

APPLICATION NOTE

MRDTect™ NPM1 and FLT3 ITD Panel

Data characterization and panel performance

Panel Overview

- **Number of genes: 2 genes**
Targeted coverage of *FLT3* ITD and *NPM1* exon 12 mutations
- **Total panel size: 307 bp**
- **Variant types: SNVs, indels, small duplications, internal tandem duplications (ITDs)**
- **Sensitive detection in the range of 10^{-4} and 10^{-5} of ITDs and *NPM1* typing variants**
Detection limit for other variants = 0.1%
- **Recommended DNA input:**
For a target VAF of 10^{-4} : 500-700 ng
For a target VAF of 10^{-5} : 700 ng
- **Sample types: DNA from whole blood or PBMCs**
- **Mapping rate: ~99.6%**
- **Percentage on-target aligned reads: ~99.2%**
- **Recommended reads per sample:**
For a target VAF of 10^{-4} : $\geq 1.2\text{M}$ paired-end reads
For a target VAF of 10^{-5} : $\geq 8.9\text{M}$ paired-end reads
- **Total assay time (from DNA to sequencer): <8.5 hrs**

ultrahigh-sensitivity next-generation sequencing (UHS-NGS) for MRD assessment in *FLT3* internal tandem duplication (ITD)-positive AML, particularly after intensive chemotherapy and before allogeneic hematopoietic cell transplantation.

NPM1 mutations and *FLT3*-ITDs are important molecular markers for AML MRD assessment, but they are commonly evaluated using different technologies. *NPM1* MRD testing has traditionally relied on highly sensitive qRT-PCR assays, which primarily target common mutation subtypes such as types A, B, and D; less common variants may require alternative or patient-specific assays. Further, RNA-based *NPM1* assays have pre-analytical variability and are not easy to integrate with DNA-based *FLT3*-ITD assays. Separate testing of the two markers leads to increased sample input requirement, laboratory burden, testing cost, and turnaround time.

FLT3-ITD MRD assessment presents its own analytical challenge because of the heterogeneous sequence and length of ITDs. Existing amplicon-based NGS methodologies typically require long-read, paired-end sequencing configurations (e.g., 2×300 bp paired-end reads) but are still limited in detection of ultra-long ITDs ($>200\text{bp}$) and often have amplification bias against longer ITDs. On the other hand, hybrid-capture-based UMI NGS approaches involve complex workflows along with longer read length configurations, that also leads to increased testing cost, workflow complexity and turnaround time. Table 1 summarizes these practical limitations and the corresponding design considerations addressed by Pillar Biosciences' MRDTect™ *NPM1* and *FLT3* ITD Panel.

The MRDTect *NPM1* and *FLT3* ITD Panel uses a single gDNA input to detect *NPM1* exon 12 mutations and a broad range of *FLT3*-ITDs. The assay is compatible with standard 2×150 bp paired-end sequencing and is designed for ultrahigh-sensitivity detection, including

Introduction

Measurable residual disease (MRD) is an important prognostic marker in acute myeloid leukemia (AML), providing information on treatment response and risk of disease recurrence beyond conventional assessment methods. Molecular MRD testing enables sensitive tracking of leukemia-associated alterations during and after treatment. The 2025 European LeukemiaNet (ELN) MRD recommendations emphasize the use of leukemia-specific molecular markers and recommend assays validated to limits of detection of 10^{-4} to 10^{-5} . Notably, the updated recommendations introduce

Consideration	ELN 2025 Guidance / Current Practice	Practical Challenge	MRDTest™ NPM1 and FLT3 ITD Panel
MRD targets and workflow	<i>NPM1</i> * MRD is typically assessed by qPCR, while <i>FLT3</i> -ITD MRD may be assessed by fragment analysis or UHS-NGS. These approaches use different analytes and workflows.	Monitoring both markers may require separate assays, RNA/cDNA and gDNA inputs, and distinct analytical workflows.	Simultaneous detection of <i>NPM1</i> mutations and <i>FLT3</i> -ITDs from a single gDNA input and workflow.
Mutation coverage	Standardized <i>NPM1</i> qRT-PCR assays primarily target common subtypes; less common variants may require patient-specific assays.	Non-common <i>NPM1</i> subtypes may not be covered by a standardized assay, and high-sensitivity testing can require substantial input material for a two-assay design.	Detects common and non-common <i>NPM1</i> exon 12 mutations together with <i>FLT3</i> -ITDs from a single gDNA input.
Analytical sensitivity	Molecular MRD assays should be validated to a LOD of 10^{-4} to 10^{-5}	UHS remains challenging for NGS	Demonstrated sensitivity of approximately $2-5 \times 10^{-5}$ VAF for both targets
FLT3-ITD detection[†]	UHS-NGS methods should reliably detect ITDs across a broad length range, including ITDs up to ~200 bp	Long ITDs can be affected by amplification and sequencing bias and require specialized assay designs	Designed to detect <i>FLT3</i> -ITDs from approximately 3 bp to >300 bp
Sequencing configuration	Conventional <i>FLT3</i> -ITD UHS-NGS approaches commonly use extended paired-end reads (e.g., 2×300) or another validated approach capable of detecting long ITDs	Longer sequencing runs can increase sequencing time, cost, and workflow requirements and restricted to specific platform/flow cell	Compatible with standard 2×150 bp paired-end sequencing

Table 1. Overview of the current landscape for *NPM1* and *FLT3*-ITD detection and advantages offered by MRDTest™ *NPM1* and *FLT3* ITD Panel. *Standard mut*NPM1* qRT-PCR assays primarily target common subtypes (A, B, and D). Approximately 5-10% of *NPM1*-mutated AML cases harbor rare variants that may require patient-specific qRT-PCR assays, and ELN 2025 mandates confirmation of diagnostic-sample detectability of the rare types. [†]*FLT3*-ITD lengths range from ~3 to >400 bp with most clinically observed ITDs being ~18–206 bp (median ~48 bp).

longer *FLT3*-ITDs from approximately 3 bp to >300 bp. By consolidating both targets into a single workflow, the panel provides a streamlined approach for longitudinal monitoring of patients with informative *NPM1* and/or *FLT3*-ITD mutations identified at diagnosis.

