DEVELOPMENT OF A REAL-TIME PCR MGMT PROMOTER METHYLATION ASSAY

by

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DEDICATION

To my loving mother, sister, and father for their continued support and understanding. To Jabil for his patience, continued encouragement, timely words of wisdom and advice during this process. I truly thank you.

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ABSTRACT

O6-methylguanine methyltransferase (MGMT) promoter methylation is associated with prolonged survival rates in patients with glioblastoma multiforme (GBM) undergoing standard therapy with alkylating agent Temozolomide (TMZ). Determining the MGMT methylation status of a patient diagnosed with GBM is crucial to triage appropriate clinical therapies. In this study, a real-time PCR assay was developed to detect the percent methylation reference (PMR) using designed primers and probes specific to methylated MGMT. The assay was developed using methylated and unmethylated commercial controls and cell lines to test specificity, sensitivity and limit of detection to further assess the assays robustness, efficacy and efficiency. This study shows this real-time PCR assay is effective and robust in determining the MGMT methylation status of a patient sample to aid physicians in their decision making for clinical therapies.

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LIST OF ABBREVIATIONS

MGMT	O6-Methylguanine Methyltransferase
ACTB	Beta-actin
FFPE	Formalin-Fixed Paraffin-Embedded
PMR	Percentage Methylated Reference
TMZ	Temozolomide
BC	Bisulfite Converted
mDNA	Methylated Millipore Control
umDNA	Unmethylated Millipore Control
PDX	Patient Derived Xenograph
DNMTs	DNA methyltransferases
O6Meg	O6-methylguanine
O6pobG	O(6)-[4-oxo-4-(3-pyridyl) butyl]guanine
O6C1G	O6-chloroethylguanine
IHC	Immunohistochemistry
MSP	Methylation Specific PCR
PCR	Polymerase Chain Reaction
GBM	Glioblastoma Multiforme
TTF	Tumor-Treating fields
NTC	No Template Control
CV	Coefficient of Variation
CT	Cycle Threshold
BLAST	Basic Local Alignment Search Tool

Tm	Melting Temperature
LOD	Limit of Detection

INTRODUCTION

Epigenetics is defined as DNA modifications that does not change the DNA sequence but can affect gene activity. Epigenetic modifications remain as cells divide and can be inherited from ancestry. Environmental influences such as diet and exposure to pollutants, can also impact the epigenome. Epigenetic changes can determine whether genes are turned on or off and can influence the production of proteins (Weinhold 2006). There are two main types of epigenetic changes that can occur in a cell; DNA methylation and histone modification. DNA methylation involves the covalent transfer of a methyl group (CH_3) to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs) creating the ability to modify the function of a gene without changing the DNA sequence. When methyl groups are added in the promoter region of a gene, that gene is silenced, and no protein is produced which represses transcription of the DNA segment. Errors in the epigenetic process, such as modifying an incorrect gene or failing to add a compound to a gene, can lead to abnormal gene activity or inactivity. Conditions including cancers, metabolic disorders, genetic disorders and degenerative disorders have been found to be related to epigenetic modifications(Weinhold 2006).

DNA methylation is essential for normal development, and plays an important role in a number of key processes including genomic imprinting, X-chromosome inactivation, and when dysregulated, contributes to diseases such as cancer (Jin et al. 2011). Cancer initiation and proliferation is regulated by both epigenetics and genetics with epigenetic modifications being identified as important targets for cancer research. DNA methylation catalyzed by DNMTs is one of the essential epigenetic mechanisms that control cell proliferation, apoptosis, differentiation, cell cycle, and transformation

(Pan et al. 2018). DNA methylation is regulated by a family of DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L.7-11. Most CG dinucleotides are methylated on cytosine residues and DNA hypomethylation and hypermethylation of CpG islands are epigenetic hallmarks of cancer (Jin et al. 2011).

MGMT is a suicide enzyme that repairs pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O6-methylguanine. DNA damage can form in response to environmental pollutants, tobacco-specific carcinogens and anticancer drugs. MGMT is key in the defense against carcinogens and a marker of resistance of normal and cancer cells exposed to alkylating therapeutics. MGMT also protects against therapy-related tumor formation caused by highly mutagenic drugs. The amount of MGMT present determines the level of repair of toxic DNA alkylation, and the MGMT expression level can indicate an individual's cancer susceptibility and their success to clinical therapies. MGMT silencing in tumors is mainly regulated epigenetically and in brain tumors this correlates with a better therapeutic response. Conversely, up-regulation of MGMT during cancer treatment limits the therapeutic response (Christmann et al. 2011: 6). Chemotherapeutic drugs and environmental compounds have the ability to alkylate DNA (Figure 1). The formation of O6-methylguanine (O6MeG), O(6)-[4-oxo-4-(3-pyridyl) butyl]guanine (O6pobG) and O6-chloroethylguanine (O6ClG) can be repaired by MGMT. MGMT repairs O6MeG by transferring the alkyl group to a cysteine residue in its active site inactivating the protein which is then ubiquitinated and targeted for proteasomal degradation (Figure 2). The longer alkyl groups O6ClG and O6pobG are repaired by MGMT by similar mechanisms (Christmann et al. 2011).

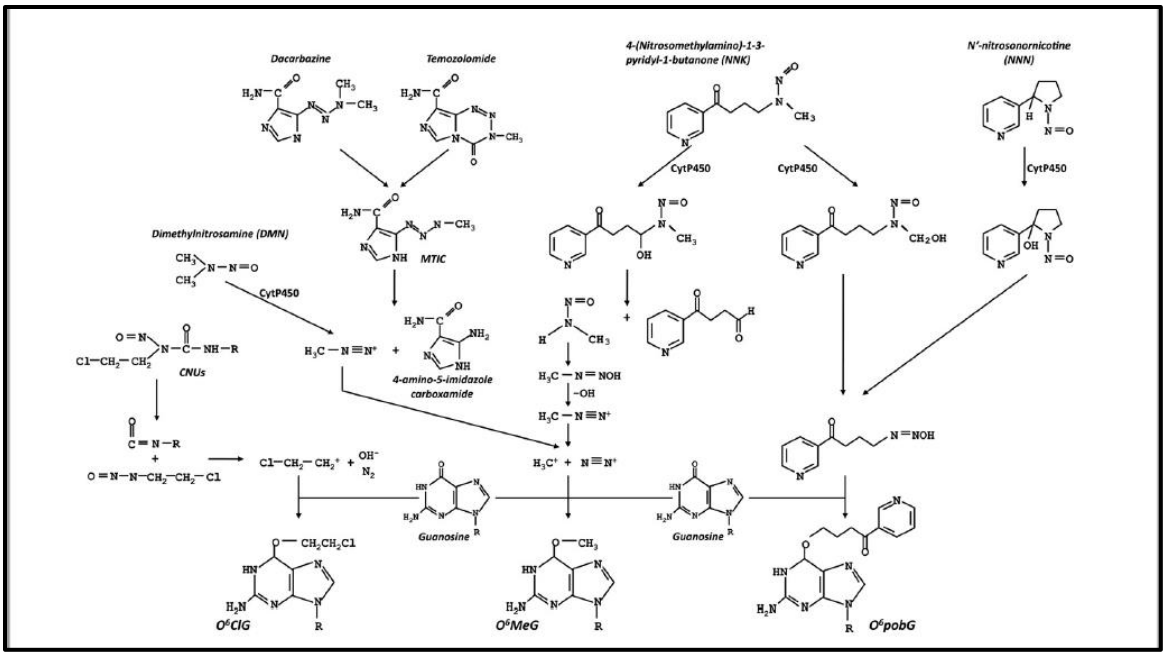

Figure 1. Mechanism of the formation of MGMT targets O6MeG, O6ClG and O6pobG in the DNA by the anticancer drugs temozolomide (TMZ), dacarbacine and chloroethylnitrosoureas (CNUs), environmental genotoxin dimethylnitrosamine (DMN) and tobacco-specific carcinogens NNN and NNK (Christmann et al. 2011)**.**

