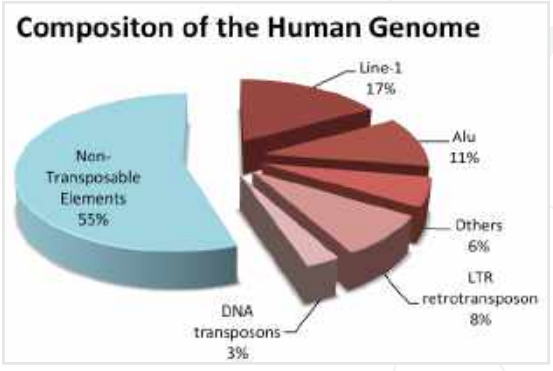
LINE-1 GLOBAL METHYLATION ANALYSIS

Introduction



Repetitive DNA sequences comprise about 45% of the mammalian genome. Long Interspersed Elements (LINE-1), a class of non-Long Terminal Repeat (LTR) retrotransposons, are amplified to more than 500,000 copies in both truncated (~0.9 kb) form or active full-length elements (~6 kb)[1]. A full-length LINE-1 contains a 5′ untranslated region (UTR), two non-overlapping open reading frames (ORF1 and ORF2), and a 3′ UTR that ends in a poly (A) tail[2]. The heavily methylated LINE-1 elements are among the host cell defense mechanisms to inhibit L1-ribonucleoprotein (L1 RNP) formation and prevent its mobilization throughout the human genome.

Hypomethylation of LINE-1 leads to genetic instability by LINE-1 transposition across the genome and disruption of gene expression[3]. Such increases in chromosomal instability contribute to cancer development and progression. Given that LINE-1 constitutes approximately 17% of the human genome, the extent of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation. Therefore, LINE-1 methylation levels are an excellent pharmacodynamics (PD) marker for hypomethylating agent therapy.

Methods

Analysis Methods – Human LINE-1 methylation assay (Assay ID: ASY3201) amplifies a 146 base pair (bp) fragment located at the 5′UTR region of the LINE-1 elements (Figure 2). The LINE-1 methylation level can be quantified by Pyrosequencing, MS-HRM, and targeted bisulfite NGS (tNGBS). These three methods use the same bisulfite PCR but measure methylation levels in different ways.

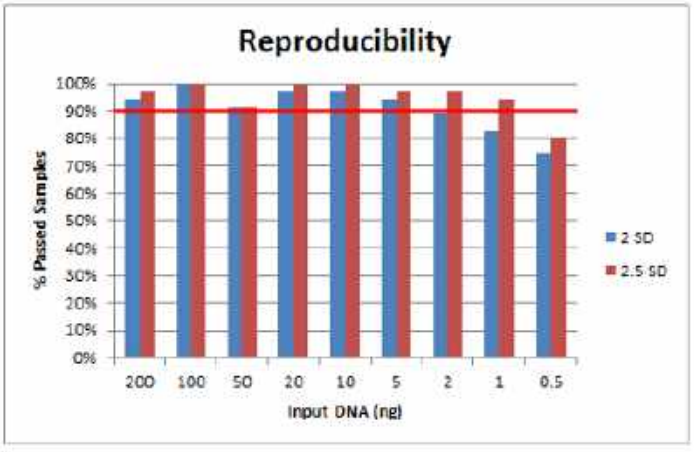
- LINE-1 Pyrosequencing Assay: Sequence a 36-bp fragment to quantify methylation level of four CpG sites
- MS-HRM Methylation Assay: Provide an average methylation level of the 146-bp region using real-time PCR.
- Targeted Bisulfite NGS (tNGBS): Sequence the entire 146-bp amplicon and quantify the methylation of 10 CpG sites with greater than 500x coverage.

Validation

Assay Specificity – The LINE-1 Assay (ASY3201) amplifies a conserved LINE-1 element throughout the genome after sodium bisulfite modification, with a PCR amplicon size of 146 bp. It is located at the 5'UTR region of the LINE-1 elements (Figure 2). Pyrosequencing and tNGBS assays demonstrated specificity to the LINE-1 repetitive element sequence.

Assay Sensitivity – Assay sensitivity was analyzed by determining the limits of detection (LOD) and quantification (LOQ). Both LOD and LOQ were set at 1 ng of input DNA.

Figure 1: Line-1 methylation assay specificity and sensitivity.



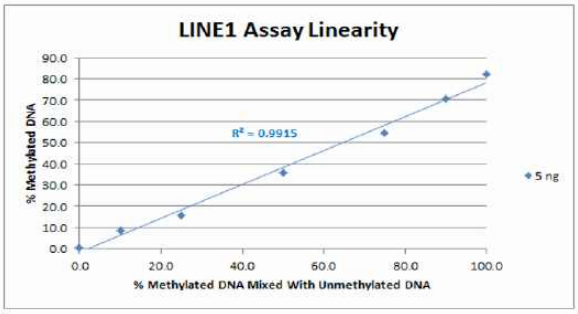
ng of DNA	Pass/Total	% Passing at 2 SD
200	33/35	94%
100	35/35	100%
50	32/35	91%
20	34/35	97%
10	34/35	97%
5	33/35	94%
2	31/35	89%
1	29/35	83%
0.5	26/35	74%

ng of DNA	Pass/Total	% Passing at 2.5 SD
200	34/35	97%
100	35/35	100%
50	32/35	91%
20	35/35	100%
10	35/35	100%
5	34/35	97%
2	34/35	97%
1	33/35	94%
0.5	28/35	80%

Assay Precision – The assay was tested for reproducibility on the same day, same operator, and same instrument (intra-assay precision), as well as reproducibility between different instruments and operators (intermediate precision). The assay was shown to be precise down to 1 ng of input DNA.