Materials and Methods

► Panel design and workflow

The MRDTest *NPM1* and *FLT3* ITD Panel was developed as a highly targeted assay for genomic DNA-based detection of *NPM1* exon 12 mutations and *FLT3* internal tandem duplications (ITDs). The assay was designed to support ultrahigh-sensitivity (UHS) MRD

testing, with targeted detection of variants demonstrated at 5×10^{-5} VAF. The panel design supports detection of *FLT3*-ITDs ranging from 3 bp to ≥300 bp.

The panel uses Pillar Biosciences' SLIMamp® multiplex PCR chemistry. Following DNA extraction, targeted enrichment and indexing are completed using standard laboratory equipment, with approximately 3.5 hours of hands-on time and less than 8.5 hours of total library preparation time; flexible stopping points are included for users with time constraints. Prepared libraries can be loaded onto a compatible sequencing system on the same day. The single-pool workflow minimizes sample handling and avoids the need to divide specimens across multiple primer pools. PiVAT® analysis subsequently processes each sample in ~3-10 minutes, generating annotated variant calls together with run-, sample-, and amplicon-level quality metrics (Figure 1).

Sequencing data are analyzed using PiVAT® (Figure 2) v2026.1.0 and beyond, which performs read alignment to GRCh37/hg19, followed by local realignment to improve paired-end read assembly and InDel detection to reduce sequencing noise. Non-uniquely mapped reads, off-target amplicons, and other low-confidence

Product	Source	Catalog No.	N
Mimix™ Myeloid Cancer Panel	Horizon Discovery	HD829	19
PL-21	DSMZ	ACC 536	13
Human Genomic DNA, male	Zyagen	GH-180M	10
Clinical	Clinical labs	N/A	57
Total Samples			99

Table 2. Specimens analyzed in this Application Note.

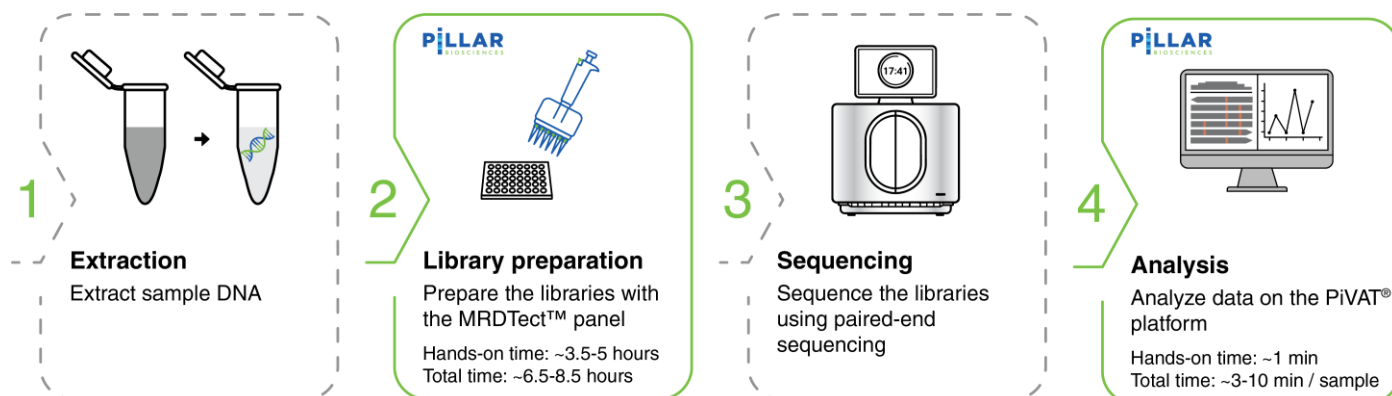


Figure 1. End-to-end workflow for the MRDTect *NPM1* and *FLT3* ITD Panel with PiVAT data analysis. The workflow consists of four sequential steps from sample processing to data interpretation. Steps highlighted in green (library preparation and data analysis) indicate components facilitated by the MRDTect™ *NPM1* and *FLT3* ITD panel and PiVAT software. **(1) DNA Extraction:** Genomic DNA is isolated from input samples. **(2) Library Preparation:** Targeted amplification is performed using an MRDTect *NPM1* and *FLT3* ITD Panel. **(3) Sequencing:** Prepared libraries are pooled and sequenced. **(4) Data Analysis using PiVAT:** Sequencing data are processed using PiVAT, which performs local realignment to generate overall sequencing and mapping metrics, and accurate SNV/indel and ITD calls.