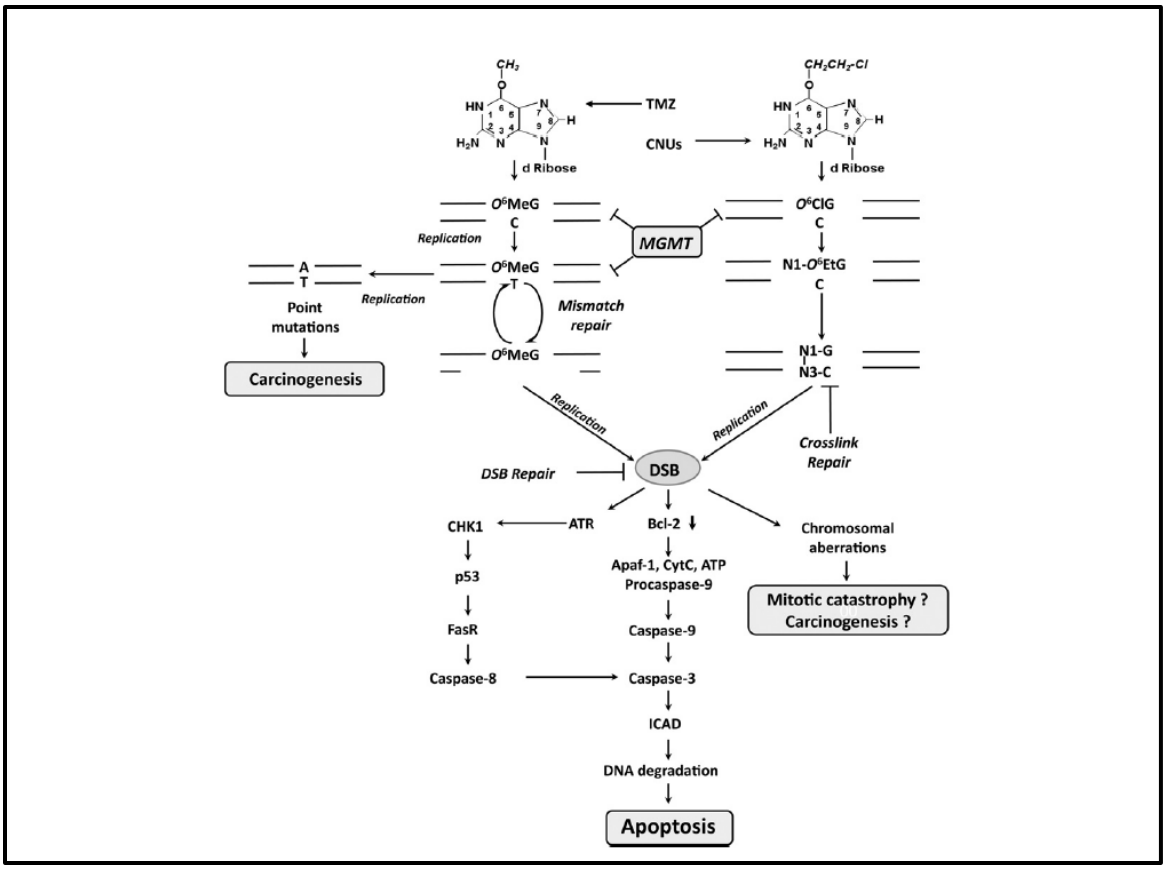

Figure 2. The repair mechanism of MGMT and processing of O6MeG and O6ClG adducts, mechanisms of conversion into DNA double-strand breaks (DSB) and triggering and execution of apoptosis (Christmann et al. 2011)**.**

MGMT is important for alkylating drug resistance of tumor cells repairing critical upstream lesions. The tumor's capability for removing DNA alkylation adducts induced by the anticancer drugs depends on the amount of MGMT expressed prior to therapy and the rate of MGMT re-synthesis. MGMT also plays an essential role in protecting the normal tissue during therapy. Therefore, interest in determining the MGMT methylation status of normal and tumor tissues are of importance because MGMT can be used as a prognostic marker for tumor resistance to O6-alkylating agents and provide a guide for therapeutic decisions (Christmann et al. 2011). Several methods are used to detect MGMT, each with its own advantages and disadvantages. Some of the detection methods

are; immunohistochemistry (IHC), methylation-specific PCR (MSP), pyrophosphate sequencing, MGMT activity test, and real-time quantitative PCR (Yu et al. 2020). MGMT expression depends on the methylation status of the gene. Specifically, the presence of 5-methylcytosine in specific CpG islands of the MGMT promoter. MGMT expression is highly regulated via promoter methylation. A total of 97 CpG islands have been identified in the MGMT promoter. Most studies focused on methylation of two CpG clusters positioned between −249 and −103 and between +107 and +196 of the gene, which have been shown to promote transcriptional silencing. Methylation of the CpG-islands results in heterochromatinization, which is accomplished by the re-arrangement and random positioning of nucleosomes and shields the transcription start site from transcription machinery. MGMT has an impact on cancer formation and therapy effectiveness, and it is frequently used as a biomarker. Therefore, the methods for determining the MGMT status of normal and cancer tissues are critical for cancer therapy effectiveness (Christmann et al. 2011).

Glioblastoma multiforme is an aggressive type of cancer that can occur in the brain or spinal cord affecting any age group but tends to occur more often in adults (Figure 3). Glioblastoma can be difficult to treat, and a cure is often not possible. Genetic predisposition and exposure to irradiation in childhood are the only established risk factors which, account for a very small proportion of glioblastomas. The first line of standard treatment for glioblastoma is surgery to allow for tissue diagnosis and to reduce tumor volume. Radiotherapy delivered to the tumor region accompanied with TMZ, followed by six cycles of maintenance chemotherapy was the first pharmacological treatment shown to prolong survival. Adding tumor-treating fields (TTF) during

maintenance and TMZ chemotherapy has been reported to prolong survival as well (Weller et al. 2019). These clinical treatments can slow the progression of the cancer and reduce signs and symptoms. Standard treatment practices for patients that are unable to see success with the therapies previous stated then reflux to clinical trials and supportive palliative care (Mayo Clinic 2019 Aug 9) .

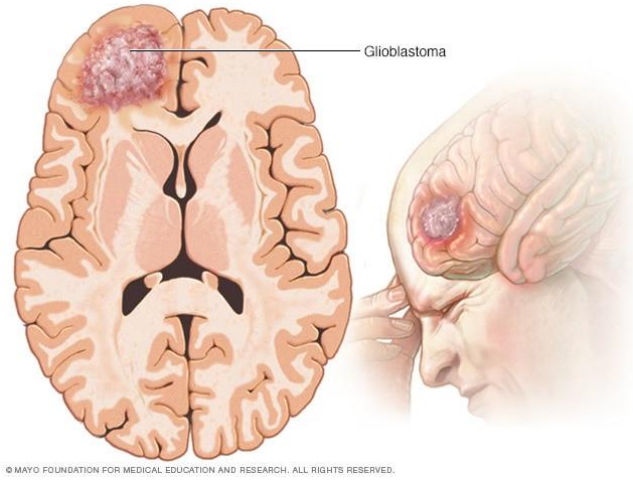

Figure 3. Glioblastoma multiforme is a type of brain cancer that begins in cells known as astrocytes that support nerve cells and can form in the brain or the spinal cord (Mayo Clinic 2019 Aug 9)

Cancer research is advancing in areas of targeted drug therapies and specialized chemotherapeutic drugs to help combat the continuation of cancer formation. TMZ is an orally administered alkylating agent used largely in the therapy of malignant brain tumors including glioblastoma and astrocytoma (Figure 4). This alkylating agent disrupts DNA replication, causing modifications and cross linking of DNA, inhibiting DNA, RNA, and protein synthesis leading to apoptosis in rapidly dividing cells (Figure 5). TMZ can cross the blood-brain barrier and has been shown to induce tumor regression and remission in patients with malignant astrocytoma and glioblastoma multiforme (Temozolomide,

2012). The capability of TMZ to cross the blood brain barrier is an important mechanism because this cellular membrane aids in blocking potentially harmful substances in the blood from entering the brain. Research has shown tumor cells can become resistant to the effects of TMZ. This resistance in most cases is correlated to higher levels of the DNA repair MGMT protein which renders TMZ less effective (Figure 5). Currently only a biopsy sample of the tumor can reveal the levels of the MGMT protein present in tumor cells. Knowing the level of MGMT in tumor cells is critical in aiding clinicians and oncologist to tailor treatment and therapy accordingly (The Brain Tumor Charity 2019)

