#151. Development of a Robust MGMT Promoter Methylation Assay for Glioblastoma Multiforme

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Abstract

The degree of methylation in the promoter region of the O-6-methylguanine-DNA methyltransferase (MGMT) gene is widely accepted as a prognostic biomarker of patient outcomes in Glioblastoma multiforme (GBM). There are a number of different assays currently available for the measurement of DNA methylation that assess methylation status by different methods and at different CpG sites in the MGMT promoter and in exon 1. There is the possibility that the various methods may produce different methylation results and thus lead to different patient treatment decisions based on the test results. Labcorp of America Holdings has provided MGMT testing services for clinical samples based upon a published assay (Kam-Morgan et al. 2009 AMP Annual Meeting; Hegi et al., 2019, Clin Cancer Res. 25:1809). Because of the need to change instrumentation this assay was re-formatted, optimized and analytically validated for possible use as an In Vitro Diagnostic Device. Formalin-fixed-paraffin-embedded (FFPE) specimens from GBM patients were evaluated using standard pathology methods, the tumor regions were identified, and the corresponding tissue tumor region from unstained slides were macro-dissected. DNA was isolated and analyzed by methylation specific real-time PCR (MSP). The copy number of methylated MGMT based on 8 CpG sites was determined and normalized to the copy number of β-Actin gene (ACTB). The MGMT MSP Assay was analytically validated to confirm that assay linearity, precision, accuracy and intra-specimen variability met pre-defined acceptance criteria. Additional analytical performance characteristics of the assay are currently being evaluated. The re-formatted MGMT MSP Assay provides a robust and reproducible measure of MGMT promoter methylation status in GBM tissue samples.

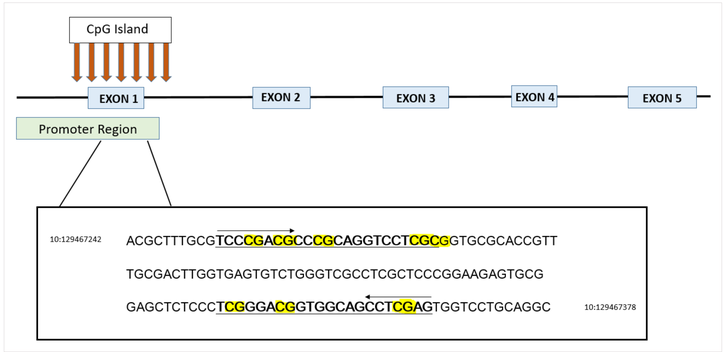


Figure 1. Schematic of the O-6-methylguanine DNA methyltransferase (MGMT) gene showing 5 exons, promoter region and CpG Island. MGMT MSP Assay forward and reverse primer binding regions are shown (underlined) and 8 CpG motifs that confer specificity to primer designs are highlighted. MGMT MSP Assay amplifies a 114 bp region within the MGMT promoter that spans exon 1 and intron 1.

Introduction

The O-6-methylguanine-DNA methyltransferase (MGMT) gene is located on the long arm of chromosome 10 (10:129461115-129777058 GRCh 38 – hg38). The MGMT MSP Assay uses a methylation specific forward primer targeting 5 CpGs and a methylation specific reverse primer targeting 3 CpGs. The MGMT MSP Assay amplifies a portion of the MGMT promoter region that spans exon 1 and intron 1. The ACTB primers amplify a region on the long arm of chromosome 7 independent of methylation status. The assay uses MGMT and ACTB calibrator plasmids to determine the number of copies of methylated MGMT and ACTB in each sample. The methylated MGMT copy number is normalized to the ACTB copy number to determine the methylation ratio. The methylation ratio and an assay specific algorithm are used to determine the methylation status. The MGMT MSP Assay is performed on the QuantStudio Dx (Thermo Fisher) platform and is intended to be marketed as an In Vitro Diagnostic Device.

Figure 2. Digitally scanned images of FFPE GBM specimen. Shown are HE stained slide (A), unstained slide pre-macrodissection (B) and unstained slide post-macrodissection (C) with tumor tissue scraped away for analysis.

Methods

The following analytical validation studies were performed and are described below:

- **Linearity** – Three clinical FFPE GBM specimens that represent methylated, unmethylated and borderline methylation ratios were tested. The 3 specimens were tested at up to 14 different DNA input levels ranging from 3.6 µg to 2.75 ng. This evaluation was performed using 2 different lots of MGMT MSP Assay Kit.
- **Precision** – Assay precision was evaluated in a single site study that included 3 operators and 3 instruments across 6 non-consecutive testing days and 1 lot of MGMT MSP Assay Kit. Ten clinical FFPE GBM specimens were tested.
- **Orthogonal Method Comparison** – Forty FFPE GBM specimens were tested with the MGMT MSP Assay and pyrosequencing assays that covered the 8 CpGs in the primer binding regions. These 40 samples were a training set to establish acceptance criteria for a larger analytical accuracy study to be performed.
- **Intra-Specimen Variability** – Twelve clinical FFPE GBM specimens had 36 sections cut and DNA isolated from sections from "front", "middle" and "back" (2 isolates each). All of the DNA isolates were tested with the MGMT MSP Assay to assess intra-specimen consistency of methylation ratio and clinical call.