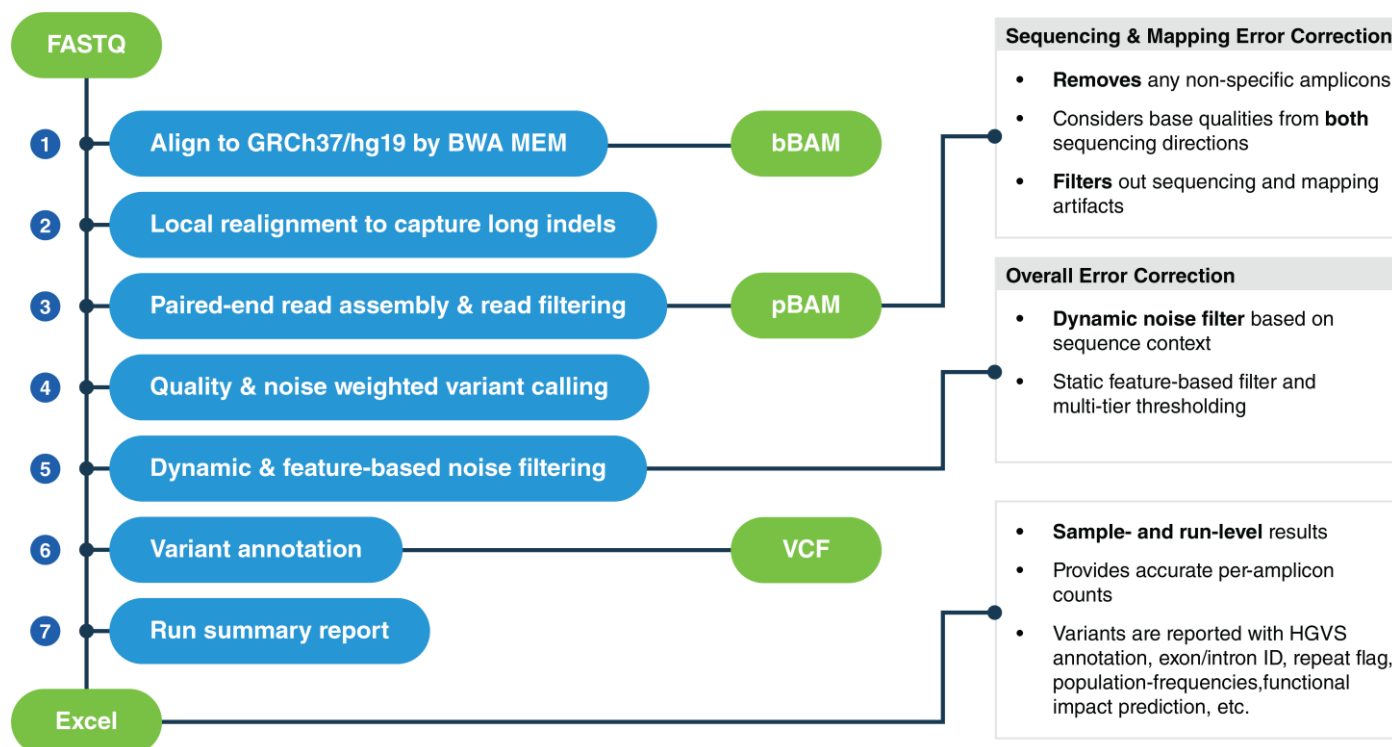


Figure 2. PiVAT analysis workflow for amplicon-based next-generation sequencing data. PiVAT implements a series of refinement steps such as local realignment, paired-end read assembly, and read filtering to improve alignment accuracy and enable robust detection of genomic variants including long indels. Multiple error-correction strategies are utilized for filtering noise, including removal of non-specific amplicons, bidirectional base-quality assessment, sequencing and mapping artifact suppression, quality- and noise-weighted variant calling, and dynamic sequence context-dependent noise filtering with complementary feature-based filtering. Variants are called with high confidence, and reported in VCF, while sample- and run-level quality control metrics, including per-amplicon performance statistics, are generated as spreadsheet reports.

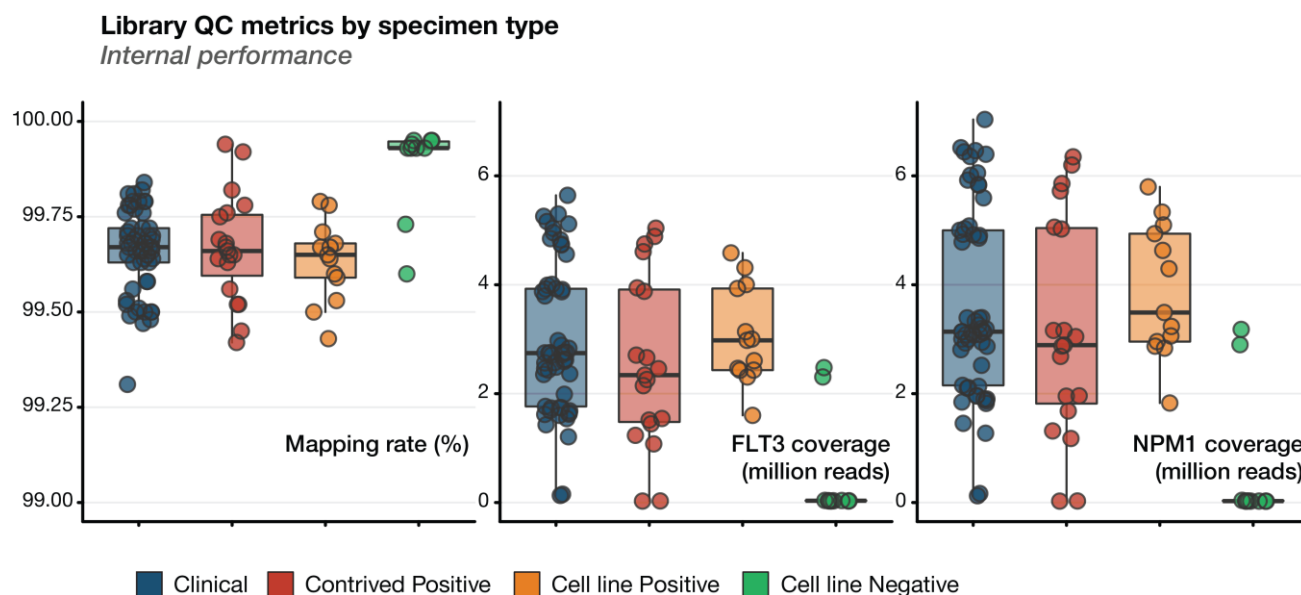


Figure 3. Library quality control (QC) metrics across specimen types (internal performance). Sequencing QC for 99 internal libraries, grouped by specimen class: dilutions from five clinical AML specimens, contrived positive (HD829), cell line positive (PL21), and cell line negative (Zyagen). Boxplots summarize the median, interquartile range (IQR), and 1.5× IQR, with individual points representing independent samples. **(Left)** Mapping rate, defined as the percentage of sequencing reads aligned to the human reference genome (GRCh37/hg19). Sequenced coverage for *FLT3* **(Center)** and *NPM1* **(Right)** amplicons across samples showed relatively consistent levels between the genes.

signals are filtered before variant calling. Candidate variants are subsequently evaluated using quality- and noise-weighted calling and post-call filtering, with sample- and run-level QC metrics generated as part of the automated analysis workflow. PiVAT also includes dedicated processing for long indels and *FLT3*-ITDs.

► Samples

Panel performance was evaluated using 99 internally generated libraries comprised of commercially available reference materials, cell-line DNA, negative controls, and clinical specimens. The study included Horizon Discovery Mimix™ Myeloid Cancer Panel material (HD829; $n = 19$), PL-21 cells containing a long *FLT3*-ITD ($n = 13$), Zyagen male genomic DNA negative controls ($n = 10$), and clinical specimens obtained from external laboratories ($n = 57$). These materials were used to evaluate sequencing quality, target coverage, analytical sensitivity, and quantitative VAF performance across both *FLT3*-ITD and *NPM1* targets (Table 2).

