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Input Matters: DNA vs RNA for NPM1 Mutation Detection in AML

Input Matters: DNA vs RNA for *NPM1* Mutation Detection in Acute Myeloid Leukemia

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Background

Human nucleophosmin 1 (*NPM1*) is a protein encoding gene involved in diverse cellular processes, including but not limited to cell proliferation, histone chaperoning and genomic stability and repair. Thus, NPM1 function is critical for normal cellular biology. Human *NPM1* is located on chromosome 5q35, consists of 12 exons and encodes at least two isoforms. This white paper will focus on the full-length protein transcript of 294 amino acids which is expressed abundantly in all tissues.

Mutations in *NPM1* are common in acute myeloid leukemia (AML), occurring in approximately 30% of adult cases. More than 50 mutation variants in *NPM1* occur in exon 12. The most frequent are A, B and D. Type A involves a TCTG duplication which represents 75-80% of cases in *NPM1*-mutated AML. Type B, with a CATG insertion, accounts for 10%, while Type D, with a CCTG insertion, constitutes 5% of cases. Type A, B and D mutations result in a frameshift and altered C-terminal sequence leading to a loss of nucleolar localization, gain of a nuclear export signal, and mis-localization of NPM1 to the cytoplasm. These changes disrupt critical cellular processes, drive *HOX* gene upregulation, and establish a leukemic state, making exon 12 mutations a primary driver of leukemogenesis in AML[1]. Other rare mutations that account for less than 1% of cases are found in exons 5, 9, and 11 which cause abnormal cytoplasmic localization of NPM1.

As the understanding of genetic variability in AML has grown, the ability to rapidly detect mutations to guide treatment decisions has become essential. Large-scale DNA sequencing, although costly, is now accessible and practical for routine use, especially at diagnosis. Next-generation sequencing (NGS) enables comprehensive gene panel testing, allowing AML patients to be screened for mutations at diagnosis and aiding WHO classification and The European LeukemiaNET (ELN) risk assessment. However, while NGS is invaluable for mapping mutations, it cannot yet meet the 24–48-hour turnaround recommended for key mutations such as *NPM1* mutations. Other methods such as fragment analysis or quantitative PCR methods can be used during the diagnosis stage for such mutations, as a fast and affordable test. Faster NGS processing is theoretically possible but remains financially challenging for widespread clinical applications[1].

Diagnosis constitutes one part of the AML patient journey; it is crucial to assess the success of the treatment. According to the ELN 2022 guidelines, *NPM1* mutations are valuable markers for the monitoring of measurable residual disease (MRD) in patients during AML treatment[2]. MRD monitoring holds prognostic value and enables the early identification of relapse through the identification and analysis of residual leukemic cells. Persistent detection of *NPM1* mutations after the second round of chemotherapy indicates an 82% relapse risk, compared to 30% in patients without detectable levels. Additionally, MRD status before allogeneic stem cell transplant is a strong predictor of outcomes, with MRD-negative patients showing significantly higher 2-year survival rates (83%) versus high-MRD patients (13%)[3]. Clinically available assays such as reverse transcriptase quantitative PCR (RT-qPCR) measured on the RNA-level, or quantitative PCR (qPCR) and droplet digital PCR (ddPCR) measured on the DNA-level are used for *NPM1* MRD assessment[1]. The use of DNA and RNA in MRD monitoring each offer unique advantages and limitations for mutation analysis.

superRCA technology enables ultra-sensitive detection of mutations, including those of the *NPM1* gene. This novel technique converts DNA into fluorescent particles using two rounds of Rolling Circle Amplification (RCA) and padlock probes to distinguish between wild-type and mutant events with extraordinary specificity. The second round of RCA, named superRCA, permits the high sensitivity of the assay due to the sheer number of repeated copies of each target available for genotype probing[4]. Assay readout is performed using conventional flow cytometers, giving a fully quantifiable assay, driving radically improved mutation detection, using a standard DNA input.

Standardized, sensitive and quality assessment assays are crucial for effective *NPM1* MRD monitoring in large testing centers. This can be achieved through either DNA or RNA assays, each offering specific advantages and limitations. This white paper compares DNA versus RNA for *NPM1* mutation detection, focusing on parameters such as stability, sensitivity, dynamic range, and functional insights.

1. Stability

DNA is highly stable and can be stored long-term without significant degradation. This allows for retrospective analyses and easy handling in clinical settings, making DNA a more robust option for mutation detection across multiple sample collection and storage stages[5]. While DNA stability is an advantage, this feature limits the ability to detect real-time cellular changes. Since DNA is not subject to rapid degradation, it cannot reflect the dynamic expression changes that occur in response to therapies or disease progression. RNA is inherently unstable and prone to rapid degradation, making it more difficult to handle in clinical workflows. Samples need to be processed quickly and stored under stringent and costly conditions to prevent RNA degradation. RNA-based assays, while less stable, offer a snapshot of the gene expression profile at the time of sample collection. This real-time insight into mutation expression can be valuable for understanding the active mutational landscape in AML[5].

2. Sensitivity

In the context of DNA and RNA based assays, sensitivity refers to the ability of the assay to detect low levels of the target nucleic acid. There are several factors that can affect assay sensitivity including the nucleic acid used, extraction and purification methods, contamination, the target sequence, and the applied amplification technology. Regarding diagnostic and prognostic workflows, sensitivity is a critical factor for accurate sample analysis.

