APPLICATION NOTE

Detection of hypermethylated circulating tumor DNA by Crystal Digital PCR™

In collaboration with:
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Hypermethylated *WIF1* and *NPY* as biomarkers in colorectal cancer

Changes in the DNA methylation status of gene regulatory regions constitute emerging biomarkers for a variety of cancers. This epigenetic alteration is biologically stable and present in circulating tumor DNA, making it suitable for early detection and noninvasive dynamic monitoring of tumor burden. In colorectal cancer (CRC), hypermethylation of *WNT inhibitory factor 1* (*WIF1*) and *neuropeptide T* (*NPY*) was found in 80% and 44.7% of metastatic and stage II/III patients, respectively [1]. To evaluate if hypermethylated *WIF1* and *NPY* can be used as a universal colorectal cancer marker and a surrogate to tumor sequence-specific mutations, a method combining bisulfite conversion (Figure 1.A) of unmethylated cytosine to uracil and Crystal Digital PCR™ was developed. A 3-color digital PCR assay targeting bisulfite converted hypermethylated promoter regions of *WIF1* and *NPY* and unmethylated Albumin gene *ALB* as a reference was optimized and tested on DNA extracted from the plasma of healthy individuals and CRC patients (Figure 1.B).

Figure 1. A

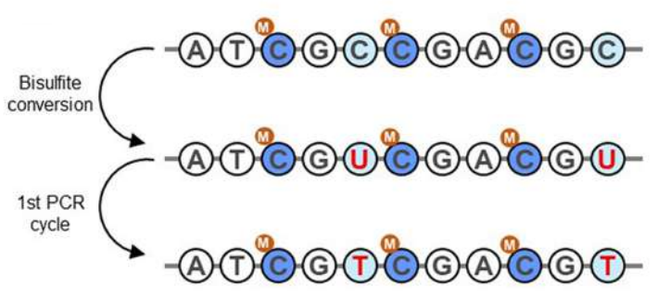

Figure 1. B

Targeted sequences	Probe Sequence (5'>3')	Forward Primer sequence (5'>3')	Reverse Primer sequence (5'>3')
Albumin reference	AGGGTTTTTATAATTTA	GGGATGGAAAGAATTTTATGTT	AAACAAACTAACCCCAAATTCT
WIF1	CGGCGTTAGGTTGC	GAGGGAGTTGTAGCGTAGTAGAGTATTTG	AAAACTCCTCGTACCGCACCTA
NPY	CGCGATTCGTTTTTTGTA	CGCGGCGAGGAAGTTTTATA	ATACTATCGAACGAACGTCTCCG

Figure 1: A. Bisulfite conversion of unmethylated cytosine (C) residues leads to their deamination to uracil (U) while methylated cytosine (M) residues (present in CpG islands) are protected from bisulfite conversion. PCR detection recognizes the uracil products of this conversion as thymine (T), thus distinguishing them from unconverted (methylated) cytosine. B. Sequences of the primers and fluorescent-labeled probes used for the 3-color Crystal Digital PCR™ assay [1]. Between 17 and 24 CpG islands displaying a similar methylation profile are targeted by the *WIF1* and *NPY* amplicons.

A 3-color Crystal Digital PCR™ assay detects hypermethylated *WIF1* and *NPY* biomarkers

The limit of blank (LOB) with a 95% confidence level of the triplex Crystal Digital PCR™ assay, defined as 3 and 12 false positive droplets for *NPY* and *WIF1*, respectively, was determined on 31 sample replicates containing unmethylated DNA ranging from 40 to 400 cp/µl (corresponding to 1000 to 10,000 copies per 25 µl reaction). To evaluate the sensitivity and the linearity of the assay, serial dilutions of chemically methylated DNA ranging from 20 to 0.2 cp/µl in a background of 400 cp/µl of unmethylated DNA (representing 5 to 0.05 % of the mutant allele fraction) were assayed in triplicate. For *NPY* and *WIF1*, a total of 0.2 cp/µl and 1 cp/µl (5 and 25 copies of methylated DNA copies per 25µl reaction) were reliably detected, respectively, corresponding to a mutant allele fraction of 0.05% and 0.25% (Figure 2).

