Enspyre MRD: Validation of an ultra-sensitive kitted MRD solution at low sequencing depth

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Abstract

Detection of molecular residual disease (MRD) post surgery and monitoring of circulating tumor DNA (ctDNA) during treatment are gaining increasing traction in patient care and drug development. First-generation assays have considerable clinical datasets but suffer from limited sensitivity due to low numbers of mutations tracked. Recent 'ultrasensitive' solutions detect ctDNA at <100 parts per million (ppm), and often as low as 10 ppm, but suffer from high sequencing requirements and costs, limiting their use to large central labs.

Enspyre is a novel sample preparation technique that enriches variant-containing DNA prior to sequencing, significantly improving signal to noise and reducing sequencing requirements. This presents the opportunity to implement ultrasensitive MRD detection and monitoring with significantly reduced sequencing and bioinformatics requirements, utilizing benchtop NGS platforms, while avoiding the need for error correction through molecular barcoding.

We present results from the analytical validation of Enspyre MRD, a kit-based MRD solution utilizing Enspyre which achieves ultrasensitive detection of ctDNA with a limit of detection below 5 ppm using ~2% of the sequencing depth of current approaches. We also present the results of inter-site concordance testing between two independent laboratory sites.

Overview of Enspyre MRD Workflow

Enspyre MRD is a tumor-informed MRD assay, utilizing a patient-specific panel of variants to enable ultrasensitive detection and monitoring of ctDNA from patient plasma.

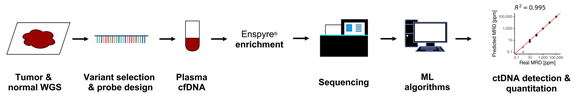

Figure 1. Workflow for the use of Enspyre in MRD detection. Whole genome sequence (WGS) data is generated from a patient tumor sample, with matched normal data generated from buffy coat. A panel of 500-2,000 patient-specific variants is identified, and proprietary algorithms are used to design optimized probes targeting each variant, as well as 5 control probes used for assay QC. These probes are subsequently used to enrich variant-containing molecules in cell free DNA (cfDNA) extracted from patient plasma. Enriched cfDNA is then sequenced, and the resulting sequence data is analyzed using machine learning algorithms to generate ctDNA detection and quantitation information.

Variant Enrichment with Enspyre

Enspyre is a novel library preparation technology that enriches variant-containing molecules prior to sequencing[1], resulting in:

- Significantly reduced sequencing & data handling/processing requirements
- Higher signal to noise
- No need for molecular-barcoding based error correction

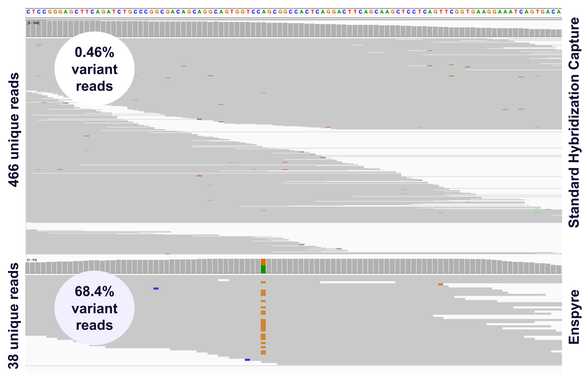

Figure 2. Enspyre reduces the sequencing depth required for variant detection. A screenshot of IGV view for variant rs1056171 (shown in orange) at 0.5% variant fraction, comparing the fraction of unique variant reads using standard hybridization capture vs. Enspyre.

Results

Limit of detection is <5 ppm

Probit regression analysis was used to estimate the LoD95 for each cell line/probe panel from hit rate data. The aggregate of these data was then used to establish an overall LoD95 for the assay at 4.85 ppm (3.75-6.03 ppm 95% CI), as shown in Figure 3.