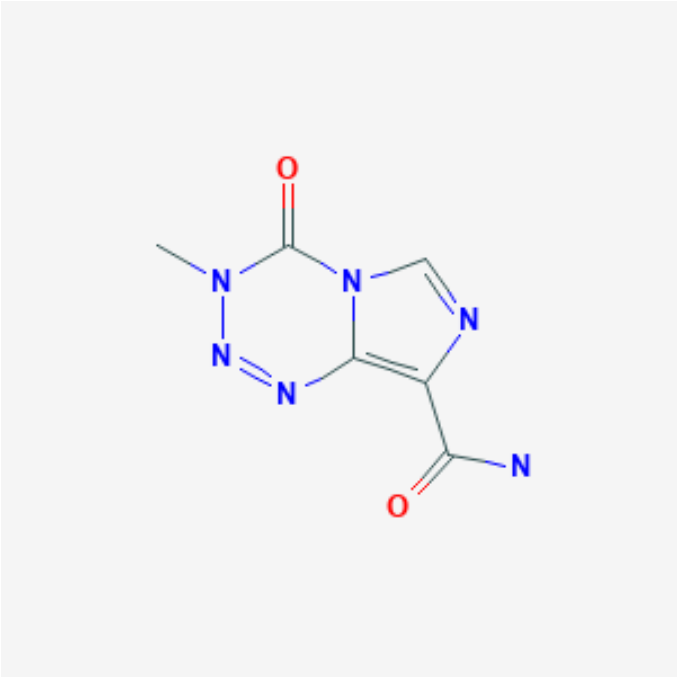

Figure 4. Chemical structure depiction of alkylating drug Temozolomide (NIH 2012)

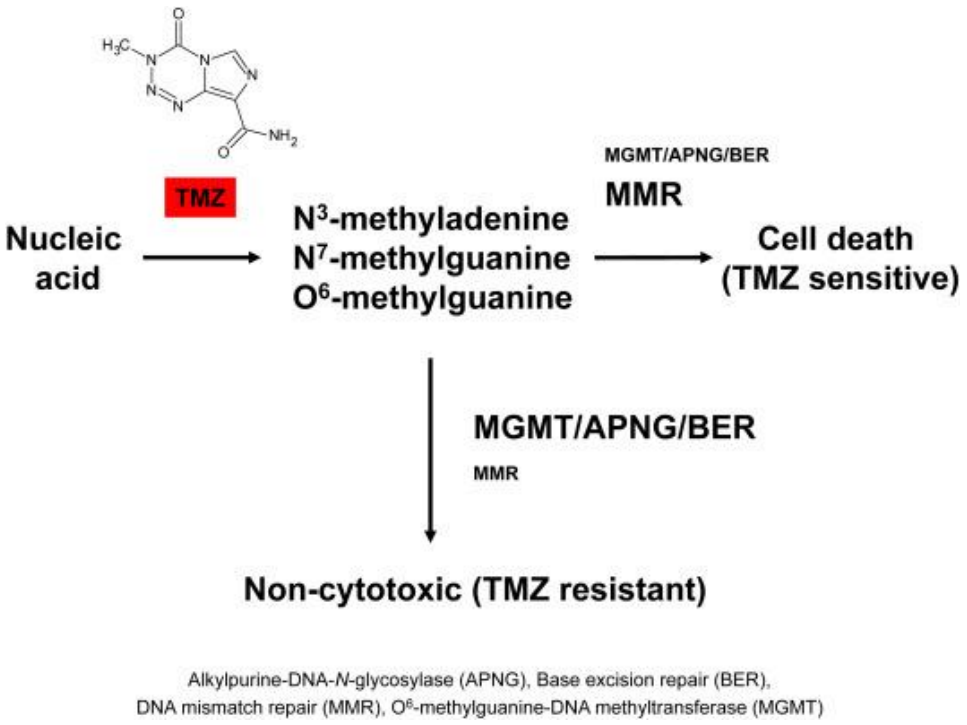

Figure 5. Mechanism of Temozolomide and Temozolomide resistance. TMZ modifies DNA at N7 and O6 sites on guanine and the N3 on adenine by the addition of methyl groups. The methylated sites can remain mutated, be fixed by DNA mismatch repair (MMR), be removed by base excision repair (BER) by the action of a DNA glycosylase such as, alkylpurine-DNA-N-glycosylase (APNG), or deakylated by the action of a demethylating enzyme such as O6-methylguanine methyltransferase (MGMT). Cells are TMZ sensitive when MMR is expressed and active (Lee 2016 Sep).

The MGMT gene is located on chromosome 10q26 and encodes a DNA-repair protein that removes alkyl groups from the O6 position of guanine which is an important site of DNA alkylation (Figure 6). When treated with alkylating chemotherapy drug TMZ epigenetic silencing of the MGMT gene by promoter methylation has been associated with longer overall survival in patients with glioblastoma. The restoration of DNA consumes the MGMT protein, which the cell must replenish. Left unrepaired, chemotherapy-induced lesions, especially O6-methylguanine, trigger cytotoxicity and apoptosis. High levels of MGMT activity in cancer cells create a resistant phenotype by blunting the therapeutic effect of alkylating agents and may be an important determinant

of treatment failure. Gene silencing by DNA methylation is an early and important mechanism by which tumor-suppressor genes are inactivated (Hegi et al. 2005).

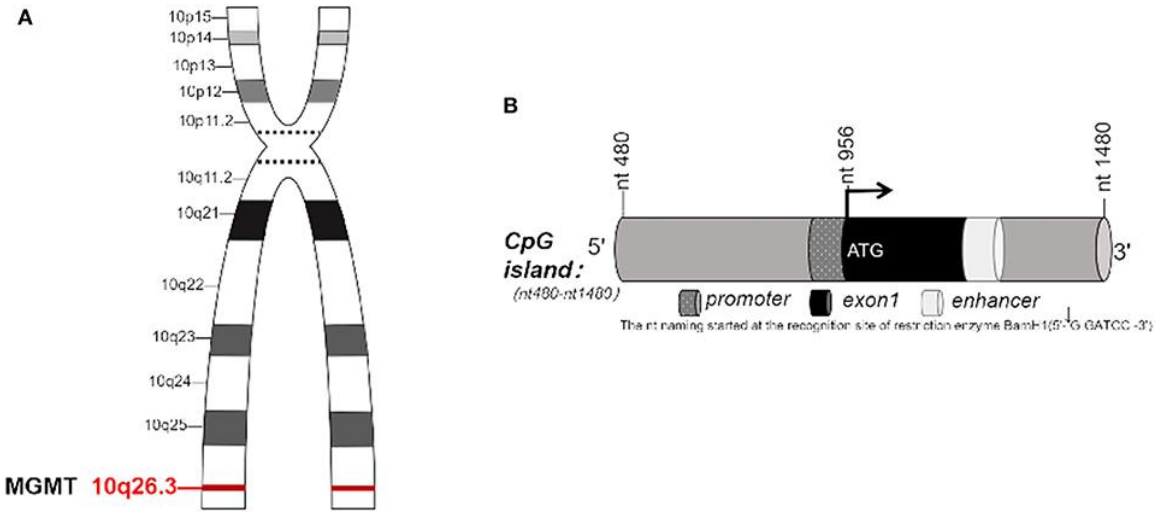

Figure 6. MGMT gene is located on chromosome 10q26.3 and the CpG island on the MGMT gene (Yu et al. 2020).

Assays to measure DNA methylation, which are important in epigenetic research and clinical diagnostics, rely on the conversion of unmethylated cytosines to uracils by sodium bisulfite. Clinical laboratories and institutions are studying DNA methylation in more detail because studies show this may be a useful marker for cancer diagnosis, screening, surveillance in high-risk individuals, monitoring of minimal residual disease, and determining optimal therapeutic options. Therefore, there are increasing demands for reliable assays to measure DNA methylation, particularly for formalin-fixed paraffin-embedded tissue (FFPE) (Ogino et al. 2006). This project will focus on the development of a duplex real-time PCR assay to detect the MGMT methylation status and percent methylated reference (PMR) for cancer patients in clinical studies. Since the testing

protocol will be established during the development of this assay, the next steps are to evaluate different sample types and testing methods to validate the assay for clinical testing.

During assay development we tested and determined the Qiagen Epitect Bisulfite Kit was a viable option for bisulfite conversion and the Qiagen MethyLight PCR Kit was a viable option for real time PCR assay. We developed and implemented a real-time PCR assay to determine the promoter methylation status of the MGMT gene instead of relying solely on IHC. This will allow for faster processing without the need to send clinical specimens out for testing at another lab which will greatly increase our turn-around time, higher control over the assay design and testing processes, and the ability to generate a clinical report after the validation of the assay to aid clinicians in treatment and therapy options for patients. DNA methylation status will be measured semi quantitively by an equation called PMR which calculates the quantity of MGMT and ACTB in a sample and control against each other multiplied by 100 to determine the methylation percentage.

