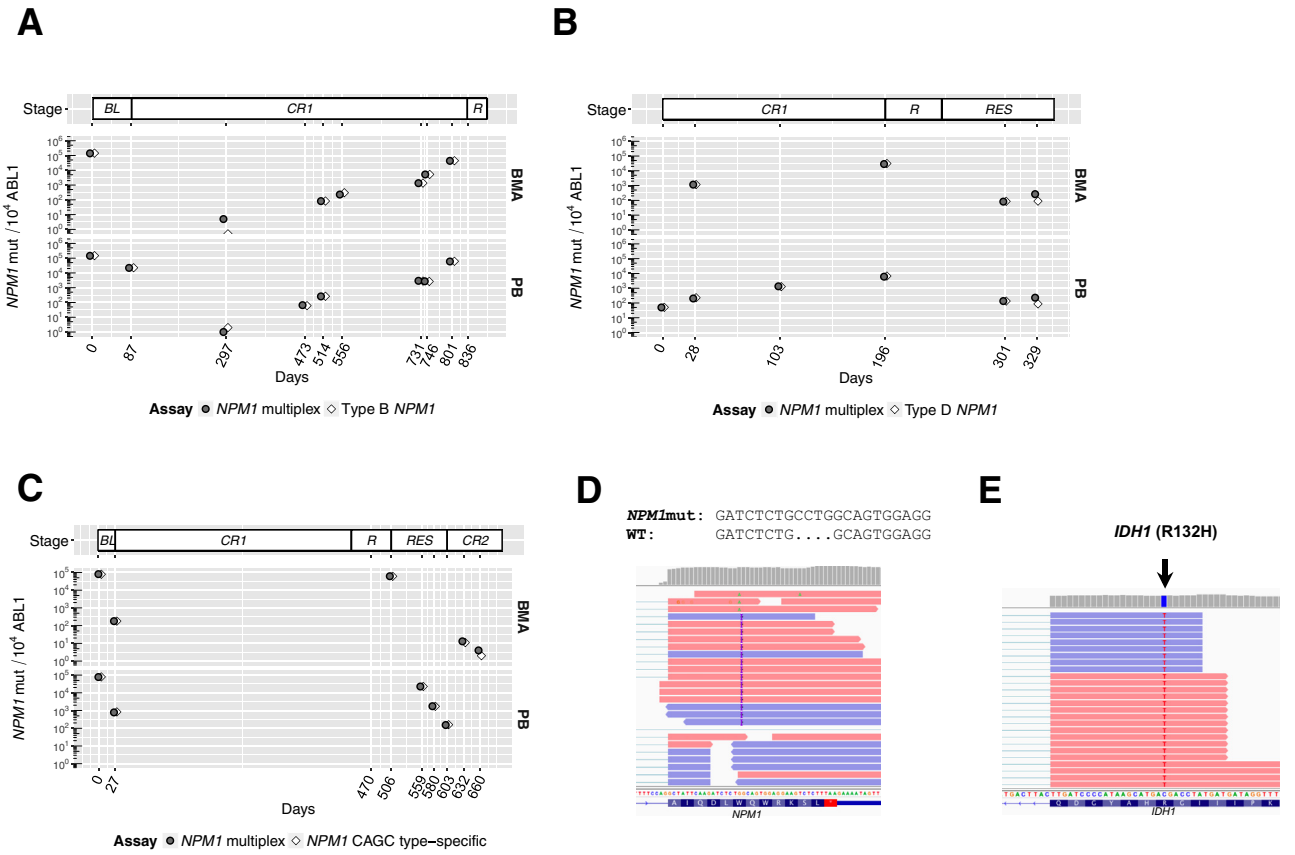

Figure 4 Monitoring of minimal residual disease in serial acute myeloid leukemia (AML) cases. **A:** Patient 1 [type B *NPM1* mutated (*NPM1*mut)]: *NPM1*mut/10^4 *ABL1* as a function of time (**top panel**). The vertical axis indicates *NPM1*mut/10^4 *ABL1*, and the horizontal axis indicates time since initial monitoring in days. Diamond points indicate results with the type B–specific assay. Circular points indicate results with the *NPM1* multiplex assay. Peripheral blood (PB) and bone marrow aspirate (BMA) samples are shown in the **bottom** and **middle panels**, respectively. Disease status is indicated in the **top panel**. **B:** Patient 2 (type D *NPM1*mut): *NPM1*mut/10^4 *ABL1* as a function of time (**top panel**). The vertical axis indicates *NPM1*mut/10^4 *ABL1*, and the horizontal axis indicates time since initial monitoring in days. Diamond points indicate results with the type D–specific assay. Circular points indicate results with the *NPM1* multiplex assay. PB and BM samples are shown in the **bottom** and **middle panels**, respectively. Disease status is indicated in the **top panel**. **C:** Patient 3 [*NPM1* c.865_866ins(CAGC)]: *NPM1*mut/10^4 *ABL1* as a function of time (**top panel**). The vertical axis indicates *NPM1*mut/10^4 *ABL1*, and the horizontal axis indicates time since initial monitoring in days. Diamond points indicate results with the CAGC type-specific assay. Circular points indicate results with the *NPM1* multiplex assay. PB and BM samples are shown in the **bottom** and **middle panels**, respectively. **D:** The insertion sequence identified by deep targeted mRNA sequencing for patient 2 above the alignment of reads supporting the type D *NPM1* insertion. **E:** Reads supporting *IDH1* (p.ArgR132His) mutation identified by deep targeted mRNA sequencing for patient 2. BL, baseline; CR1, complete remission 1; CR2, complete remission 2; R, relapse; RES, resistant disease; WT, wild-type.

favorable outcomes if undergo transplantation without detectable MRD, as measured by immunophenotype.[40] Thus, MRD monitoring in general, and MRD monitoring of patients with *NPM1*mut AML in particular need to become more widely accessible tools for prognostication and treatment planning in general practice not only for patients in clinical trials.

