Validation of the EntroGen MGMT Promoter Methylation Detection Kit

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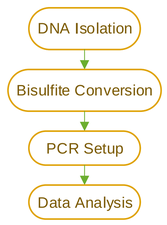
INTRODUCTION

O^6-Methylguanine-DNA methyltransferase (MGMT) has been shown to undergo epigenetic silencing by promoter methylation, resulting to lower expression and enzymatic activity that can predict drug response. Studies have shown improved response to alkylating agents (e.g. TMZ, carmustine) in glioblastoma tumors with MGMT promoter methylation. Therefore, measuring MGMT promoter methylation has become an important diagnostic tool in the clinical management of patients with glioblastoma diagnosis considered for treatment with TMZ or other alkylating cytotoxic agents. EntroGen's MGMT Methylation Detection Kit (MSPCR, Cat. No. MGMT-RT44) is a real-time PCR-based assay for detection of MGMT promoter methylation within the DMR2 locus. Its target region starts at chr10:131265513 (hg19 genome build) in the MGMT promoter region and covers CpG sites 75-86. The assay uses ACTB gene on chromosome 7 for DNA input and quantitates the MGMT promoter methylation levels by taking the methylated MGMT copies over ACTB copies, multiplied by manufacturer provided conversion factor, and divided by the tumor fraction (if known). The calculation formula is presented below.

$$Methylation\ ratio\ = \frac{(methylated\ MGMT\ copies \div ACTB\ copies)}{Tumor\ fraction} \times CF$$

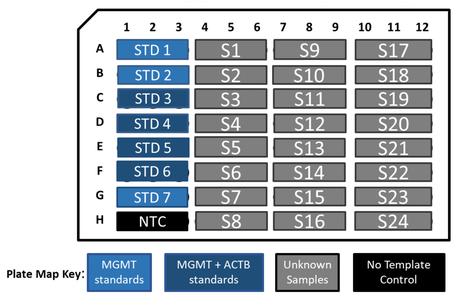
The assay involves a 4-step process (Figure 1):

Figure 1. MGMT promoter methylation detection workflow



Each assay run requires 7 MGMT and 4 ACTB standards (included). Samples are loaded in triplicate (Figure 2).

Figure 2. Suggested PCR plate layout



OBJECTIVE

The objective of this study was to validate the EntroGen real-time PCR-based MGMT Methylation Detection Kit (MSPCR, Cat. No. MGMT-RT44) for diagnostic use by establishing the analytical cutoff of the assay using glioblastoma samples previously tested by an orthogonal method (KDL-Pyro).

METHODS

DNA from glioblastoma tumor specimens was isolated using a silica-membrane based nucleic acid extraction method. The isolated genomic DNA underwent bisulfite conversion using the EpiTect® Fast Bisulfite Conversion Kit (Qiagen; Cat. No. 59824). A total of 89 unique samples (75 GBM tumor samples, 14 controls) underwent 121 sample runs with the MSPCR assay on a Roche® LightCycler 480 real-time PCR system. Methylated MGMT promoter and ACTB copy numbers were calculated based on the provided control standards. The MGMT:ACTB copy ratio was log2 transformed for further analysis. Statistical analysis was performed using R package *mclust* (http://www.r-project.org). Results were compared to data from a previously validated MGMT pyrosequencing detection method at KDL.

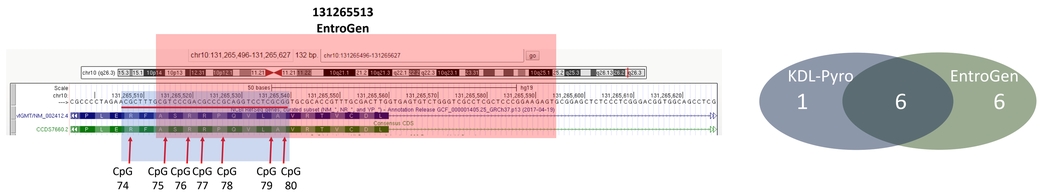
REFERENCES

1. Dunn J, Baborie A, Alam F, Joyce K, Moxham M, Sibson R, Crooks D, Husband D, Shenoy A, Brodbelt A, Wong H, Liloglou T, Haylock B, Walker C. Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. Br J Cancer. 2009 Jul 7;101(1):124-31.
2. Vlassenbroeck I1, Califice S, Diserens AC, Migliavacca E, Straub J, Di Stefano I, Moreau F, Hamou MF, Renard I, Delorenzi M, Flamion B, DiGuiseppi J, Bierau K, Hegi ME. Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma. J Mol Diagn. 2008 Jul;10(4):332-7
3. Stichel D, Ebrahimi A, Reuss D, Schrimpf D, Ono T, Shirahata M, Reifenberger G, Weller M, Hänggi D, Wick W, Herold-Mende C, Westphal M, Brandner S, Pfister SM, Capper D, Sahm F, von Deimling A. Distribution of EGFR amplification, combined chromosome 7 gain and chromosome 10 loss, and TERT promoter mutation in brain tumors and their potential for the reclassification of IDHwt astrocytoma to glioblastoma. Acta Neuropathol. 2018 Nov;136(5):793-803.