The Qiagen EpiTect Bisulfite Conversion Kit was evaluated and tested during assay development. Genomic DNA was subjected to sodium bisulfite to determine the methylation status of the MGMT DNA sequence of a particular sample. The incubation of the genomic DNA with sodium bisulfite will result in the conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment will give rise to different DNA sequences for methylated and unmethylated DNA (Figure 7). The correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. This is achieved by incubating DNA in high bisulfite salt concentrations at high temperatures and low pH. Purification is

necessary to remove bisulfite salts and chemicals used in the conversion process that inhibit downstream procedures. The genomic DNA will undergo bisulfite-mediated conversion of unmethylated cytosines using the Bisulfite Mix and Protect Buffer followed by the incubation on a thermal cycler with predetermined cycling conditions provided in the protocol. The binding of converted single-stranded DNA to the membrane of an EpiTect spin column was accomplished using Buffer BL. Washing of the membrane bound DNA was accomplished using Buffer BW to remove residual sodium bisulfite. Desulfonation of membrane-bound DNA was done using Buffer BD. Washing of the membrane-bound DNA to remove the desulfonation agent was done using Buffer BW. The elution of pure converted DNA from the spin column was done using Buffer EB (Figure 8). The bisulfite thermal cycling program provided an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent sulfonation, cytosine deamination, enabling high cytosine conversion rates of over 99%. Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure (Qiagen 2014 Dec).

	Original sequence	**After bisulfite treatment**
Unmethylated DNA	N-C-G-N-C-G-N-C-G-N	N-U-G-N-U-G-N-U-G-N
Methylated DNA	N-**C**-G-N-**C**-G-N-**C**-G-N	N-**C**-G-N-**C**-G-N-**C**-G-N

Figure 7. Bisulfite treatment allows for two different DNA sequences for methylated and unmethylated DNA. The methylation status of a DNA sequence can be determined by using sodium bisulfite. Incubation of the DNA with sodium bisulfite results in the conversion of unmethylated cytosine residues into uracil which leaves methylated cytosines unchanged (Qiagen 2014 Dec).

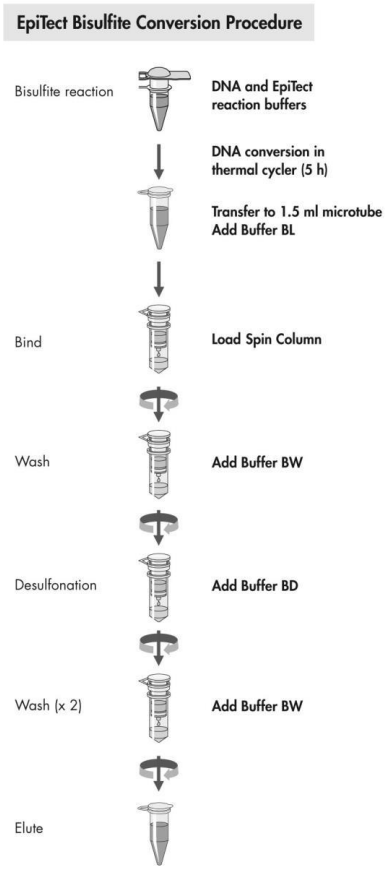

Figure 8. The Qiagen EpiTect Bisulfite Conversion Procedure. The mixture of extracted DNA with EpiTect reaction buffers is performed followed by the incubation on a thermal cycler. The cleanup of bisulfite converted DNA with reagent buffers is followed by wash steps leading to the eluate of purified converted bisulfite DNA (Qiagen 2014 Dec).

The Qiagen EpiTect Methylight PCR Kit was evaluated and tested during assay development for the detection of methylated MGMT. The EpiTect Methylight PCR Kit contains a HotStarTaq Plus DNA Polymerase that is a modified form of a DNA Polymerase (recombinant 94 kDa DNA polymerase) which is isolated from Thermus aquaticus, cloned into *E. coli*. The enzyme is activated by a 5-minute, 95°C incubation step. The EpiTect Probe PCR Buffer is a highly sensitive methylation quantification Buffer of bisulfite converted DNA targets which includes multiplex-

PCR–enabling Factor MP. dNTP Mix which includes dATP, dCTP, dGTP, and dTTP of ultrapure quality. Lastly, the kit includes 50x ROX Dye Solution which is a passive reference dye for normalization of fluorescent signals on specific PCR Systems (Qiagen 2011 Aug). Selection of the genetic regions of interest for MGMT and ACTB was determined for the primer and probe design of this real-time PCR methylation assay. The primer and probe design for MGMT and ACTB was accomplished by referencing the published papers by Ida et al and Vlassenbroeck et al. These sequences were further analyzed and optimized using IDTs recommendations based on melting temperature (Tm), GC content, oligonucleotide size, and other factors. The concentrations of the real-time PCR mix and cycling conditions were established by predetermined parameters provided in the Qiagen EpiTect Methylight PCR protocol. The standard curves were evaluated for efficacy and background amplification. The primers and probe design for bisulfite converted MGMT and ACTB were tested for sensitivity and specificity. The specificity of the primers and probe for MGMT and ACTB were tested with bisulfite converted DNA and genomic DNA that was not subjected to bisulfite conversion. The primers did not amplify genomic DNA that was not subjected to bisulfite conversion but did amplify bisulfite converted DNA indicating that the primers and probes are specific to bisulfite converted DNA. The primers and probes for MGMT and ACTB was tested for sensitivity with methylated and unmethylated manufactured commercial controls and cell lines. ACTB primers/probes will always amplify since the region selected is devoid of CpG sites and will be used as an internal control. MGMT primers/probes should only amplify samples that are methylated and should not amplify samples that are unmethylated. The MGMT primers/probe only amplified control samples that are

methylated and not the control samples that are unmethylated indicating that the primers/probe are sensitive to methylated MGMT.

MGMT promotor methylation maybe a useful marker for predicting prognosis and monitoring efficacy of adjuvant treatment with TMZ in cancer patients and for evaluating risks for tumor recurrence and surveillance in high and low risk individuals. Assays to measure MGMT promoter methylation are being developed for FFPE tissue and rely on sodium bisulfite treatment of genomic DNA from tumor tissue to reflux into real time PCR testing to determine methylation status (Ogino et al. 2006). Studies show patients with glioblastoma containing a methylated MGMT promoter benefit from TMZ, whereas those who do not have a methylated MGMT promoter did not benefit from TMZ (Hegi et al. 2005).

MATERIALS AND METHODS

MGMT primers were design based on the publication titled “Validation of Real-Time Methylation-Specific PCR to Determine O6-Methylguanine-DNA Methyltransferase Gene Promoter Methylation in Glioma” (Vlassenbroeck et al. 2008). The MGMT probe design was based on the publication titled “Real-Time Methylation-Specific Polymerase Chain Reaction for MGMT Promoter Methylation Clinical Testing in Glioblastoma” (Ida et al. 2017). The ACTB primers and probe were designed from the Ida et al paper. Primers were optimized and synthesized at 100µmole by Integrated DNA Technologies (IDT). Primers and probes were assigned internal laboratory identifiers based on standard operating procedures.

CpGenome Universal Methylated DNA (Millipore, Cat no. S782) and CpGenome Universal Unmethylated DNA (Millipore, Cat no. S7822) were used as controls to develop the standard curve, test LOD, sensitivity and specificity, and were used for positive and negative controls for this assay. Methylated cell line K562 (ATCC, CCL-243) and unmethylated cell line RKO (ATCC, CRL-2577) were used for FFPE testing for LOD, sensitivity and specificity, and were used for positive and negative controls for this assay.

Real-time PCR thermocyclers used throughout the project include the Applied Biosystems Veriti 96-Well Thermal Cycler (ThermoFisher Scientific, Cat no. 4375786). Real-Time PCR machine used throughout the project include Applied Biosystems ViiA7 Real-Time PCR System with 96-Well Block (ThermoFisher Scientific, Cat no. 4453534).

Samples were subjected to bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen, Cat no. 59104). DNA at 50ng (2.5ng/µL at 20µL) was added to the bisulfite mix and protect buffer. DNA and EpiTect reaction mix were subjected to bisulfite conversion using Veriti thermal cycler with pre-established cycling conditions. Thermal cycling conditions were the following denaturing and incubation steps: 95°C for 5 minutes, 60°C for 25 minutes, 95°C for 5 minutes, 60°C for 85 minutes, 95°C for 5 minutes, 60°C for 175 minutes, and hold at 20°C indefinitely. Samples were transferred to a 1.5mL tube with Buffer BL and carrier RNA then loaded to a spin column. Samples were washed with Buffer BW followed by desulfonation with Buffer BD. Samples were then washed further (2x) with Buffer BW followed by elution in 20µL with Buffer EB. Samples were not quantitated after bisulfite conversion but proceeded immediately to EpiTect Methylight (Qiagen 2014 Dec).

