Use of Internal Standards to Develop Improved Methylation Detection in cfDNA

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Detection of aberrant levels and patterns of methylation in circulating free genomic DNA (cfDNA) may be useful in predicting the presence of malignancy in a particular tissue or in monitoring known malignancies for recurrence or treatment resistance. The goal of this study is to develop methods to improve accurate and sensitive detection of methylation in cfDNA utilizing synthetic spike-in internal standards (IS) that enable target- and assay-specific limits of detection and control for technical error. Initial testing of an off-the-shelf IS yielded promising results but, technical error increased as methylation increased, likely due to suboptimal IS design and experimental conditions. Next, a mixture of optimized IS for five targets known to be altered in cancer, SOX2, CDO1, TAC1, SOX17, and HOXA7, was created. Each IS was designed to be spiked into DNA, bisulfite-treated and measured in either hybrid capture or amplicon-based next generation sequencing libraries. Sequence changes (either A>T or T>A to avoid changes caused by bisulfite treatment) were introduced approximately every 50 bp to allow the IS to be distinguished from endogenous DNA. IS were cloned into plasmids, linearized, quantified and combined to create the IS mix. This IS mixture will be combined with a set of reference human genomic DNA samples obtained from the National Institute of Standards and Technology known to have differing levels of methylation such that the ratio of IS mixture:genomic DNA varies. These will be bisulfite-treated, used to prepare amplicon libraries and sequenced. Methylation, technical error and limit of detection will be assessed.

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