For external performance evaluation, additional clinical specimens were prepared and sequenced at four partner laboratories. These datasets encompassed 111 externally prepared libraries and included a range of dilutions, DNA inputs, and sequencing depths, enabling

assessment of assay performance under different laboratory and sequencing conditions.

Analytical sensitivity was assessed using serial dilutions of positive control and clinical specimens prepared with 500-700 ng DNA input. Dilutions extended from 1% VAF to 0.001% VAF, allowing performance to be evaluated across and below the target MRD range of 10^{-4} to 10^{-5} VAF. Samples tested in the study had *FLT3* ITDs in the range of 54bp to 300bp.

Results

► Sequencing performance across sample types

Internal performance assessment

Across 99 internally generated libraries, mapping rates remained consistently high across clinical specimens, positive controls, cell-line controls, and negative controls (Figure 3). The median mapping rate exceeded 99% across specimen classes. Coverage of the *FLT3* and *NPM1* amplicons was also maintained across specimen classes, with a median *NPM1/FLT3* coverage ratio of 1.2.

External performance assessment

Assay performance was further evaluated using 111 libraries prepared and sequenced independently by four external partner sites. DNA input and sequencing depth varied between sites, providing an assessment of assay robustness across different laboratory conditions. Despite these differences, all four sites demonstrated consistently high mapping rates, while both *FLT3* and *NPM1* achieved substantial sequencing coverage across the clinical specimen sets (Supplementary Figure 1). Differences in absolute target coverage largely reflected site-specific differences in total sequencing depth.

Observed VAFs were also compared with orthogonal values supplied by partner laboratories (Supplementary Figure 2). Across 17 measurements encompassing both *FLT3*-ITD and *NPM1* variants, panel-derived VAFs were highly correlated with orthogonal measurements from the lab's approved diagnostic tests over more than four orders of magnitude, from 0.0014% to 62% VAF. Pearson correlation on log10-transformed VAFs was 0.999 (95% CI, 0.998-1.000). These results show strong quantitative agreement within the evaluated specimens and VAF range.

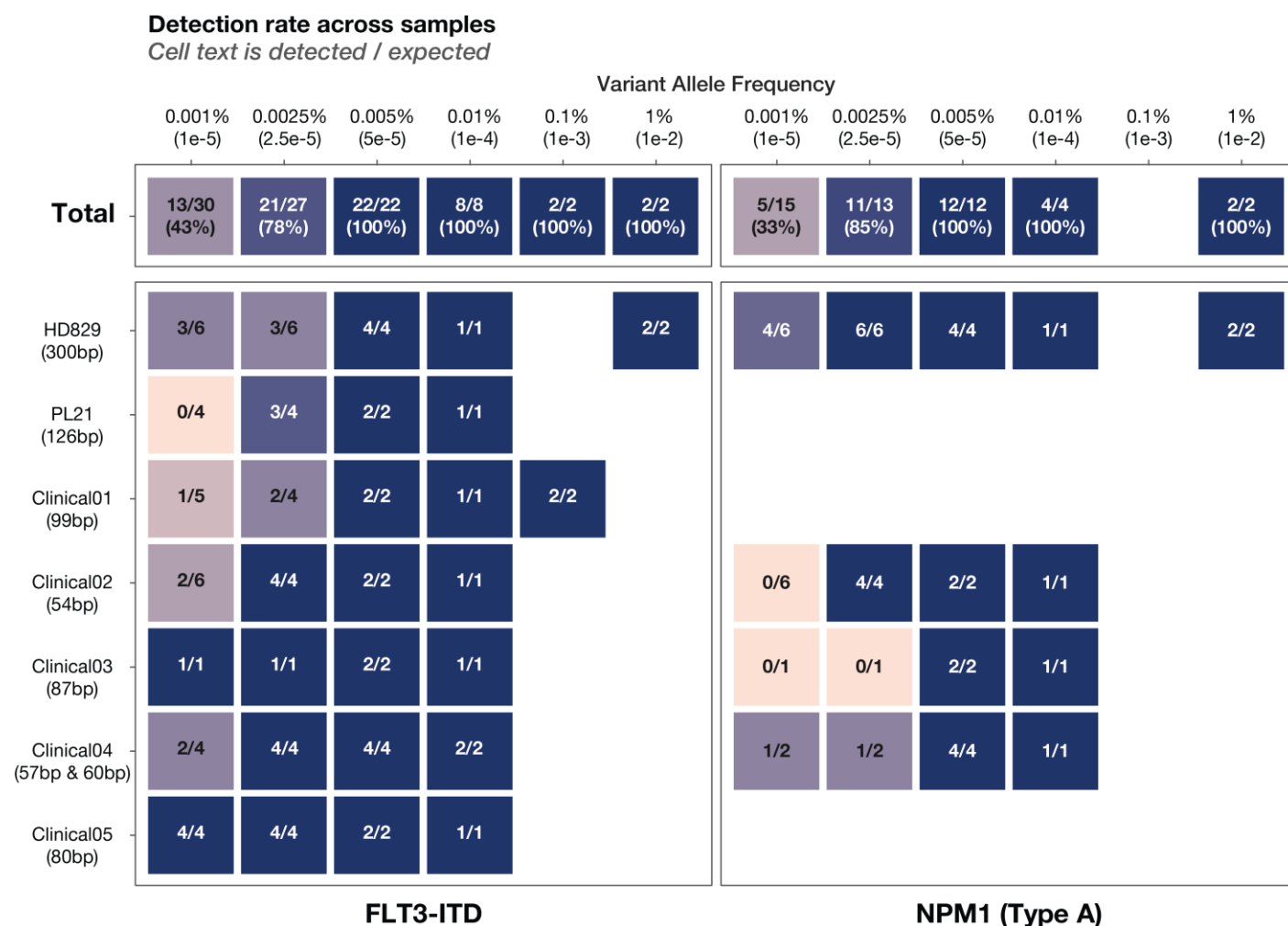


Figure 4. Detection of *FLT3*-ITD and *NPM1* mutations across serial dilutions. Positive specimens were serially diluted to nominal VAFs from 1% (1×10^{-2}) down to 0.001% (1×10^{-5}) and sequenced in replicate (82 libraries). Each cell shows the number of replicates in which the expected variant was detected over the number tested. Cell shading scales with the detection rate. The length under each sample (e.g., 300bp under HD829) shows the length of the expected ITD. Clinical04 carries two *FLT3*-ITDs (57 bp and 60 bp), and each is listed separately in its cell. The Total row sums every expected call at each dilution. Both variant classes were detected in 100% of replicates at 0.005% and above. Blank cells are dilutions that were not tested for that specimen.