All real-time PCR reactions were completed using 2X EpiTect MethyLight Master Mix with 50X ROX Dye Solution added separately (Qiagen, Cat no. 59496) and nuclease free water. Final reaction volume was 20µl total including a 5µl sample volume. Sample DNA input was 12.5ng (2.5ng/µL at 5µL) assuming full recovery from the bisulfite conversion. Real-time PCR cycling conditions were initial PCR activation step at 95℃ for 5 minutes followed by 45 cycles of denaturation at 95℃ for 15 seconds and annealing and extension at 60℃ for 1 minute (Qiagen 2011 Aug). Coefficient of variation (CV) was calculated as the ratio of the standard deviation to the Ct average. CV values ≤ 3% were considered acceptable, indicating that the data points included in the CV calculation have acceptable variation from the mean. For a standard curve with five

serial dilution points, our aim of acceptable criteria was R^2 value ≥ 0.95 and an efficiency ranging between 80%-120%.

Percent Methylated Reference (PMR) was calculated with all samples in each PCR run with controls to determine the percentage of MGMT methylation in each sample. Q stands for the quantity value calculated on the Applied Biosystems ViiA7 software which is then input in this calculation. PMR calculation:

PMR= (Qsample/QMethylCtrl)MGMT/(Qsample/QMethylCtrl)ACTB)*100

RESULTS

Millipore Methylated DNA Controls were purchased for the generation of a quantitative standard curve. Methylated controls were bisulfite converted and eluted at 2.5ng/µL at 20µL then serially diluted 1:2 with nuclease free water generating five standard curve points to input into the Applied Biosystems ViiA7 QuantStudio software including a no template control (Table 1).

Table 1. Millipore Methylated DNA Controls were used to generate assay standard curve for quantitation.

Sample Name	ng Total	Sample Volume (µL)	Diluent Volume (µL)
Millipore Methylated Control	12.5	40	NA
	6.25	20	20
	3.125	20	20
	1.56	20	20
	0.78	20	20

Primers and fluorescently labeled probes were designed for the methylated sequence of the gene of interest MGMT and internal control ACTB. Both probes were TaqMan probes. MGMT probe was labeled with a FAM fluorophore and ACTB probe was labeled with a HEX fluorophore (Table 2).

Table 2. MGMT and ACTB primers and probes selected for development of methylation assay.

	ACTB	MGMT
Forward Primer	GGG GTG TTT GGT TTA GGT GTT ATG T	TTC GAC GTT CGT AGG TTT TCG C
Reverse Primer	CTA CCC TAA AAC AAC CCC ACT CC	CGA AAC TAC CAC CGT CCC GA
Probe	HEX-CAA ATA CCA /ZEN/ ACC AAC CCC AAT-3IABkFQ/	56-FAM/AAA CGA CCC /ZEN/ AAA CAC TCA CCA AAT CGC-3IABkFQ/

Highly methylated MGMT region of interest DNA sequence was used to design methylated specific primers and probe. MGMT forward primer interrogates five CpG islands, reverse primer interrogates three CpG islands and probe interrogates two CpG islands. If the MGMT gene is methylated the base pair cytosine will remain a cytosine after bisulfite conversion. The MGMT methylated specific primers and probe will only bind to the complimentary sequence containing cytosines after bisulfite conversion. If the MGMT gene is unmethylated the base pair cytosine will change to uracil after bisulfite conversion. The MGMT methylated specific primers and probe will not bind to the complimentary sequence containing thymine after bisulfite conversion (Figure 9).

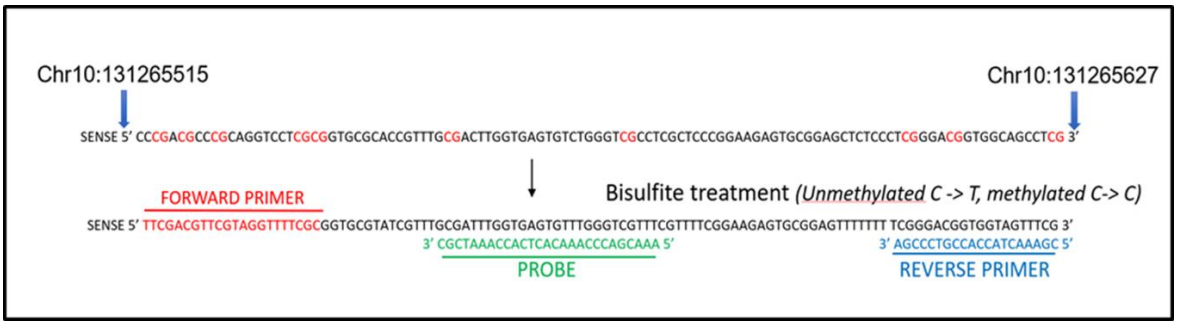

Figure 9. MGMT DNA region of interest bisulfite converted generating altered MGMT DNA sequence used to develop methylated primers and probe.

ACTB region of interest DNA sequence was used to design primers and probe as an internal control. ACTB forward primer, reverse primer, and probe DNA sequence are all devoid of CpG islands. Regardless if a sample is methylated or unmethylated ACTB will amplify to test that the PCR reaction is working appropriately based on established PCR conditions and mechanics and to ensure we are amplifying MGMT methylation when present. Since the ACTB gene is devoid of CpG islands the base pair cytosine will change to thymine after bisulfite conversion. The ACTB primers and probe will bind to the complimentary sequence after bisulfite conversion (Figure 10).

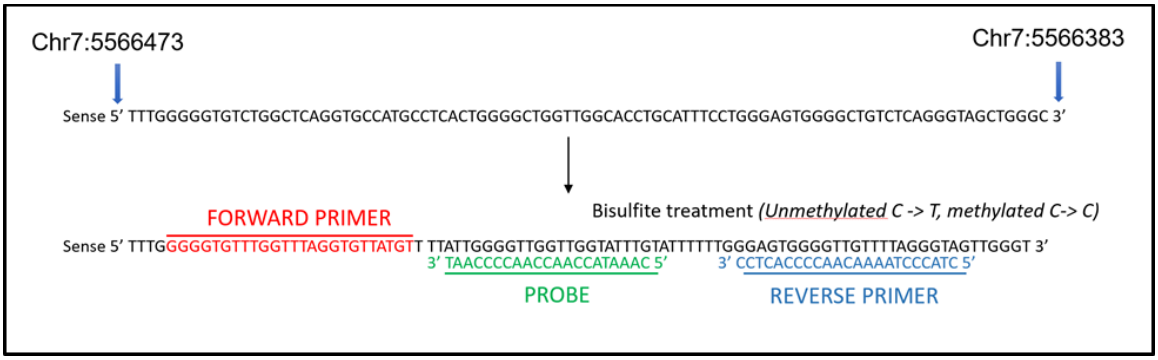

Figure 10. CpG devoid ACTB DNA region of interest bisulfite converted generating unaltered ACTB DNA sequence used to develop ACTB primer and probe design.

The 10X primer-probe mix consisted of 4µM forward primer, 4µM of reverse primer, and 2µM of probe. The PCR reaction contained a final primer concentration of 0.4µM and probe concentration of 0.2µM for both MGMT and ACTB (Table 3).

Table 3. MGMT and ACTB final primer concentration of 0.4µM and a final probe concentration of 0.2µM.

ACTB	PCR concentration (µM)
Forward Primer	0.4
Reverse Primer	0.4
Probe	0.2
MGMT	PCR Concentration (µM)
Forward Primer	0.4
Reverse Primer	0.4
Probe	0.2

This experiment tested the initial primer and probe design for MGMT and ACTB at varying DNA inputs for methylated DNA. The following questions were interrogated: determine if primer and probe design work efficiently and determine the appropriate/approximate DNA input for the assay. The DNA input was decided to be approximately 10ng, but further experiments would need to be performed to lock down a specific input. The CV value was ≤ 3% for each DNA input, a laboratory standard for every real-time assay designed (Table 4). Based on these results one could infer that the primers and probes for MGMT and ACTB were efficient but after reviewing the PCR amplification curves the samples did not indicate a sigmoidal curve indicative of an efficient PCR reaction for the MGMT primer/probe design. The PCR amplification

curves for the ACTB primers and probe did indicate a sigmoidal curve indicative of an efficient PCR reaction.