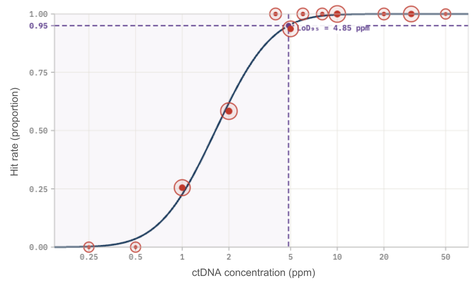

Figure 3. Limit of detection; probit regression analysis of aggregated hit rate data across 3 independent sample types/panels and 6 independent reagent batches shows an assay LoD95 of <5 ppm.

Limit of blank is 0 ppm with 100% specificity

Numbers of replicates and results for each of four panels assessed are shown in Table 1. No false positive calls were observed across 136 replicates using ctDNA extracted from 25 variant-free healthy donors. The false positive rate was therefore 0% (0-2.68% Clopper-Pearson 95% CI) and the LoB was zero. 8 samples were excluded from the analysis, where 1 sample did not yield sufficient library quantity and 7 were qualified as drop-outs by in-assay QC.

Probe panel	Healthy donor replicates	False positives
TE1065	32	0
TE1066	31	0
TE1068	20	0
TE1069	53	0
Total	**136**	**0**

Table 1. Limit of blank; zero false positives were observed across 4 independent probe panels using cfDNA extracted from healthy donor plasma.

Flexible panel size tunes sensitivity at consistent read depth

Reads were down-sampled from >30M to 5M read pairs per sample and for each sequence depth 10 random draws of the probe panel were assessed. Limits of detection were found to be consistent between 10M and >30M read pairs (Figure 4), with one panel size showing reduced sensitivity at 5M read pairs, supporting the use of 10M read pairs as standard. LoD was found to scale pseudo-linearly with panel size, supporting the use of variable panel sizes to address different applications or indications requiring different levels of sensitivity, while also suggesting that larger panels may enable further improvements to sensitivity in the future.

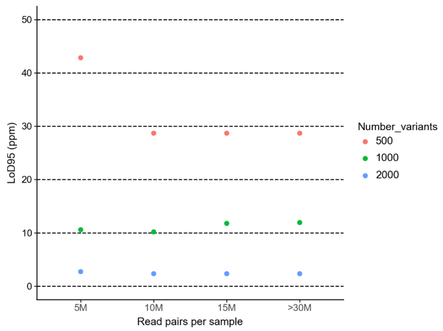

Figure 4. Effect of panel size & sequence depth; consistent LoD95 was observed from 10-30+ million read pairs. Assay sensitivity can be tuned through panel size.

High degree of linearity across 5 orders of magnitude

The assay was found to be highly linear over the range of ctDNA concentrations tested (5-100,000 ppm), resulting in a linear fit with R^2=0.998 (Figure 5).

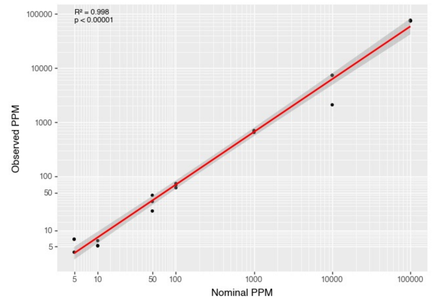

Figure 5. Linearity; a very high degree of linearity was observed across the range of ctDNA concentrations assessed.

Assay success rate is >97.5%

Over a total of 488 samples included in validation studies, 12 samples were invalidated, giving an assay success rate of 97.5%. 4 samples were invalidated due to insufficient library yield, while 8 were invalidated due to in-assay QC failure.

Limit of Quantitation is 5 ppm

Total error was calculated for each of the ctDNA concentrations assessed using the root mean square model as defined in CLSI EP17-A2. Total allowable error was defined as 50%, in line with the change in ctDNA quantitation deemed to be clinically significant during therapy monitoring[2]. Using this threshold, the LoQ of the assay was found to be 5 ppm.

Repeatability & reproducibility is 100%, consistent inter-site results

All samples were called positive over a range from 4-20 ppm across two operators and two reagent batches, with runs performed on two different days, demonstrating 100% repeatability and reproducibility of the assay. Serial dilutions assessed at two independent laboratory sites produced consistent detection and quantitation results both between sites and operators (Figure 6).