Assay Accuracy – The accuracy of the LINE-1 assay was determined by comparing the methylation data obtained from the validation test samples to the expected values of the control DNA with known methylation levels. An R-square value of greater than 0.9 at the LOQ indicates that the assay is linear and free of PCR Bias. The average R-square for each input DNA amount was calculated and the results are shown in Figure 2. All seven input amounts, down to as low as 0.5 ng of starting DNA, show R-square values above 0.9. An example of the 5 ng input DNA amount is also shown in the figure.

Figure 2: LINE-1 Assay Accuracy and Linearity

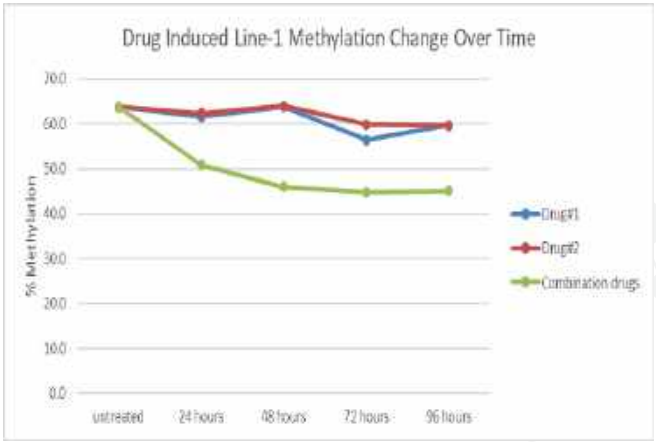


Input DNA (ng)	R-Square Mean
200	0.9989
100	0.9985
50	0.9972
20	0.9966
10	0.9917
5	0.9915
2	0.9842
1	0.9835
0.5	0.9868

Applications

A Biomarker in Cancer Research – LINE-1 hypomethylation has been found in various cancer types, such as bladder cancers[4], liver cancer[5], and gastrointestinal cancers[6]. LINE-1 methylation loss can be used as a surrogate marker for cancer diagnosis and prognosis. Genomic DNA isolated from whole blood, buffy coat, buccal cells, serum, FFPE tissue, and other biological specimens is suitable for quantifying LINE-1 methylation level using targeted bisulfite NGS, Pyrosequencing, or MS-HRM.

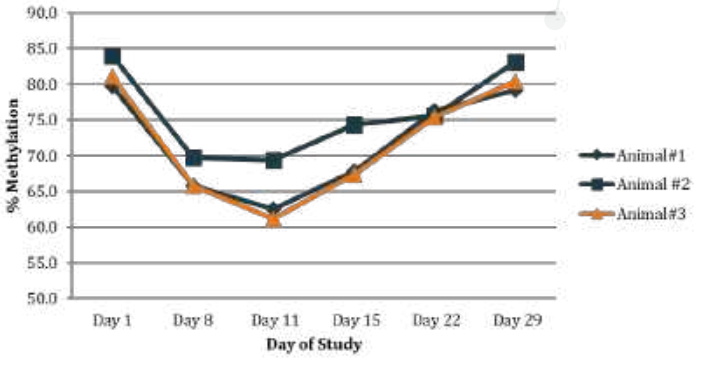
Figure 3: HMA drug treatment of H9 cells at different time points



High-Throughput Drug Screening – Hypomethylating agents (HMAs) such as azacitidine and decitabine are FDA-approved for use in the United States in myelodysplastic syndrome and are being investigated for use in a number of tumors. The Line-1 Pyrosequencing methylation assay has been used for drug screening of compounds that influence global DNA methylation states[7]. Figure 2 shows the drug-induced Line-1 methylation change at different treatment time points at a selected dose.

A Surrogate Pharmacodynamic (PD) Marker – Line-1 Pyrosequencing methylation analysis has been used as a pharmacodynamic (PD) marker for several clinical trials in cancer drug development[8-9]. Line-1 methylation can be used as a predictor of clinical response. The time course of LINE-1 methylation following drug administration may be related to HMA concentration in blood or plasma. Simulated LINE-1 matrices can develop a PK/dose-response model which can be used to optimize the dose regimen and to evaluate the dynamic of HMAs.

Figure 4: PD Analysis of non-human primates



Line -1 Global DNA Methylation Analysis - Results in % Methylation						
Animal ID	Day 1	Day 8	Day 11	Day 15	Day 22	Day 29
1	79.7	65.8	62.5	67.8	76.3	79.1
2	84.0	69.7	69.4	74.3	75.6	83.1
3	81.1	65.8	61.2	67.3	75.4	80.4

References

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