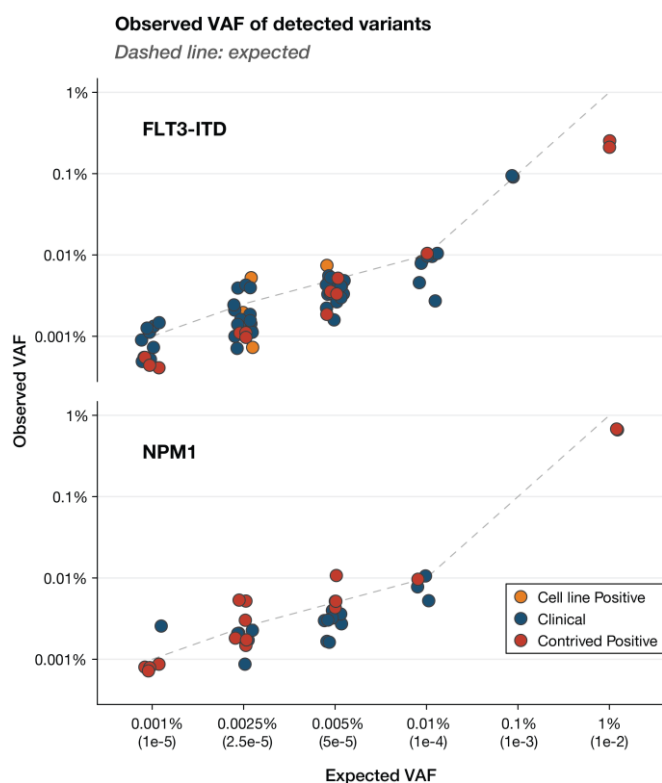


Figure 5. Observed variant allele frequency and detection rate across serial dilutions. Observed VAF of every detected *FLT3*-ITD and *NPM1* call, plotted against nominal dilution and colored by specimen class. The dashed line marks the expected VAF (observed = nominal). Points are jittered horizontally for visibility. Undetected replicates are not shown.

► Sensitive and accurate detection at low VAF

Analytical sensitivity was evaluated using serial dilutions of positive control and clinical specimens prepared with 500–700 ng DNA input. Across the internal dilution series, both *FLT3*-ITD and *NPM1* variants were detected in 100% of tested replicates at 0.005% VAF (5×10^{-5}) and above (Figure 4). Detection became stochastic below this level, consistent with the expected behavior of ultralow-frequency variant detection near the assay limit. These results support an estimated LoD below 5×10^{-5} VAF for both targets under the tested conditions.

Observed VAFs followed the expected dilution trend across both variant classes. In the internal dilution

series, measured tracked the expected dilution trend across both variant classes over several orders of magnitude, supporting quantitative consistency in addition to qualitative detection (Figure 5).

External dilution-series testing provided an independent assessment of low-VAF performance. Across partner datasets, all primary variants with an inferred expected VAF $\geq 0.005\%$ were detected, including 32/32 *FLT3*-ITD calls and 19/19 *NPM1* calls. Observed and inferred expected VAFs remained correlated across the dilution series, with log-scale Pearson correlation coefficients of 0.94 (Supplementary Figure 3).

When results were pooled across partner sites, 68 of 70 primary *FLT3*-ITD calls and 49 of 52 primary *NPM1* calls were detected (Supplementary Figure 4). All *FLT3*-ITD calls with an assigned VAF were detected across the evaluated VAF bins, and all three *NPM1* misses occurred at or below 0.0025% VAF. Together, these results are consistent with 100% detection at and above the estimated 0.005% LoD across both internally generated and externally generated datasets; detections were also observed below this level.

Summary

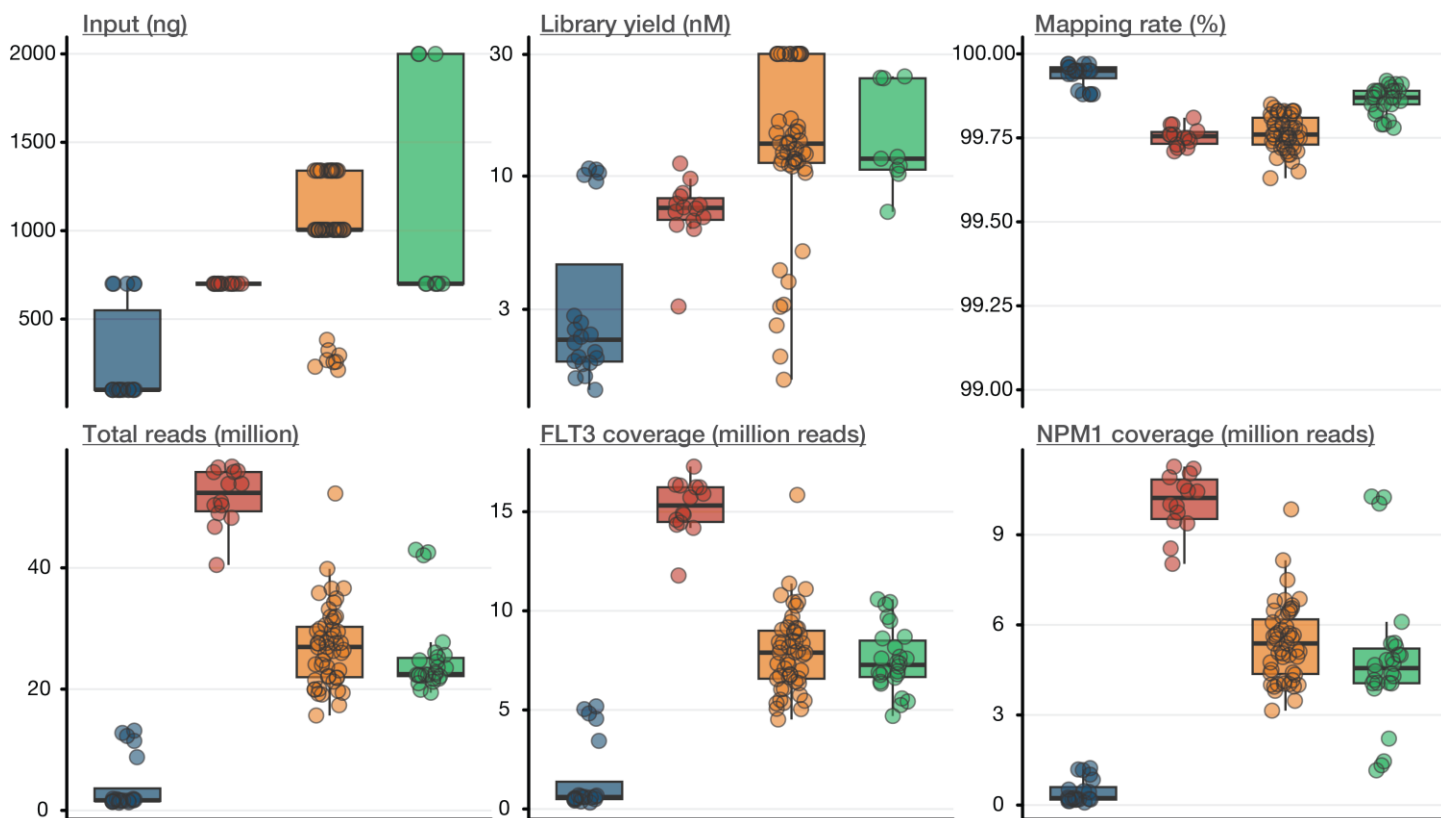
The MRDTect NPM1 and FLT3 ITD Panel combines *NPM1* exon 12 and *FLT3*-ITD detection in a single gDNA-based workflow compatible with standard 2×150 bp sequencing. Across internal and external evaluations, the assay maintained high mapping rates and balanced target coverage, with strong agreement between panel-derived and orthogonal VAF measurements.

