A methylation state specific targeted background depletion technique for enrichment of ctDNA fraction

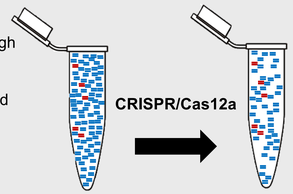
Authors: Caitlin Gilley, Miguel Williams, Sarah Falotico, Emily Neaga, Kade Pettie and Anthony Shuber

AACR Liquid Biopsy November 2024 | Poster #A014

Harbinger Health

BACKGROUND

Liquid biopsy continues to be promising in early cancer diagnostics due to the non-invasive nature of the specimen collection, but low levels of circulating tumor DNA (ctDNA) still present a challenge. High levels of DNA methylation at many genomic loci are known cancer biomarkers, but unmethylated, non-cancer DNA background can obfuscate methylated, cancer DNA. Harbinger Health has developed a sequencing-based, non-invasive, multicancer assay, HHx, that leverages the methylation signal associated with the early carcinogenesis within cell-free DNA (cfDNA). However, ctDNA accounts for as low as 0.003% of the total cfDNA[1]. To aid in the isolation of the low ctDNA fractions, we developed a novel CRISPR/Cas12a technology for targeted depletion of unmethylated background at the CpG level. The resulting ctDNA enriched product can be used in conjunction with either PCR or sequencing-based methods. Here, we describe the use of our CRISPR/Cas12a depletion methodology in both a gblock model system and patient derived cfDNA using both qPCR and sequencing HHx to enrich cancer-derived methylation signal by reducing non-cancer background.



Target hypomethylated DNA to cut and enrich for hypermethylated DNA

Figure 1: Depletion schematic. Hypermethylated cancer-derived DNA (red) becomes a larger fraction of the total DNA when hypomethylated background DNA (blue) is depleted via CRISPR/Cas12a.

METHODS

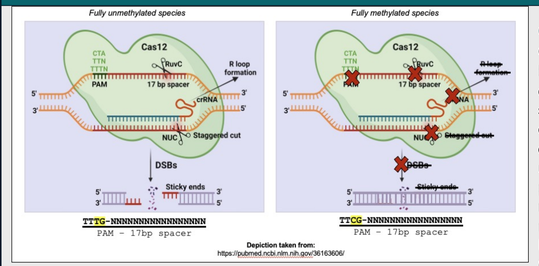


Figure 2: CRISPR/Cas12a mechanism. Cas12 only requires crRNAs to generate double-stranded breaks and cleaves target region approximately 17 bp upstream target PAM sequence (CTA, TTN, or TTTN). *Hillary, et a;. Molecular Biotechnology.* 2023;65:311-325

crRNA Design

Guide RNAs were designed to bind sites within sequences of contiguously unmethylated CpG sites regions informative of cancer status. crRNAs were designed to have the unmethylated "TG" in the protospacer adjacent motif (PAM) recognition site to differentiate methylated from unmethylated DNA post bisulfite conversion (Figure 2)[2].

Specificity Evaluation with gBlock Dilution Series and Multiplexing with cfDNA

For initial evaluation of the specificity of the CRISPR/Cas12a, a model bisulfite converted gBlock dilution series was synthesized to test via qPCR to detect potential Ct delay in hypomethylated gBlock. We assessed depletion in conjunction with the HHx assay, which involves library construction and enrichment via hybrid capture and includes target regions with crRNAs, the same gBlock dilution series was used to observe depletion of unmethylated DNA post sequencing. Depletion of unmethylated DNA was visualized using the Integrated Genomics Viewer and plotted the number of total reads versus the number of methylated reads within the gBlock region.

Finally, we began an initial assessment of multiplexing a 6-plex crRNA with cfDNA in patient cancer and noncancer samples with the HHx assay. We evaluated the delta in the number of reads containing fully methylated contiguous CpGs, denoted "meth hepitype" (Formula 2) and the total number of reads containing the relevant CpGs (Formula 1), denoted, "total hepitype" in CRISPR/Cas12a cut DNA versus uncut DNA, then calculated the fold change by taking the ratio of the meth hepitype fraction in cut and uncut of the same sample (Formula 3).