RESULTS

The EntroGen MSPCR assay covers CpG sites between 75 and 86 (12 sites), while the KDL-pyro assay covers CpG sites between 74 and 80 (7 sites). The KDL-Pyro assay covers one (1) site (CpG 74) that is not covered by the EntroGen MSPCR assay, while six (6) additional sites are covered by the EntroGen MSPCR assay and not covered by the KDL-Pyro assay (Figure 3).

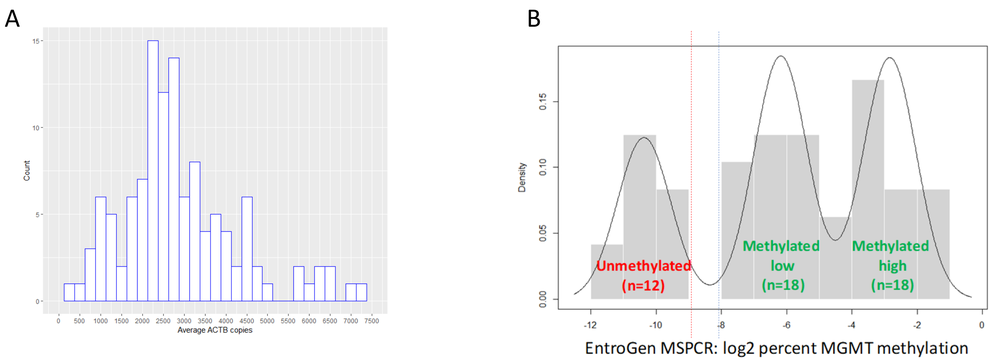
Figure 3. MGMT promoter CpG site coverage by EntroGen MSPCR & KDL-Pyro.



Establishing cutoffs for MSPCR

A total of 120 sample runs across 88 unique samples met the minimum ACTB copy requirement (>200 copies). Samples included GBM patient tumor samples, Zymo methylated DNA controls, CAP proficiency testing samples, and normal brain tissue (Figure 4A). Analysis of the MSPCR data revealed the best fit into a trimodal distribution model (Figure 4B) with three (3) distinct peaks; one for unmethylated (peak at 0.1%), one for low methylated (peak at 1.5%), and one for high methylated (peak at 12.5%) samples.

Figure 4. (A) Average ACTB copies across 120 sample runs; (B) trimodal distribution model of the EntroGen MSPCR data depicting the upper and lower cutoffs (0.17%-0.55%).

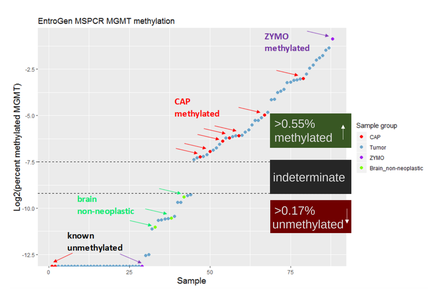


Sample classification using the established cutoffs

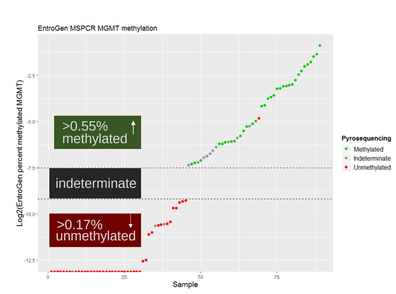
The classification of 88 unique samples (including ZYMO and CAP PT controls) is depicted in Figure 5A below. The horizontal lines show the indeterminate range. When compared to KDL-Pyro, 33/34 (97.1%) samples classified as methylated with KDL-Pyro were also classified as methylated by EntroGen MSPCR, and 41/42 (97.6%) of samples classified unmethylated by KDL-Pyro were classified as unmethylated by EntroGen MSPCR.

Figure 5. (A) Classification of 88 unique samples; (B) Comparison with results of KDL-Pyro.

A



B



CONCLUSIONS

- Trimodal distribution model had the best fit for the EntroGen MSPCR MGMT % methylation.
- Cutoffs established based on the trimodal model had the highest concordance with an orthogonal method.
- There were no indeterminate classifications of clinical samples when using the EntroGen MSPCR assay.