Both variant classes were detected in 100% of internally tested replicates at 0.005% VAF and above, with additional detections below this level. The panel also detected *FLT3*-ITDs spanning a broad size range, including variants >300 bp. Together, these data support the use of this focused workflow for high-depth monitoring of informative *NPM1* and *FLT3*-ITD variants identified at diagnosis.

Supplementary Figures and Tables

Library QC metrics by partner site

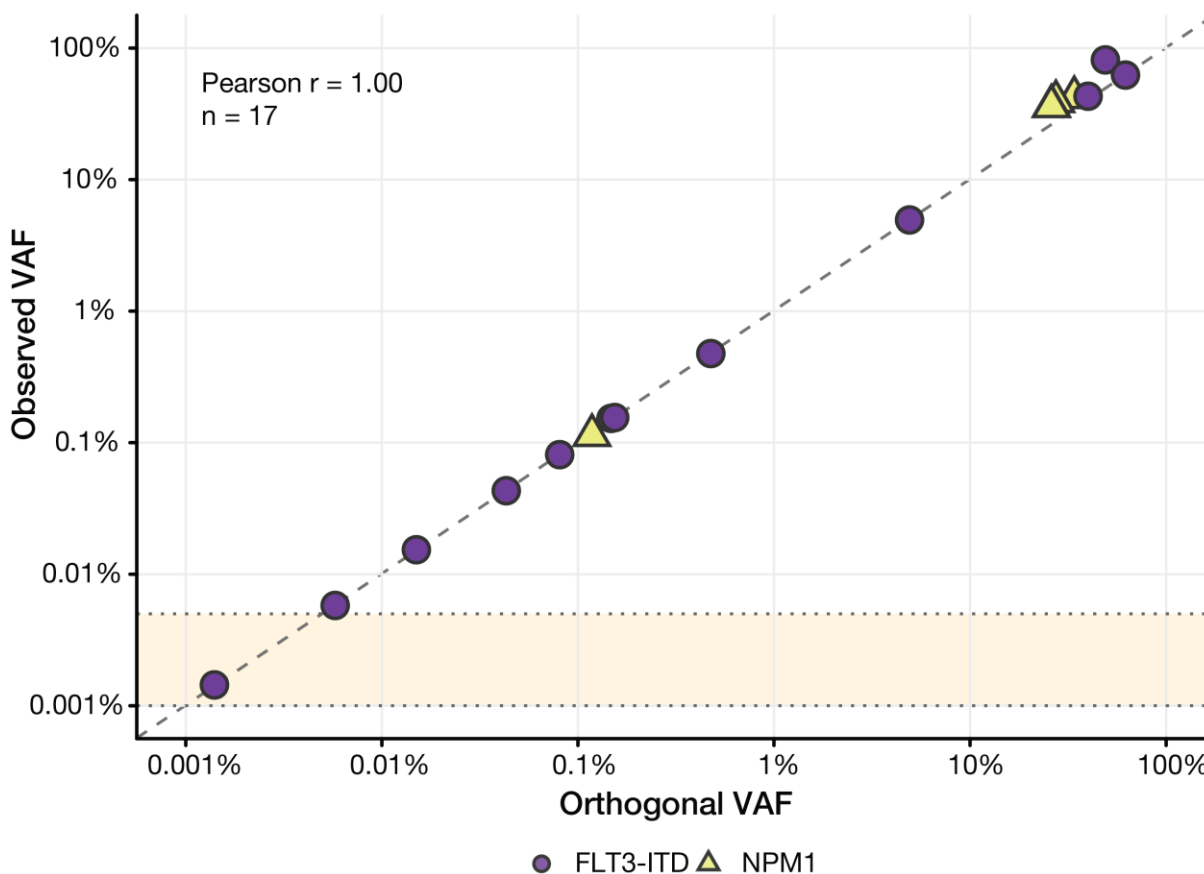
External Performance on 111 libraries



Supplementary Figure 1. Library quality control (QC) metrics across clinical specimens. Distribution of key sequencing quality metrics for clinical specimens, prepared and sequenced externally by four partner sites: Partner #1 (blue; n = 20), Partner #2 (red; n = 14), Partner #3 (orange; n = 51) and Partner #4 (green; n = 26). Boxplots summarize the median, interquartile range (IQR), and 1.5× IQR, with individual points representing independent samples. **Panels:** DNA input (ng), library yield (nM, log scale), total reads (millions), mapping rate (%; defined as the percentage of sequencing reads aligned to the human reference genome GRCh37/hg19), and *FLT3* and *NPM1* coverage (million reads). All partner site data demonstrated consistently high library and sequencing metrics, indicating robust assay performance across diverse sample types, while differences in read depths primarily reflect variations in sequencing depths between partner sites.