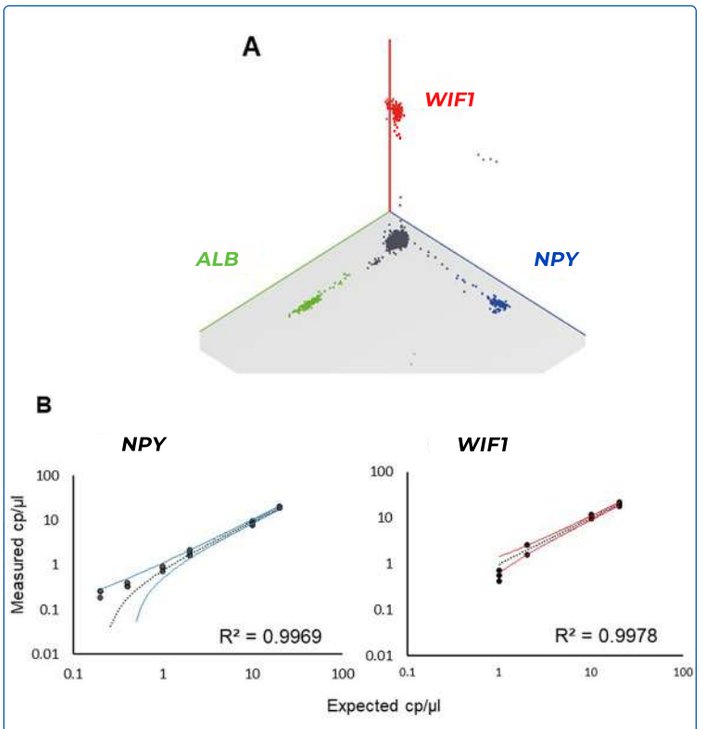

Figure 2: A. Crystal Miner 3D dot-plot of the 3-color Crystal Digital PCR™ assay for the detection of hypermethylated *WIF1*, *NPY* and *ALB* on chemically methylated DNA. B. Standard curves obtained by measuring serial dilutions of methylated DNA in a background of unmethylated DNA. To determine whether the observed mutant quantity was significantly above the LOB, a Bayesian approach based on false positive probability was used. For each result, the number of positive partitions was corrected by deducting eventual false-positive partitions weighted by their probability distribution. A sample was considered as negative when the lower bound of the corrected 95% confidence interval included zero, in which case it is not represented on the graph. For more information, please see https://www.gene-pi.com/wp-content/uploads/2019/11/GenePi_-Memo_LOB_correction_method-1.pdf

Detection of hypermethylated *WIF1* and *NPY* in cancer patients and healthy individuals

A total of 10 and 5 DNA samples derived from plasma of stage III or IV CRC patients and healthy individuals, respectively, was tested using the 3-color Crystal Digital PCR™ assay. All plasma DNA samples from CRC patients scored positive for hypermethylated *WIF1* and *NPY*, whereas hypermethylated *WIF1* and *NPY* were not detected in healthy individuals. The fraction of hypermethylated plasma DNA was calculated by reporting the *WIF1* and *NPY* concentration to the unmethylated *ALB* reference concentration. The hypermethylated *WIF1* fraction in plasma DNA ranged from 8% to 93%, while that of hypermethylated *NPY* ranged from 0.1% to 78% (Figure 3). The lowest quantity of hypermethylated *WIF1* and *NPY* detected in plasma samples was 5.1 and 1.2 copies per µl of bisulfited DNA eluate, respectively.

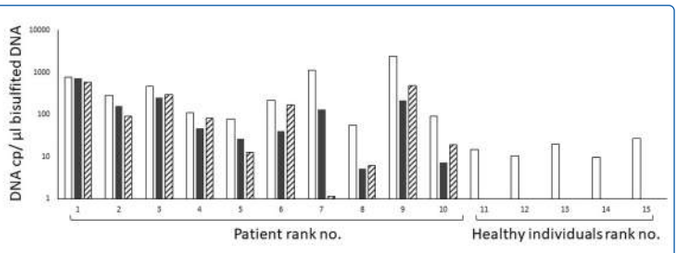

Figure 3: Concentration of *ALB* (white bars), hypermethylated *WIF1* (black bars) and hypermethylated *NPY* (hashed bars) in plasma of CRC patients and healthy individuals. DNA concentration is expressed in copies per µl of bisulfited DNA eluate. Depending on the DNA concentration of the sample, 20ng or 20µl of DNA sample extracted from the patients was bisulfite-treated in a thermocycler at 98 °C for 10 minutes and 53 °C for 4 hours using the EZ DNA Methylation-Gold kit (Zymo Research) and eluted in 10µl final volume.

Application Note Highlights

- Bisulfite conversion followed by 3-color Crystal Digital PCR™ enables the reliable detection of down to 25 and 5 copies of hypermethylated *WIF1* and *NPY* DNA, respectively, per 25µl reaction
- Crystal Digital PCR™ detection of hypermethylated *WIF1* and *NPY* can be used as a universal colorectal cancer marker and a surrogate to tumor-specific mutations
- Hypermethylated *WIF1* and *NPY* were detected in all 10 stage III/IV colorectal cancer patient plasma samples, while hypermethylated *WIF1* and *NPY* were not detected in any of the 5 healthy individuals.

To learn more about digital PCR, please visit Stilla Technologies' Learning Center at www.gene-pi.com

REFERENCES

[1] Garrigou S, Perkins G, Garlan F, Normand C, Didelot A, Le Corre D, Peyvandi S, Mulot C, Niarra R, Aucouturier P, Chatellier G, Nizard P, Perez-Toralla K, Zonta E, Charpy C, Pujals A, Barau C, Bouché O, Emile JF, Pezet D, Bibeau F, Hutchison JB, Link DR, Zaanan A, Laurent-Puig P, Sobhani I, Taly V. A Study of Hypermethylated Circulating Tumor DNA as a Universal Colorectal Cancer Biomarker. Clin Chem. 2016 Aug;62(8):1129-39.

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