SentryDx MRD Detection Platform from ctDNA Achieves Extreme Sensitivity and Low Cost

A standardized low-cost solution for detecting the presence or absence of cancer DNA

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INTRODUCTION

Circulating tumor DNA (ctDNA) detection has great value for clinicians, drug developers and patients. Currently, NGS is the primary technology used for detecting ctDNA, but high costs prevent broad adoption and frequent testing. Additionally, NGS tumor informed tests are customized to each patient, making regulatory approval difficult.

SentryDx has developed a ctDNA detection assay with (1) high sensitivity (2) high specificity (3) standardization (4) integrated quality controls and (5) low cost. The SentryDx approach achieves these qualities using a novel blocking strategy, allowing for the specific amplification of target ctDNA in positive samples followed by low-cost Sanger sequencing to verify the presence of the targeted ctDNA sequence. With this strategy, single copy ctDNA is enriched to over 99% in the final sample, removing DNA sequencing as a source of error. Further, the production of a 'normal' sequence amplicon when the target ctDNA is absent results in an integrated quality control lacked by past blocking methods.

METHODS AND MATERIALS

Modeling Expected Performance: The expected hybridization percentage of the blocker to the target variant was calculated using IDT Oligo Analyzer Tool. The sample start with 1 variant DNA copy and 9999 normal DNA copies (0.01% variant allele frequency [VAF]). The model assumes 50% replication efficiency for each PCR cycle. The model is limited due to predictions being made using standard DNA rather than LNAs, which are predicted to have a great difference in binding strength.

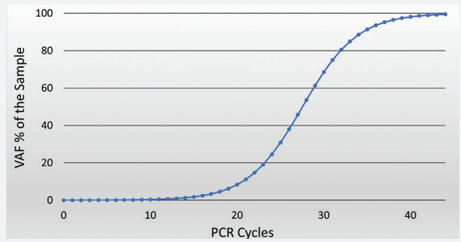
Process Control Development: KRAS Wild Type (HD710) and KRAS G13D 50% (HD290). 50 ng of genomic KRAS WT was added to each sample. KRAS G13D DNA was serially diluted and added to each sample. The samples were enriched using a LNA blocker during the 1st PCR. A second PCR reaction was used to further amplify the target. Version 1 – 1st PCR cycling conditions: denature (95 0C), blocker anneal (80 0C), and primer anneal (60 0C) for 55 cycles. Version 2 – 1st PCR cycling conditions: denature (95 0C), blocker anneal (80 0C), and primer anneal (60 0C) for 35 cycles followed by denature (95 0C), and primer anneal (60 0C) for 30 cycles. Samples were run on a 1.5% agarose gel and visualized. DNA was obtained from Horizon Discovery.

Combining High Sensitivity and High Specificity: Genomic wild type and variant DNA was obtained from Horizon Discovery. 0.0125% variant DNA samples were prepared by diluting variant DNA (50% VAF stock) to 0.0125 ng/ µl and combined at equal volume with 50 ng/µl of wild type DNA. Each sample contained an estimated 1.8 variant DNA copies. 100% normal DNA contained 50 ng of wild type DNA. PCR reactions used Q5 2x Master Mix from NEB. PCR reactions were purified using AMPure XP Beads. Samples were sequenced using Sanger Sequencing. Sanger traces for each nucleotide at each position were calculated using Indigo (gear-genomics.com).

Sensitivity Comparison Using cfDNA: 0.1% VAF Horizon Discovery cfDNA was previously tested on 5 different assays (Williams et al., 2021). 0.1% VAF Horizon Discovery multiplexed cfDNA was purchased (HD777) and 100% wild type Horizon Discover multiplexed cfDNA was purchased. 20 ng was added to each sample. 14 replicates were run on the SentryDx KRAS 12/13 assay and 14 replicates were run on the SentryDx EGFR 790 assay. Sanger sequencing variant analysis was performed using Indigo (gear-genomics).

RESULTS

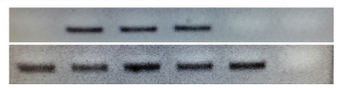
Modeling Enrichment Using a Blocker Starting at 0.01% Variant Allele Frequency (VAF)



Blocking of DNA replication occurs during PCR when the blocker hybridizes to the DNA strand prior to the primer. This results in linear replication of one strand of wild type DNA, but exponential replication of variant DNA. The model was based off hybridization rate using IDT's Oligo Analyzer tool. The model predicts that a sample starting at 0.01% VAF is enriched to >50% after 29 cycles and >99% after 45 cycles.

Developing a Process Control

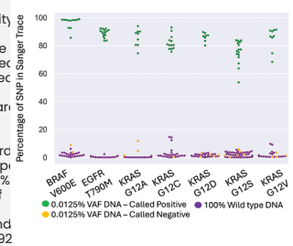
Lane	1	2	3	4	5	6
Amount of KRAS WT DNA (ng)	50	50	50	50	50	N/A
KRAS G13D Copies Approx.	0	~72.5	~7.25	~0.725	~0.072.5	N/A
Approximate KRAS G13D (%)	0%	0.5%	0.05%	0.005%	0.0005%	N/A



Version 1: blocking prevents a sample with 100% wild type DNA from being visualized on an agarose gel (Lane 1) and could not be distinguished from a sample lacking DNA or process failure (Lane 6). **Version 2/Bicycle PCR:** allows for sequencing confirmation of 100% wild type sample (Lane 1) while a sample lacking DNA could still not be visualized (Lane 6). Sanger sequencing of Version 2 showed the 13D variant was present in Lanes 2, 3 and 4 and the wild type was present in Lanes 1 and 5.