Table 4. Results of sample Ct values for Methylated Millipore Control at varying DNA inputs for initial primer/probe design.

	ACTB				MGMT			
Sample Name	Ct Value	Average	Standard deviation	Coefficient of variation	Ct Value	Average	Standard deviation	Coefficient of variation
mDNA.1ng	29.99	29.72	0.24	0.81	31.76	31.89	0.12	0.36
	29.64				31.98			
	29.53				31.92			
mDNA.5ng	27.54	27.58	0.08	0.31	29.94	29.85	0.10	0.33
	27.68				29.86			
	27.53				29.75			
mDNA.10ng	26.75	26.74	0.05	0.20	28.90	29.01	0.10	0.35
	26.79				29.07			
	26.69				29.07			
mDNA.20ng	25.64	25.69	0.05	0.19	28.13	28.19	0.06	0.21
	25.73				28.19			
	25.71				28.24			
mDNA.40ng	24.48	24.45	0.06	0.23	27.09	27.05	0.04	0.16
	24.48				27.06			
	24.39				27.00			

The PCR amplification curves of MGMT for these samples do not indicate a sigmoidal curve indicative of an efficient PCR reaction. The PCR amplification curves for ACTB primers and probe do indicate a sigmoidal curve indicative of an efficient PCR reaction. Based on these results the primer design for MGMT was revised to provide a sigmoidal curve indicative of an efficient PCR reaction (Figure 11). IDT's recommendations were to decrease the melting temperatures (Tm) of the forward and reverse primer and increase the Tm of the probe for MGMT. This was achieved by keeping the existing bases to the original MGMT forward primer, adding two more bases to the original MGMT reverse primer, and adding eleven bases to the original MGMT probe. Adding higher input into the PCR reaction begins to rescue the sigmoidal curve at 20ng and 40ng but this input is not ideal for a PCR reaction.

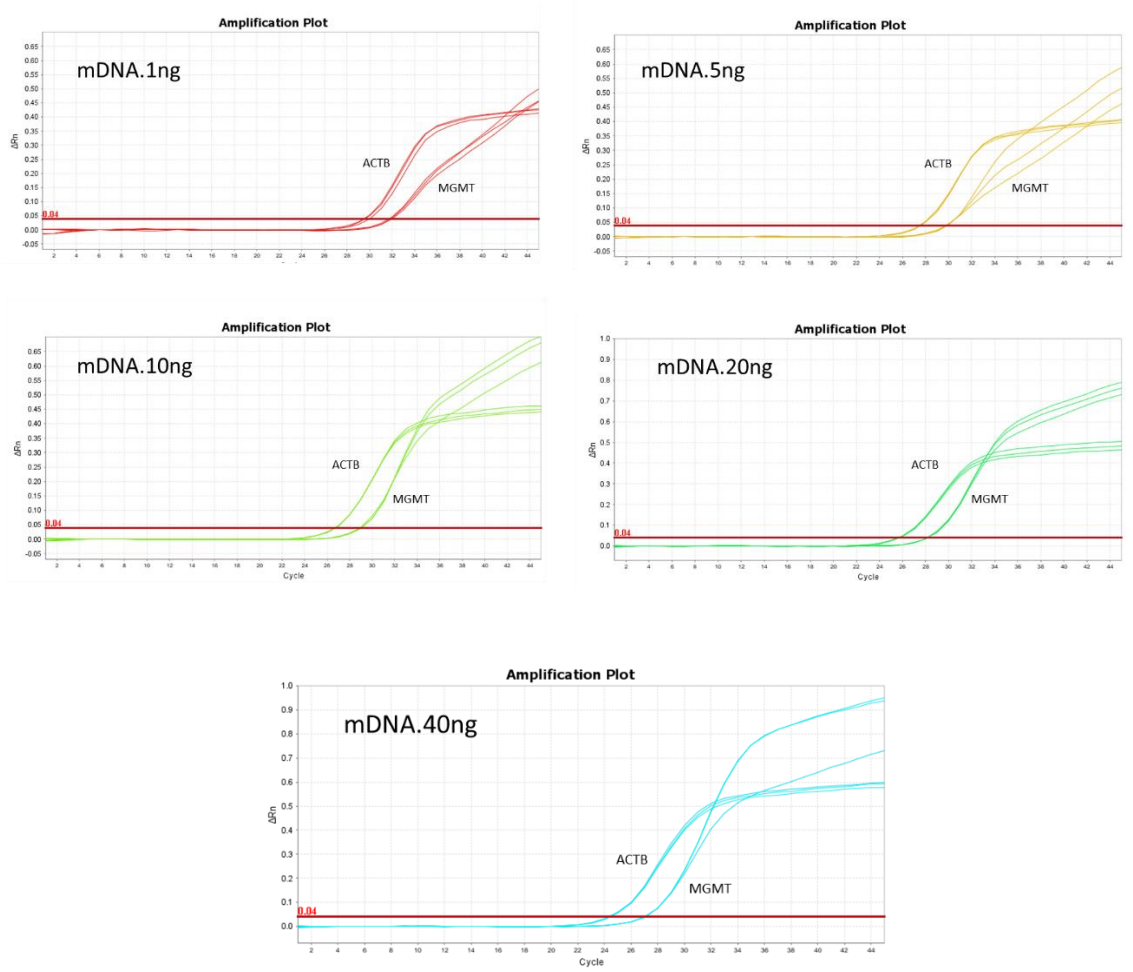

Figure 11. Amplification curves of the Methylated Millipore control at varying DNA inputs for initial primer/probe design.

This experiment tested initial primer and probe design for MGMT and ACTB at varying DNA inputs for unmethylated DNA. The following questions were interrogated: determine if primer and probe design is specific to methylated MGMT DNA and will not bind and amplify unmethylated MGMT. After reviewing the results, the MGMT primers and probe are specific for methylated MGMT. Unmethylated Millipore control should indicate no methylation in this control sample. The primer and probe design for MGMT were undetermined due to its inability to bind unmethylated sequences and the ACTB

internal control amplified as expected. The CV value was ≤ 3% for each DNA input of ACTB, a laboratory standard for every real-time assay designed (Table 5).

Table 5. Results of sample Ct values for Unmethylated Millipore Control at varying DNA inputs for initial primer/probe design.

	ACTB				MGMT			
Sample Name	Ct Value	Average	Standard deviation	Coefficient of variation	Ct Value	Average	Standard deviation	Coefficient of variation
umDNA.1ng	29.72	29.71	0.05	0.18	Undetermined	NA	NA	NA
	29.66				Undetermined			
	29.76				Undetermined			
umDNA.5ng	27.63	27.64	0.04	0.14	Undetermined	NA	NA	NA
	27.62				Undetermined			
	27.69				Undetermined			
umDNA.10ng	27.16	27.13	0.05	0.20	Undetermined	NA	NA	NA
	27.07				Undetermined			
	27.16				Undetermined			
umDNA.20ng	26.07	26.05	0.03	0.12	Undetermined	NA	NA	NA
	26.02				Undetermined			
	26.08				Undetermined			
umDNA.40ng	24.83	24.70	0.15	0.60	Undetermined	NA	NA	NA
	24.74				Undetermined			
	24.54				Undetermined			

The redesigned primer and probe for MGMT was interrogated in this experiment after the non-sigmoidal curve was observed in the previous experiment. The CV values for mDNA and umDNA for MGMT and ACTB were ≤ 3%, a laboratory standard for every real-time assay designed. Based on these results the redesigned primers and probes for MGMT were inferred to be efficient but further review of the PCR amplification curves of mDNA for MGMT would need to be conducted along with the CV values to confirm if a sigmoidal curve was achieved confirming an efficient and effective PCR reaction for the redesigned MGMT primers and probe. As mentioned before the PCR amplification curves for ACTB primers and probe did indicate a sigmoidal curve in a previous experiment therefore, no redesign was necessary. There was late amplification

with umDNA for MGMT at a Ct value of 38.65. Based on the MGMT primer design there should be no amplification with the MGMT primer and probes with unmethylated DNA. Two out of the three replicates for umDNA for MGMT were undetermined. Based on this result the amplification curve for the second replicate for the umDNA was concluded to be technician contamination during pipetting the PCR plate or nonspecific binding which can occur late in the PCR reaction as reagents are exhausted. This late amplification was not indicative of nonspecific binding of the MGMT primer and probe design (Table 6).