Formula 1:

$$\Delta total\ hepitype = \frac{Total\ hepitype\ counts\ over\ target\ region\ of\ cut\ sample}{Total\ hepitype\ counts\ over\ target\ region\ of\ uncut\ sample}$$

Formula 2:

$$\Delta meth\ hepitype = \frac{meth\ hepitype\ counts\ over\ target\ region\ of\ cut\ sample}{meth\ hepitype\ counts\ over\ target\ region\ of\ uncut\ sample}$$

Formula 3:

$$Fold\ Enrichment\ (X) = \frac{Cut\ Meth\ Hepitype\ Fraction}{Uncut\ Meth\ Hepitype\ Fraction}$$

RESULTS

Cas12a Depletion Shows Specificity for Hypomethylated gBlock

CRISPR/Cas12a demonstrated specificity for depletion of unmethylated gBlock by qPCR. Comparing hypomethylated gBlock that underwent Cas treatment (positive control) with hypermethylated gBlock (specificity control) shows an almost 4.5 Ct delta, indicating that hypo gBlock was cut and non-amplifiable (Figure 3).

Figure 3. Depletion in gBlock via qPCR

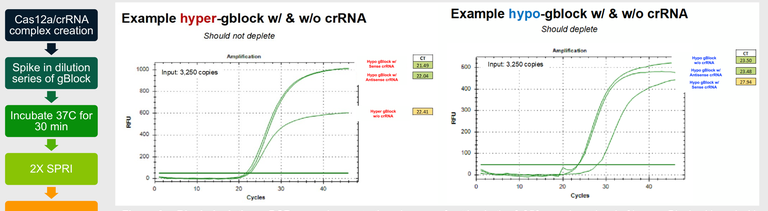


Figure 3: gBlock depletion. qPCR curve demonstrating absence of cutting in the 3,250 copy input level of hyper gBlock with and without crRNA, but cutting still occurs with specificity control containing hypo gBlock (left). Cas12a cutting in the 3,250 copy input level of hypo gBlock, but neither hypermethylated glock nor no Cas negative control (right). Threshold for qPCR was 47.16.

Figure 4. Depletion in IGV and Meth Hepitype Enrichment by Counts

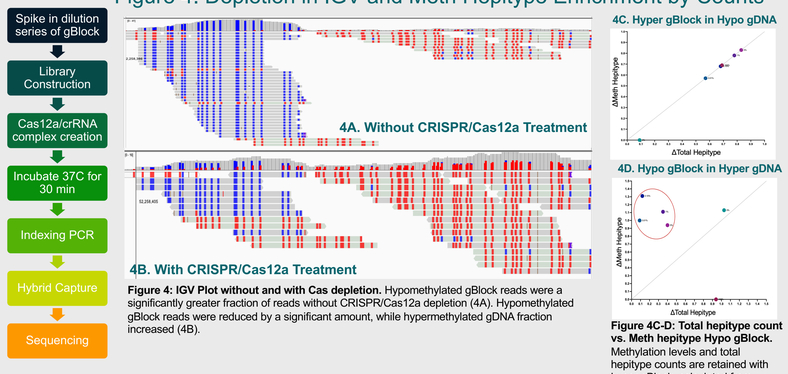


Figure 4: IGV Plot without and with Cas depletion. Hypomethylated gBlock reads were a significantly greater fraction of reads without CRISPR/Cas12a depletion (4A). Hypomethylated gBlock reads were reduced by a significant amount, while hypermethylated gDNA fraction increased (4B).

Figure 4C-D: Total hepitype count vs. Meth hepitype Hypo gBlock. Methylation levels and total hepitype counts are retained with hyper gBlock, calculated from NovaSeq data (4C). Less total hepitype counts indicative of Cas cutting, but methylation levels are maintained. (4D).

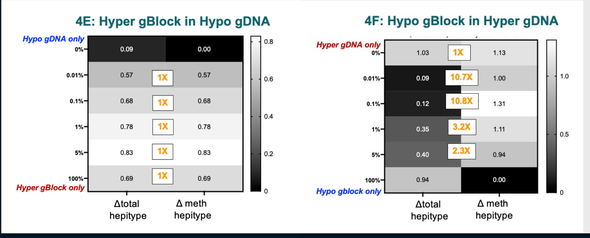


Figure 4E-F: Fold enrichment at each gBlock dilution levels. No fold enrichment of meth CpG fraction in bisulfite converted hyper gBlock spike-in dilution series from NovaSeq data, further demonstrating specificity of CRISPR/Cas12a (4E). There was up to 10.8X fold enrichment, with greatest efficiency of cutting at 0.01% and 0.1% hypo gBlock spiked into hyper gDNA.