Combining High Sensitivity and High Specificity

82 out of 107 samples with 0.0125% variant DNA were called positive for a sensitivity of 76.7% across 7 targets. 16.5% of samples would have 0 copies of variant DNA based on Poisson distribution. Called positives averaged a SNP signal of 85.1% with a standard deviation of 8.5. Called negative averaged a SNP signal of 2.4% with a standard deviation of 2.9. 100% wild type averaged a SNP signal of 1.6% with a standard deviation of 2.2. The one-tailed p-value between Called positives and Wild type samples is 1.5*10-92



Comparing Sensitivity Using 0.1% VAF cfDNA

Sensitivity Results for 0.1% Variant Allele Frequency (VAF) of Cell-Free DNA

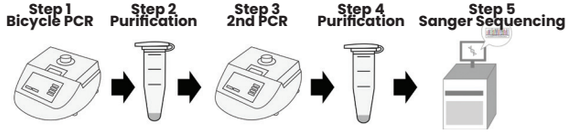
Company	Technology	Positive Samples	Total Samples	Sensitivity
SentryDx	Blocking	24	28	85.7%
NIST	ddPCR	3	24	12.5%
Belfer	ddPCR	12	27	44.4%
Belfer	NGS	0	20	0.0%
AZ	NGS	4	24	16.7%
MoCha	NGS	22	24	91.7%

For KRAS G12D, the SentryDx assay detected 13 out of 14 samples (92.3%). For EGFR T790M, the SentryDx assay detected 11 out of 14 samples (78.6%). Data for NIST, Belfer, AZ and MoCHa assays were separately and independently generated (Williams et al., 2021). Comparison is limited as the tests used different stock materials with different input requirements.

SENTRYDX PROCESS STEPS

Bicycle PCR™

Two Cycles Allows for Internal Process Control



SentryDx Process: Bicycle PCR contains two separate cycling conditions within the first PCR. The first utilizes a blocker annealing temperature for high efficiency blocking. The second lacks this step for low efficiency blocking. By sequencing high and low efficiency blocking conditions within the first PCR, samples lacking target ctDNA SNPs produce a wild type product that is sequenced and serves as an internal process control. The process requires only a thermal cycler PCR cleanup, and Sanger sequencing, resulting in low cost and high throughput.

DISCUSSION

Modeling Expected Performance: The model predicts a blocker can be used to enrich a sample from 0.01% to >99% in just 45 PCR cycles. Previous attempts at blocking have not been able to achieve that level of enrichment. ICE-COLD PCR was only able to enrich from 0.1% to 10.4% (Milbury et al., 2011). XNA Molecular Clamps on average enriched from 0.25% to 7.5% (Zhang et al., 2020). The model suggests that previous approaches have not been able to reach the predicted efficiency enrichment of blocking.

Process Control Development: A sample containing no target ctDNA SNP needs to generate signal to ensure detectability of a process failure. Consequently, a blocker needs to be highly effective to allow for targeted enrichment of a single copy of target ctDNA while allowing for normal DNA replication in a negative sample. One key advantage of the SentryDx method is the use of two cycling conditions that vary in blocking effectiveness during a single PCR. This integrated quality control allows for the detection of process failures.

Combining High Sensitivity and High Specificity: Oncologists require high sensitivity and high specificity tests when making treatment-based decisions. For ctDNA testing, an assay with high statistical confidence between a sample containing 0 or 1 variant copies of DNA would be high clinical value. These data suggests that the SentryDx method can provide this statistical confidence with its large signal:noise ratio. One key observations is the level of signal separation between green points (0.0125% VAF called negative) and the orange points (0.0125% called positive). For a sample with 0.0125% VAF, Poisson distribution predicts most samples would contain 0, 1, 2 or 3 copies of variant DNA. The 0 variant copy event from a 0.0125% sample should look like a 100% wild type sample, which is seen here (Called Negatives and Wild Type DNA samples have same signal). No samples with intermediate signals were observed.

Sensitivity Comparison Using cfDNA: The sensitivity of the SentryDx method with contrived 0.1% VAF cfDNA was determined. This data suggests the SentryDx method is more sensitive compared to droplet digital PCR and comparable to NGS. This comparison is limited because the tests were done on different lots of Horizon Discovery cfDNA, by different organizations, and different protocols. The key takeaway is the SentryDx method performed at high sensitivity using a contrived 0.1% VAF cfDNA reference standard. Reference standards are an important performance control for future FDA approval.

CONCLUSION

Detection of cancer-specific SNPs at extremely low prevalence (0.0125%) in genomic DNA and at 0.1% in contrived cfDNA serves as a foundation upon which new diagnostics will be developed and optimized. The method uniquely combines high sensitivity and processing controls that produce signal with negative samples. The method also provides high statistical confidence between 0 and 1 copy of variant DNA, producing no samples with intermediate signal, and providing the high accuracy needed to make treatment decisions. The low cost also means patients can be tested more frequently. Widespread adoption of an assay with this level of performance would be expected to improve patient outcomes and increase patient's quality of life.

REFERENCES

Milbury, C. A., Li, J., & Makrigiorgos, G. M. (2011). Ice -COLD-PCR enables rapid amplification and robust enrichment for low-abundance unknown DNA mutations. Nucleic Acids Research, 39(1), e2–e2.

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SentryDx is overcoming the trust issues in detecting low frequency cancer

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