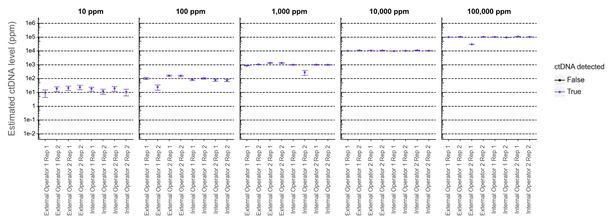

Figure 6. Inter-site concordance; consistent results were obtained between two independent sites, with 100% ctDNA detection across the assessed range.

Discussion

As clinical utility of MRD testing is increasingly established, a growing need exists for accessible and cost-effective solutions for ultrasensitive detection of ctDNA to guide adjuvant treatment and to monitor patients for treatment resistance and disease recurrence. The emerging generation of ultrasensitive MRD assays is generating compelling clinical data, but suffers from high costs, centralization, and a challenging IP landscape.

Enspyre MRD is a kit-based MRD solution that achieves detection of ctDNA with an LoD95 of <5 ppm, using a fraction of the sequence depth of current approaches and without the need for molecular barcoding. This enables the implementation of cost-effective, ultrasensitive, and more accessible MRD detection and monitoring.

Materials & Methods

Samples
cfDNA from plasma obtained from healthy donors was extracted using the Qiagen QIAamp Circulating Nucleic Acid kit and quantified using Qubit 1X dsDNA High Sensitivity assay.

Three independent probe pools were designed, each targeting a panel of ~2,000 variants present in one of three cell lines (A549 NSCLC; NA24631, NA24385). DNA from these cell lines was fragmented through ultrasonication, yielding a fragmentation profile mimicking cfDNA with an average fragment length ~150bp. NA24694 DNA was used as background for serial dilution of A549 and NA2463, while NA24149 was used as background for serial dilution of NA24385. Background DNA was also fragmented using ultrasonication. Four additional probe panels targeting variants found in clinical samples not used in this study were designed for use in limit of blank studies.

Limit of blank (LoB)
cfDNA from 25 healthy donors was used to process a total of 144 assay replicates using 6 independent reagent batches and 4 independent probe panels. DNA input masses of 20ng (120 replicates) and 5ng (24 replicates) were assessed, with the workflow performed by 4 independent operators.

Limit of detection (LoD)
For each of 3 probe panels, a serial dilution of cell line DNA was prepared. A total of 344 replicates of Enspyre MRD were performed across dilutions and panels using 6 independent reagent batches and 5 operators.

Linearity
Using a single probe pool, 3 replicates were performed at each of 8 ctDNA fractions spanning 5 orders of magnitude between 5 and 100,000 ppm.

Limit of quantitation (LoQ)
48 repeats at each of 5 ctDNA fractions from 1 to 30 ppm were assessed using a single probe pool and used to calculate LoQ.

Repeatability, reproducibility and consistency between sites
Two operators each performed a total of 50 assay replicates across 5 ctDNA fractions and two independent reagent batches across two days to assess repeatability and reproducibility.

A single probe pool was used against a serial dilution of cell line DNA across two independent laboratory sites and the results used to assess inter-site consistency.

Effect of panel size and sequence depth on sensitivity
In-silico down-sampling of numbers of probes and sequence reads was used on a single probe pool dataset to assess the impact of panel size and sequence depth on assay sensitivity.

Summary

In this study we have validated a novel tumor-informed MRD assay utilizing the Enspyre variant enrichment technology.

The assay requires just 10M read pairs for the analysis of plasma-derived cell free DNA (~2% of the sequencing depth used in standard hybridization capture approaches[3]), enabling ultrasensitive ctDNA detection and quantitation using benchtop sequencing platforms.

The limit of detection (LoD95) of the assay was established as <5 parts per million ctDNA, and the specificity as 100%. The assay is quantitative, with a high degree of linearity across 5 orders of magnitude, and delivers repeatable and reproducible results across reagent batches, operators, and laboratory sites.

Enspyre reagents are available for research use; for details please contact customersupport@biofidelity.com

References

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