Observed vs Orthogonal VAF

Dashed: identity-line. Orange band: 0.001% calling threshold to 0.005% est. LoD.

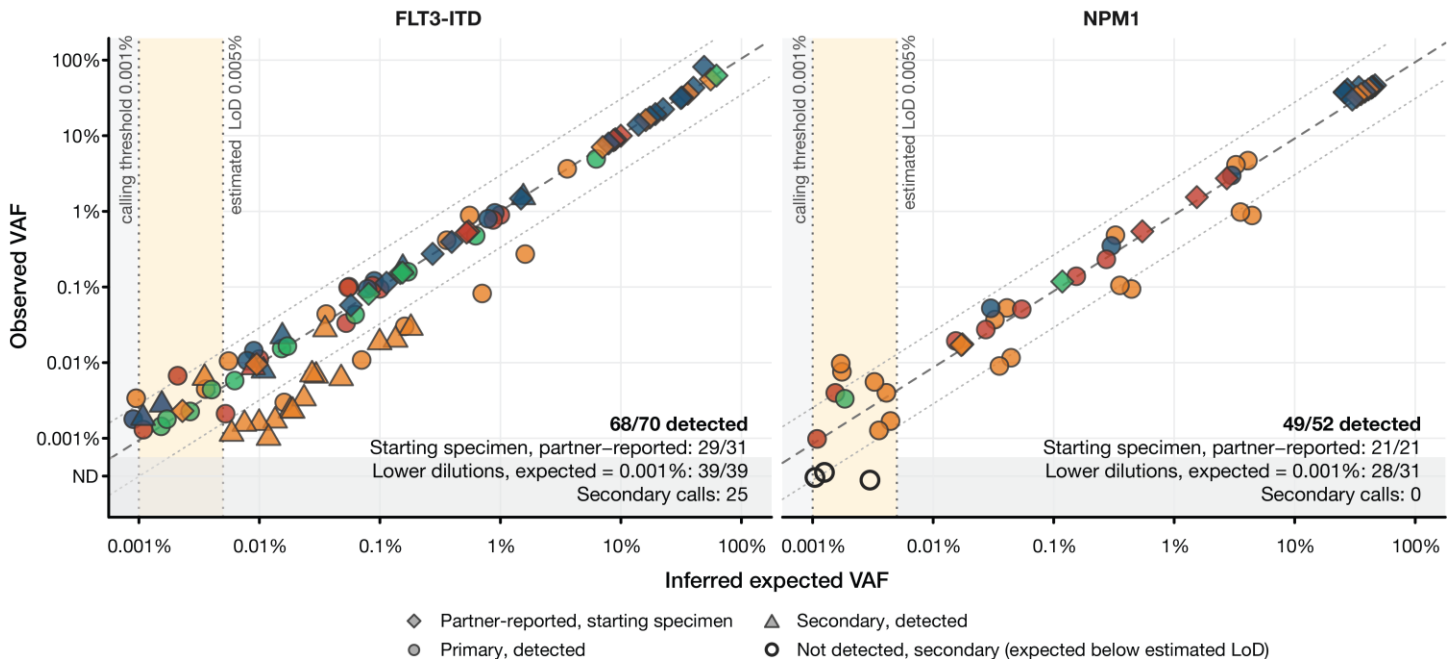


Supplementary Figure 2. Concordance between panel-measured observed variant allele frequencies (VAF) and partner-supplied orthogonal test VAF. For each partner-reported *FLT3*-ITD or *NPM1* variant with a partner-supplied VAF, the VAF measured by the panel (y-axis) is plotted against the partner's orthogonal test value for the same library (x-axis). If the partner site had indicated presence of the variant (e.g., by non-NGS methods) but had not provided any VAF, those variants were not included in this figure. Both axes are log₁₀-scaled. The 17 measurements come from two sites: (1) Partner #1: six neat clinical specimens. Two carry *FLT3*-ITDs (45 bp and 84 bp) and four carry *NPM1* insertions (Type A or D). (2) Partner #4: eleven libraries from three dilution series which contain a 24bp ITD, a 117bp ITD and Seracare's Seraseq® Myeloid Mutation DNA Mix reference material. Points are colored and shaped by gene (circles for *FLT3*-ITD, triangles for *NPM1*). The dashed diagonal marks perfect agreement (panel's observed VAF = partner supplied orthogonal VAF). The shaded orange band spans the panel's 0.001% calling threshold to its estimated 0.005% limit of detection, with dotted lines at each boundary. Panel and partner VAFs were highly correlated across more than four orders of magnitude, from 0.0014% to 62% (Pearson r on log₁₀-transformed VAFs = 0.999, 95% CI 0.998-1.000, $p \sim 0$, $n = 17$).