Selected patients with *NPM1*mut AML are currently ineligible for quantitative MRD monitoring because their *NPM1*mut is rare or their insertion sequence is unknown because detection was performed using capillary electrophoresis. It is possible to use qPCR difference-in-cycle thresholds[1] as an alternative for rare *NPM1*muts, but this approach is sensitive to input RNA and does not express MRD in *NPM1*mut per 10,000 *ABL1* copies, making cross-study comparisons challenging, especially when patient materials are limiting. The development of many individual patient-tailored tests is also a possibility but may pose challenges in the clinic, where each type-specific PCR assay and its corresponding quantitative standard require rigorous quality control and regulatory approval that may not be feasible in agency-regulated clinical laboratories, such as those encountered in the United States. Moreover, site-to-site differences in tests developed at different laboratories for rare mutations may further fragment studies across sites and raise the complexity of interpreting results across centers. Finally, AML is a dynamic and heterogeneous disease that can evolve. Reports demonstrating intrapatient *NPM1* heterogeneity and type switching indicate the possibility of MRD underestimation or misdiagnosis in some patients with type-specific assays, which can result in adverse patient outcomes when used to guide patient care.

The multiplex assay described in this work was effective across a range of diverse common and rare *NPM1*muts, with

overall concordance with type-specific assays. The efficacy of the new multiplex assay was demonstrated in a total of 14 common and rare subtypes and accurately measured *NPM1*mut even in an extremely challenging heterogeneous pool of 256 subtypes, illustrating the extreme robustness of the approach. The observed background was higher compared with type-specific assays but still well below proposed cutoffs for treatment decisions.[38] In addition, the assay successfully quantified *NPM1*mut in a mixture of different mutant subtypes, illustrating its utility in cases of clonal heterogeneity. *NPM1* levels are expressed by the new assay in the same units as previous *NPM1* studies, thus enabling comparison to concurrent and historical work.[10,11,15] Importantly, the multiplex test remained effective and quantitative in the absence of sequence information at diagnosis and matched the upward and downward MRD trend in serially monitored patients seen with *NPM1*mut MRD assays. Overall, these data indicate that a single, easily deployed *NPM1*mut test can effectively simplify *NPM1*mut MRD testing capabilities for laboratories, while reducing the potential complexities associated with *NPM1* quantification.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2017.03.005*.

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