Table 6. Results of Ct values for Millipore Controls after the redesign of MGMT primer/probe.

	ACTB				MGMT			
Sample Name	Ct Value	Average	Standard deviation	Coefficient of variation	Ct Value	Average	Standard deviation	Coefficient of variation
mDNA	27.02	27.04	0.08	0.28	29.91	29.81	0.09	0.30
	26.98				29.77			
	27.13				29.74			
umDNA	27.13	26.99	0.12	0.44	Undetermined	38.65	NA	NA
	26.92				38.65			
	26.93				Undetermined			

The PCR amplification curve of MGMT for the mDNA sample after the redesign does indicate a sigmoidal curve indicative of an efficient PCR reaction. The PCR amplification curves for ACTB primers and probe continue indicate a sigmoidal curve. Based on these results the redesigned MGMT primers and probe with IDT's Tm recommendation were able to rescue the previous amplification curves that did not show a sigmoidal curve. The primer/probe design for MGMT and ACTB were concluded to be efficient, specific and optimized therefore, these primers and probes for ACTB and MGMT were used in all subsequent experiments. (Figure 12).

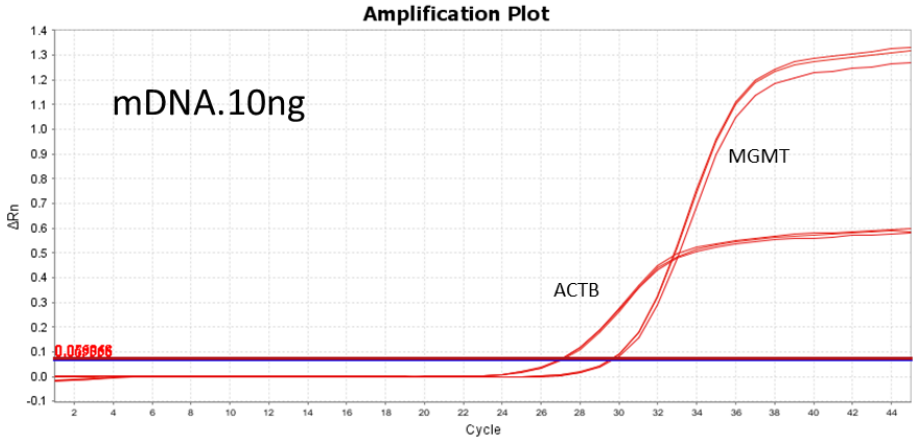

Figure 12. Amplification curve of the methylated Millipore control at 10ng input.

After determining the ACTB and MGMT primers and probes are specific and functioning efficiently and effectively a more comprehensive experiment was conducting to test sensitivity and specificity of the MGMT and ACTB primer and probe design. This experiment tested that the primer and probe design for both genes are specific for both methylated/bisulfite converted DNA. This was conducted by testing methylated and unmethylated DNA subjected to BC along with genomic DNA not subjected to BC. The CV values for all samples tested in this cohort were ≤ 3%. Each genomic DNA sample (gK562, g-umRKO, g-mDNA, and g-umDNA) tested did not indicate any sample amplification confirming the primer and probe design for both genes are specific for bisulfite converted DNA. Each unmethylated sample (umRKO and umDNA) that was bisulfite converted indicated ACTB amplification at an average Ct value between 26 and 27 confirming the internal control is working effectively. Each unmethylated sample that was bisulfite converted indicated no MGMT amplification confirming primer and probe specificity to methylated DNA sequences. Each methylated sample (mK562 and mDNA)

that was bisulfite converted indicated ACTB amplification at an average Ct value between 26 and 27 confirming the internal control is working effectively. Each methylated sample (mK562 and mDNA) that was bisulfite converted indicated MGMT amplification at an average Ct value between 29 and 30 confirming sensitivity to MGMT methylation in these samples (Table 7). NTC was tested in this experiment and no amplification was indicated.

Table 7. Ct values of Millipore Controls and cell lines of BC DNA and genomic DNA.

	ACTB				MGMT			
Sample Name	Ct Value	Average	Standard deviation	Coefficient of variation	Ct Value	Average	Standard deviation	Coefficient of variation
mK562	26.904	26.85	0.05	0.18	29.14	29.16	0.05	0.16
	26.845				29.22			
	26.809				29.10			
gK562	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
mRKO	26.698	26.65	0.05	0.18	Undetermined	NA	NA	NA
	26.631				Undetermined	NA	NA	NA
	26.607				Undetermined	NA	NA	NA
gRKO	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
mDNA	27.097	27.18	0.07	0.26	30.40	30.41	0.09	0.29
	27.224				30.51			
	27.216				30.33			
g-mDNA	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
umDNA	27.248	27.20	0.06	0.22	Undetermined	NA	NA	NA
	27.224				Undetermined	NA	NA	NA
	27.136				Undetermined	NA	NA	NA
g-umDNA	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA
	Undetermined	NA	NA	NA	Undetermined	NA	NA	NA

The initial standard curve dilution scheme was a 1:5 serial dilution using Millipore Methylated control subjected to bisulfite conversion. Standard curve included four dilution points for assay quantitation and provided linear regression for both ACTB and MGMT genes (Table 8).

Table 8. Initial standard curve serial dilution scheme.

Sample Name	ng Total	Sample Volume (µL)	Diluent Volume (µL)
Millipore Methylated Control	12.5	28	NA
	2.5	5	20
	0.5	5	20
	0.1	5	20

ACTB standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for ACTB shows a linear relationship indicated by the R^2 value $\geq$ 0.96 and efficiency $\geq$ 87% (Table 9).

Table 9. ACTB linear regression analysis with metrics over five replicates with initial standard curve dilution scheme.

ACTB Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.32	-3.40	-3.68	-3.33	-3.37
Y-Inter	30.01	30.74	30.75	31.00	30.73
R^2	1.00	1.00	0.98	0.96	1.00
Eff%	99.98	96.88	87.01	99.71	98.15
Error	0.07	0.06	0.15	0.22	0.06

MGMT standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for MGMT did not consistently show a linear relationship in four out of the five replicates. Rep-1, Rep-3, Rep-4 and Rep-5 indicated by the R^2 values $\leq$ 0.95 but the efficiencies ranged from 90% - 106%. Based on literature, the aim of this assay was to have a R^2 value ≥ 0.95 and PCR efficiency range between 80%-120% (Table 10).

Table 10. MGMT linear regression analysis with metrics over five replicates with initial standard curve dilution scheme.

MGMT Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.41	-3.29	-3.56	-3.24	-3.17
Y-Inter	33.28	32.88	33.94	33.78	33.53
R^2	0.83	0.98	0.94	0.92	0.79
Eff%	96.33	101.55	90.97	103.77	106.86
Error	0.50	0.16	0.29	0.35	0.57

Each of the standard curve dilution points tested shows reproducibility and a CV value ≤ 3% for STD 1 and STD 2 for both MGMT and ACTB. STD 3 and STD 4 do not show reproducibility and a CV value ≤ 3% for MGMT and ACTB. Three out of five assay replicates for STD 3 for MGMT showed a CV value ≥3%. Two of out five assay replicates for STD 4 for MGMT a CV was unable to be calculated due to only one replicate amplifying during the PCR reaction. One out of the five assay replicates for STD 4 for MGMT shows a CV value ≥ 3%. One out of five assay replicates for STD 4 for ACTB show a CV value ≥ 3%. Based on these results it was concluded that more dilution points needed to be added between 12.5ng and 2.5ng. The data was not reproducible at the lower ends of this dilution series which would affect downstream data analysis of PMR values and linear regression calculations (Table 11).