RT-qPCR can be sensitive to low-frequency mutations, particularly those that are actively expressed, offering a better understanding of mutation dynamics in cancer cells. This makes RNA a good option for detecting MRD and rare mutations. However, RNA's sensitivity is compromised by its instability and degradation, which can affect the quality of the assay, especially in samples with low RNA yield. cDNA is usually synthesized from RNA to enable analysis; this adds an extra step to the workflow compared to DNA which can be analyzed directly. The technical biases in reverse transcription can lead to under-representation of certain mutations. On the other hand, DNA-based approaches may overlook certain low-frequency mutations if they do not occur in sufficient numbers of cells. Additionally, mutations that do not have an immediate functional impact may be detected, leading to potential over-interpretation of clinically irrelevant findings.

NPM1 has been established as a reliable MRD marker for AML[2]. In this context, RT-qPCR is generally used but detection is limited to specific mutation variants. DNA has been investigated as an alternative, owing to its stability and lack of bias in variant detection. In a study by Vonk *et al.*, NGS was applied on a cohort of cDNA and DNA patient samples with *NPM1* mutations in remission post chemotherapy treatment[6]. The NGS VAF frequency of 0.01% corresponded to the threshold of RT-qPCR, concluding that DNA offers a robust

alternative to RT-qPCR for monitoring *NPM1* MRD in AML. In another comparative study, 110 samples of *NPM1* mutated AML were retrospectively analyzed. DNA based assays qPCR, ddPCR and targeted deep sequencing were compared to RNA based RT-qPCR. Using a DNA MRD cutoff point determined from RT-qPCR values that suggested a prognostic relevance, the three DNA methods exhibited strong correlation to RT-qPCR, with qPCR showing the highest correlation[7]. qPCR results in peripheral blood matched the RT-qPCR analysis in 95% of the samples as opposed to 85% in bone marrow, indicating superior sensitivity of RT-qPCR. However, leukemic DNA was detected in roughly 10% of samples using qPCR, with transcripts undetectable using RT-qPCR, suggesting there is a benefit for some patients to undergo DNA analysis. The authors concluded that transcript analysis is more complex than assuming transcript numbers reflect remaining disease burden and predict the course of disease.

In addition, the superRCA *NPM1* mutation assay has been compared to RNA-based RT-qPCR and DNA-based deep sequencing in a study of 86 bone marrow and blood samples from AML patients. superRCA showed strong correction to the two methods and when using detectable signals as a cutoff in the bone marrow and blood samples - sensitivity and specificity to RT-qPCR was 89% and 84%, respectively[8].

4. Mutation detection, functional Insights and gene expression

RNA-based approaches detect mutations that are actively transcribed, providing insight into which mutations are functionally expressed allowing clinicians to assess the functional relevance of the mutations. This allows for more targeted treatment, as only expressed mutations may be relevant for therapy. The dynamic nature of RNA expression means that some mutations may not be detected if they are not being actively transcribed at the time of sampling. *NPM1* mutations in exon 12 are functionally expressed[1] and thus detectable at both RNA and DNA level. However, RNA expression levels can vary significantly between different phases of the disease or treatment, and the MRD-levels may differ between different patients[9].

DNA captures the entire genome regardless of gene expression; therefore, its analysis enables the detection of all mutations present in the genome, including those that are silent or not expressed. This makes DNA assays highly accurate in identifying the presence or absence of mutations without concern for their downstream effects. Naturally, DNA analysis cannot capture whether the mutation is expressed or functional. This limitation can be significant in AML, where not all mutations found in DNA result in disease-driving changes.

Conclusions

DNA and RNA each provide valuable but distinct insights into the detection of *NPM1* mutations in AML. DNA-based methods like qPCR, ddPCR, and deep-seq provide a more stable measurement unaffected by RNA expression variability, making them a reliable alternative when RNA stability is compromised. In addition, DNA-based assays offer ease of use, and direct mutation detection, making them ideal for routine diagnostics. However, they cannot provide real-time functional insights. While less stable and more prone to variability, RNA assays such as RT-qPCR allow for the detection of expressed mutations, offering a more dynamic view of mutation impact on disease progression. However, advances in technology and further studies have pointed to DNA based analysis as a suitable alternative for RNA analyses that have additional workflow step and a less stable starting material.

The study and implementation of ultra-sensitive and DNA methods, such as superRCA[4], to investigate *NPM1* mutations at the DNA level in AML enables a future where it is possible to surpass the current gold standard of NPM1 assay sensitivity. Higher sensitivity is essential for early prediction and potential prevention of relapse of the disease, giving a window of opportunity for treatment decision making that otherwise would not be possible.

Table 1: Summary of the key differences between DNA and RNA analysis and the conclusions that can be drawn from each.

DNA	RNA
✓ Detects coding and non-coding mutations	✓ Detects expressed mutations exclusively
✓ Stable – suitable for retrospective sample analysis	✓ Identifies functional gene fusions and splice variants
✓ Work with DNA directly, no reverse transcription step required	✓ Reflects current cellular activity
× Does not reveal gene expression	× Requires fresh samples to achieve high quality RNA
Get the full mutation landscape	**Get a dynamic snapshot of gene function**

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