RESULTS

Hypo gBlock was spiked into hyper gDNA and run through HHx assay with CRISPR/Cas12a to assess compatibility with our current workflow. IGV plots demonstrated clear cutting of hypo gBlock with Cas depletion, with hyper gDNA remaining uncut (Figure 4A). Hypo gBlock remained uncut in library that did not undergo Cas depletion (Figure 4C). We also demonstrate that Cas treatment does not affect total hepitype counts (Figure 4B), but methylation levels are retained (Figure 4D), further confirming specificity with sequencing. Fold enrichment of hypo gBlock ranged from 2.3 to 10.8X, with highest enrichment at 0.01% and 0.1% gBlock methylated tumor fraction models (Figure 4E). These results are consistent with the level of specificity seen in the qPCR melt curves.

Enrichment of cfDNA Tumor Fraction in 5-plex of Targets

Table 1. 5-plex Depletion of Cancer Patient cfDNA Fold Difference by Target

Region	Cas Treatment	Total Hepitype Count	Meth Hepitype Count	Meth Hepitype Fraction	Fold Change
1	Yes	1619	5	0.31%	1.08
	No	1699	5	0.29%	
2	Yes	1173	6	0.51%	1.70
	No	1326	4	0.30%	
3	Yes	943	5	0.53%	1.89
	No	1075	3	0.28%	
4	Yes	1563	0	0.00%	0.00
	No	1607	1	0.06%	
5	Yes	1087	3	0.28%	1.08
	No	1161	3	0.26%	
Average of 5-Plex	Yes	1277	3.8	0.33%	1.43
	No	1374	3.2	0.23%	

Table 1: Methylated Fold Change Across 5 Multiplexed Targets in Cancer cfDNA. The ratio of total hepitype counts and meth hepitype counts within each region was calculated to obtain the methylation count fraction. Fold enrichment was determined by first calculating the delta of meth hepitype reads over total hepitype reads, followed by the ratio of meth hepitype/total hepitype reads in CRISPR/Cas12a depleted aliquot versus no treatment aliquot.

To show the ability to multiplex crRNAs, a 5-plex of targets was tested with CRISPR/Cas12a depletion in one breast cancer patient cfDNA sample. This patient was called positive for cancer with our current standard HHx assay with an estimated tumor content of 0.0027%. The amount of enrichment observed varied by target, with an average fold difference across the 5 regions of 1.42, demonstrating multiplexing capabilities (Table 1). This variability is due to the frequency of fully methylated hepitypes present in the original library and the depth of coverage. As these targets reflect approximately 0.003% of the total library, the enrichment observed here would be expected to increase if sequencing was restricted to these target sites.

CONCLUSIONS

Harbinger Health's Cas12a-mediated depletion of background unmethylated takes advantage of the high specificity of CRISPR used in tandem with crRNAs directed against both sequence and methylation state specific target regions. Our approach effectively depletes unmethylated "non-cancer" signal without affecting methylated "cancer", demonstrated in both model methylated DNA and patient derived cfDNA. This method can be multiplexed and used with both qPCR and sequencing based workflows to improve sensitivity of rare methylation events in bioassays.

REFERENCES

1. Stejskal, P., Goodarzi, H., Srovnal, J., Hajdúch, M., van 't Veer, L. J., & Magbanua, M. J. (2023). Circulating tumor nucleic acids: Biology, release mechanisms, and clinical relevance. Molecular Cancer, 22(1). https://doi.org/10.1186/s12943-022-01710-w
2. Hillary, V. E., & Ceasar, S. A. (2022). A review on the mechanism and applications of CRISPR/Cas9/CAS12/CAS13/CAS14 proteins utilized for Genome Engineering. Molecular Biotechnology, 65(3), 311–325. https://doi.org/10.1007/s12033-022-00567-0