Results

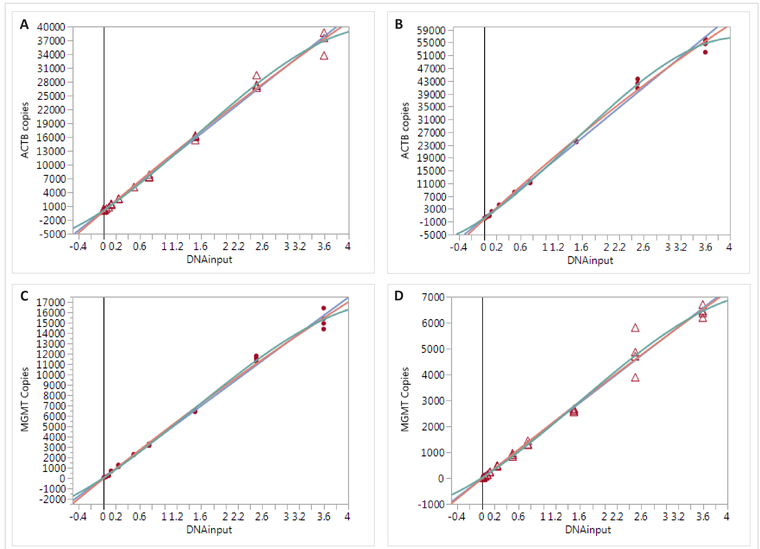


Figure 3. The linear ranges were identified in linear regression models with significant slopes and intercepts between 2.75 ng and 2500 ng DNA input of highly methylated sample. As shown in charts A-D, linearity of the MSP MGMT Assay was determined to range from 4 to 4782 copies of MGMT and 20 to 27649 copies of ACTB. Charts A and C are from MGMT MSP Assay Kit lot 1 and Charts B and D are from MGMT MSP Assay Kit lot 2. The MGMT MSP Assay was determined to have a wide linear range that encompasses clinically relevant DNA input levels.

Conclusions

- The MGMT MSP Assay has been suitably re-formatted, optimized and transferred to the QuantStudio Dx platform.
- The MGMT MSP Assay has been analytically validated for linearity, precision, orthogonal test method comparison and intra-specimen variability.
- Upon completion of additional analytical validation studies, the MGMT MSP Assay will be suitable for submission as an In Vitro Diagnostic Device.

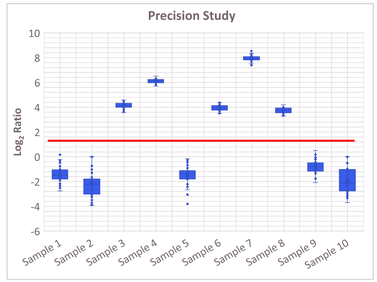


Figure 4. Box and Whisker plot of Log_2 methylation ratio of 10 clinical FFPE GBM samples. Red line represents clinical cut-off and demonstrates 100% agreement of qualitative call seen throughout study.

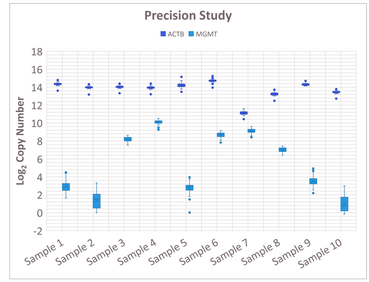
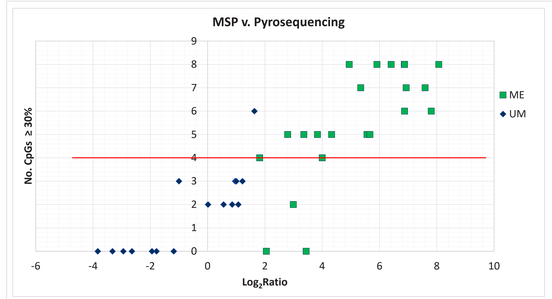


Figure 5. Precision of MGMT and ACTB copy number as demonstrated by single site precision study. Sample to sample variation in MGMT is representative of biological variability in methylation of MGMT promoter regions in 10 clinical FFPE GBM samples used in study. Sample to sample precision of ACTB copies demonstrates normalized DNA input into bisulfite conversion reaction.



MSP

	Pyrosequencing		
	ME	UM	Total
ME	19	3	22
UM	1	16	17
Total	20	19	

Figure 6. Comparison of the MGMT MSP Assay with pyrosequencing assays that cover the 8 CpGs in the primer binding region. Forty clinical FFPE GBM specimens were tested of which 39 gave valid results in both assays. The highest level of agreement was seen when comparing the MGMT MSP Assay result with the number of CpGs with ≥ 30% methylation via pyrosequencing. These results were used as pilot study for a larger analytical accuracy study to be performed.

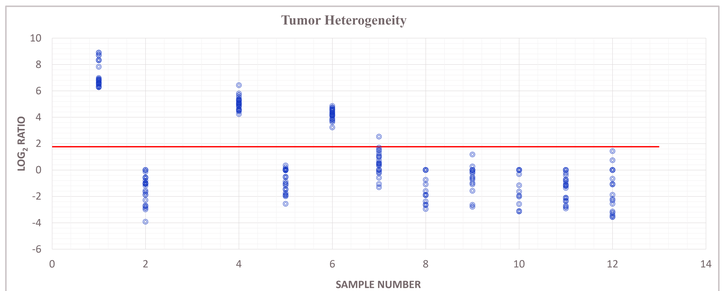


Figure 7. Tumor Heterogeneity study from 12 clinical FFPE GBM specimens of which 11 gave valid results. Red line denotes clinical cut-off. Each of 11 the specimens had 24 PCR data points. Only 1 switch in qualitative (methylated/unmethylated) call was observed in these 264 data points.

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