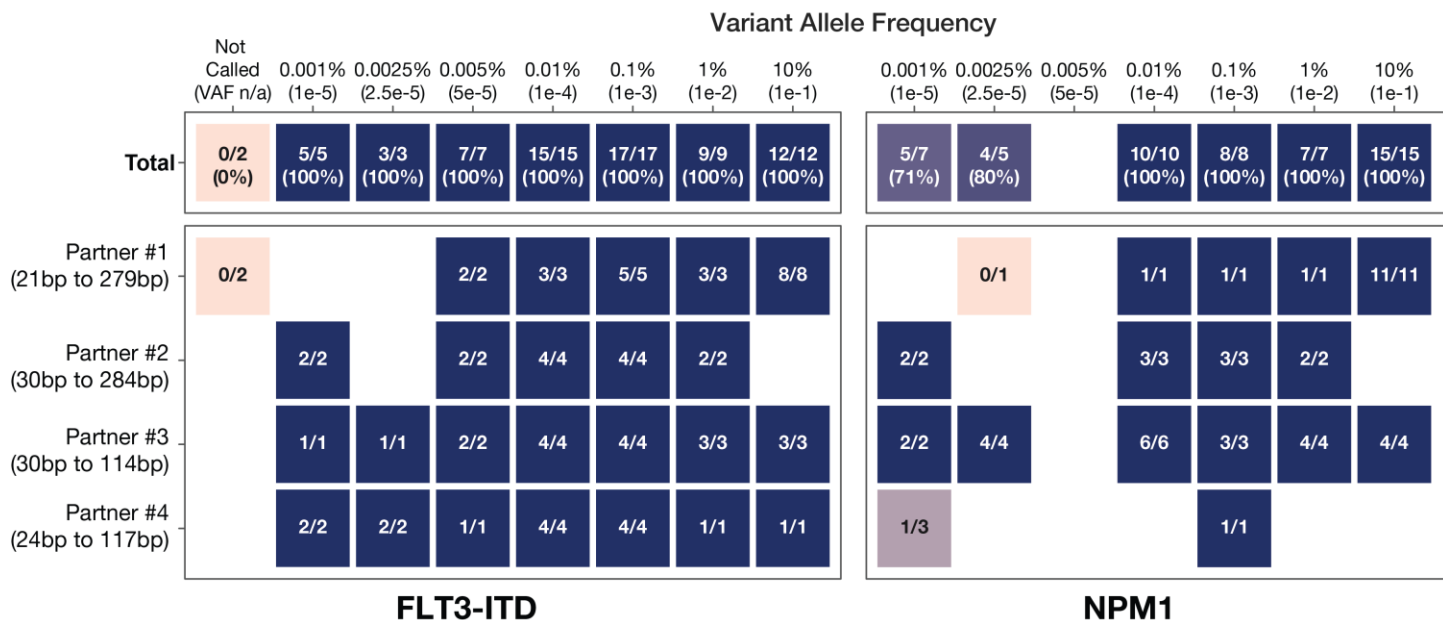
Observed vs inferred expected VAF across partner dilution series

Dashed: observed = expected. Dotted: 3-fold.

Orange band: below the 0.005% estimated LoD, where dropouts are expected.

**Supplementary Figure 3. Observed versus inferred expected variant allele frequency (VAF) across partner dilution series.**

Partner sites diluted positive specimens into negative background (typically a GIAB cell line). Orthogonal VAF for the primary *FLT3*-ITD and *NPM1* variants was available for some samples previously sequenced on other NGS assays. It was not available when the comparator method was capillary electrophoresis (CE) based; these variants are also marked as primary calls in this figure. Additional (secondary) variants were detected at lower VAF in the supplied samples, owing to the higher sensitivity of the MRDTect NPM1 and FLT3 ITD Panel, and orthogonal VAFs for these were also not available. Because observed VAF was highly concordant with orthogonal VAF where both were available (Supplementary Figure 2), expected VAFs for all diluted samples were inferred as follows: (1) in the least-diluted sample, the observed VAF of the variant (ITD or *NPM1*) was used as the anchor VAF; (2) the expected VAF at each lower dilution was inferred as anchor VAF $\times$ (dilution / anchor dilution). Partner-reported variants in the starting specimen are plotted at the orthogonal VAF where supplied, and otherwise at the panel VAF. *FLT3*-ITD was detected in 68/70 scored calls (29/31 in the starting specimen and 39/39 at lower dilutions), and *NPM1* in 49/52 (21/21 and 28/31). Every primary variant with expected VAF $\geq 0.005\%$ was detected at lower dilutions (*FLT3*-ITD 32/32, *NPM1* 19/19). The two *FLT3*-ITD misses were partner-reported ITDs not called in the starting specimen; they have no VAF and are not plotted. The three *NPM1* misses are in the 0.001-0.005% range (right panel; open circles at the bottom). Primary calls detected below the 0.001% threshold (*FLT3*-ITD 5, *NPM1* 7) are not shown. Observed and expected VAF were well correlated across all detected calls (Pearson r on $\log_{10}$ VAF = 0.94, $p < 2 \times 10^{-16}$, $n = 154$). The dashed line marks identity (observed = expected), and dotted lines mark ± 3 -fold. The grey band lies below the 0.001% calling threshold, and the orange band spans the threshold to the estimated LoD (0.005%), where dropouts are expected. Diamonds are partner-reported variants in the starting specimen, circles are primary variants at lower dilutions, and triangles are secondary variants, shown only when detected; all points are colored by site: Partner #1 (blue), Partner #2 (red), Partner #3 (orange) and Partner #4 (green). Primary variants are those reported by the partner; in series with no partner report, the highest VAF call per gene is treated as primary. Secondary variants are additional calls not reported by the partner.

Detection rate across partner sites*Cell text is detected / expected*

Supplementary Figure 4. Detection of *FLT3*-ITD and *NPM1* mutations across partner sites. Detection rate of primary *FLT3*-ITD and *NPM1* calls, pooled across all partner sites. Partner-reported variants in the starting specimen at their observed VAF, plus primary variants at lower dilutions at their inferred expected VAF. Each call is placed in the highest VAF level at or below its VAF (levels 0.001%, 0.0025%, 0.005%, 0.01%, 0.1%, 1% and 10%). Two partner-reported variants (unknown VAFs) were missed by the panel and are placed under “Not Called” column. *FLT3*-ITD calls were detected in 68 of 70 calls, 100% at every VAF level. *NPM1* calls were detected in 49 of 52 calls. All three misses were at or below 0.0025% VAF.

Detailed Panel Content

Gene	Transcript	Full CDS exons	Codons	Covered* Mutations
FLT3	NM_004119	14-15	569-648	ITDs, SNVs/indels in the juxtamembrane domain (JMD)
NPM1	NM_002520		283-295	Type A (860_863dup aka 863_864insTCTG), Type B (863_864insCATG), Type D (863_864insCCTG)

**Variants covered by the MRDTect NPM1 and FLT3 ITD Panel include, but are not limited to, those listed in this table. For a complete list of positions covered by the panel, please refer to the codons covered for each gene.*

Ordering Information

Panel	Part Number
MRDTect™ NPM1 and FLT3 ITD Panel	HDA-MY-1011-24

Pillar Index Kit Options	Reactions	Part Number
Pillar Biosciences LBx Indexing Kit A	24 Combinations, 96 reactions	IDX-PI-1013-96
Pillar Biosciences LBx Indexing Kit B	24 Combinations, 96 reactions	IDX-PI-1014-96

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