Table 11. Results of initial standard curve dilution scheme.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV	CT Mean	CT SD	CV (%)
STD 1	1	26.37	0.09	0.33	29.70	0.11	0.38
	2	27.03	0.13	0.47	29.45	0.20	0.68
	3	26.84	0.11	0.42	30.09	0.09	0.29
	4	27.32	0.16	0.60	30.37	0.19	0.63
	5	27.02	0.08	0.29	29.93	0.20	0.68
STD 2	1	28.72	0.07	0.23	31.72	0.18	0.58
	2	29.36	0.09	0.30	31.37	0.10	0.33
	3	29.31	0.08	0.27	32.35	0.46	1.44
	4	29.64	0.08	0.25	32.35	0.42	1.30
	5	29.43	0.16	0.56	31.97	0.12	0.38
STD 3	1	30.90	0.24	0.77	34.22	0.24	0.70
	2	31.75	0.24	0.77	33.76	0.48	1.42
	3	31.45	0.23	0.72	35.16	1.62	4.60
	4	32.18	0.34	1.05	34.63	1.36	3.94
	5	31.72	0.16	0.52	35.44	1.79	5.05
STD 4	1	33.39	0.31	0.93	36.82	2.96	8.04
	2	34.15	0.25	0.72	36.38	0.74	2.04
	3	34.70	0.59	1.71	37.45	0.28	0.74
	4	34.17	1.50	4.40	37.42	NA	NA
	5	34.10	0.26	0.76	35.08	NA	NA

Millipore methylated and unmethylated controls were diluted in ratios to mimic patient samples with a specific MGMT methylated percentage to interrogate and apply the PMR calculation. Samples were diluted to the following MGMT methylated percentages: 100%M, 100%U, 50%M, 30%M, 20%M, 10%M, 5%M, 4%M, 3%M, 2%M and 1%M. This experiment attempted to test the PMR calculation of diluted controls at specific percentage ratios and the limit of detection of this assay. One finding that was unexpected was the unmethylated control (umNegCtrl) and the unmethylated sample (100%U) indicating methylation for MGMT. Previous experiments did not indicate any MGMT methylation with undetermined amplification curves and the specificity and sensitivity was tested and concluded to work effectively. After reviewing lab notes the unmethylated control Vial B was used as the diluent to the methylated control to achieve pre-determined methylation percentage ratios. The Millipore unmethylated control contains two vials from different sources (Vial and Vial B). Vial A contains human genomic DNA and Vial B contains genomic DNA isolated from a human fetal cell line. This experiment was the first test to use Vial B and though the Ct values for both samples were ≥37 it was concluded that there was slight methylation in Vial B and this could not be used in future experiments because this would skew the PMR calculations downstream and show methylation in a negative control sample. Samples 100%U, 5%M, 4%M, 3%M, and 1%M had CV values ≥ 3% when using the Ct mean for each assay replicate in the calculation. After reviewing the data using 10% methylation cutoff as our limit of detection for this assay could be inferred based on the CV values but further testing would need to be performed to confirm this observation. Lessons learned from this experiment was to use more dilution points between 12.5ng and 2.5ng for standard curve

and not use Vial B for unmethylated sample and diluent for methylation percentage ratios (Table 12). NTC was tested in this experiment and no amplification was indicated.

Table 12. Results for varying methylated samples for each assay replicate using Unmethylated Millipore control Vial B and initial standard curve dilution scheme.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV	CT Mean	CT SD	CV (%)
100% M	1	26.31	0.05	0.17	29.70	0.13	0.44
	2	26.91	0.03	0.11	29.22	0.29	0.98
	3	26.53	0.01	0.02	29.83	0.16	0.55
	4	26.79	0.06	0.24	29.59	0.03	0.12
	5	26.84	0.08	0.31	29.51	0.06	0.21
100% U	1	27.69	0.10	0.36	43.88	NA	NA
	2	28.42	0.05	0.16	39.77	1.65	4.15
	3	28.55	0.08	0.29	42.32	NA	NA
	4	28.93	0.12	0.40	42.79	1.58	3.70
	5	28.58	0.07	0.26	43.05	NA	NA
50% M	1	26.88	0.08	0.31	30.73	0.20	0.65
	2	27.35	0.08	0.27	30.25	0.22	0.72
	3	27.56	0.05	0.19	31.18	0.30	0.96
	4	27.68	0.06	0.20	30.66	0.30	0.99
	5	27.83	0.14	0.51	30.97	0.19	0.61
30% M	1	27.15	0.06	0.20	31.63	0.31	0.97
	2	27.63	0.04	0.15	30.72	0.23	0.75
	3	27.76	0.11	0.39	32.10	0.42	1.30
	4	28.06	0.11	0.41	31.40	0.18	0.59
	5	27.85	0.12	0.45	31.60	0.11	0.35
20% M	1	27.54	0.06	0.22	32.35	0.22	0.68
	2	27.58	0.07	0.24	31.18	0.28	0.89
	3	27.97	0.05	0.17	32.44	0.44	1.37
	4	28.06	0.05	0.16	31.97	0.21	0.64
	5	28.11	0.12	0.42	31.76	0.52	1.65
10% M	1	27.27	0.04	0.13	33.65	0.75	2.24
	2	27.98	0.01	0.04	32.40	0.42	1.30
	3	28.27	0.10	0.34	33.84	0.55	1.62
	4	28.45	0.04	0.16	32.96	0.33	0.99
	5	28.56	0.06	0.22	33.50	0.34	1.02
5% M	1	27.70	0.10	0.38	33.94	0.28	0.84
	2	28.23	0.06	0.22	35.77	2.34	6.54
	3	28.20	0.08	0.27	34.71	0.13	0.36
	4	28.78	0.08	0.28	34.17	0.71	2.07
	5	28.45	0.25	0.87	35.02	1.06	3.03
4% M	1	27.59	0.11	0.41	34.17	0.67	1.95
	2	28.04	0.07	0.25	34.19	0.35	1.03
	3	28.31	0.08	0.28	34.43	0.29	0.84
	4	28.80	0.20	0.69	34.72	1.05	3.03
	5	28.69	0.01	0.04	34.54	0.58	1.68
3% M	1	27.88	0.08	0.30	34.63	0.53	1.52
	2	28.26	0.05	0.17	36.30	1.41	3.89
	3	28.35	0.08	0.29	35.23	0.48	1.37
	4	28.71	0.18	0.62	34.59	1.21	3.49
	5	28.48	0.07	0.24	35.03	0.36	1.02
2% M	1	27.89	0.05	0.17	35.31	0.95	2.69
	2	28.41	0.12	0.41	34.52	0.07	0.22
	3	28.39	0.06	0.20	35.28	0.85	2.41
	4	28.91	0.08	0.29	35.53	0.61	1.73
	5	28.59	0.05	0.17	34.61	0.54	1.55
1% M	1	27.43	0.02	0.07	35.84	1.10	3.07
	2	28.44	0.07	0.26	35.60	1.18	3.30
	3	28.50	0.08	0.30	37.31	2.12	5.69
	4	29.27	0.20	0.70	40.41	3.32	8.22
	5	28.59	0.10	0.35	36.20	1.04	2.88
mPosCtrl	1	26.43	0.06	0.22	29.80	0.10	0.35
	2	27.07	0.07	0.26	29.27	0.17	0.57
	3	26.90	0.10	0.36	30.08	0.19	0.64
	4	27.28	0.11	0.40	30.01	0.16	0.54
	5	26.90	0.02	0.08	29.81	0.28	0.94
umNegCtrl	1	27.79	0.11	0.41	42.11	NA	NA
	2	28.45	0.10	0.35	37.63	NA	NA
	3	28.47	0.04	0.15	38.97	NA	NA
	4	28.91	0.04	0.13	40.95	NA	NA
	5	29.07	0.05	0.19	39.32	2.23	5.67

PMR was calculated for each sample tested in this cohort to determine if percent methylation could be determined based on this PCR assay. PMR calculation uses the quantity values calculated on the QuantStudio software of the ViiA7 for MGMT and ACTB mPosCtrl against the quantity values of MGMT and ACTB of each sample tested multiplied by 100. Each percentage point for each assay replicate had a higher PMR value than what was prepared in the lab due to the unmethylated Millipore control Vial B being used which indicated levels of methylation. This unmethylated control was used as the diluent to prepare each methylated percentage point. Therefore, it was concluded this experiment needed to be repeated using Unmethylated Millipore control Vial A which was tested and proven to be unmethylated for MGMT in previous experiments (Table 13).

Table 13. PMR calculations for methylated samples prepared at specific percentage ratios using Unmethylated Millipore control Vial B and initial standard curve dilution scheme.

PMR (%)						
Sample Name	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Mean of PMR
100M%	98.28	93.48	92.99	95.76	116.93	99.49
100U%	0.02	0.22	0.09	0.04	0.02	0.08
50%	73.60	60.89	74.70	83.48	80.34	74.60
30%	48.62	53.06	43.66	63.88	51.16	52.08
20%	38.94	37.20	43.47	42.51	57.00	43.82
10%	14.40	21.09	21.57	28.00	21.40	21.29
5%	14.99	4.16	11.20	15.80	7.70	10.77
4%	7.49	6.23	14.57	11.60	11.38	10.25
3%	10.90	2.17	9.06	12.79	6.65	8.31
2%	7.59	6.23	9.76	6.45	10.04	8.01
1%	3.99	3.64	3.81	1.23	3.52	3.24
mPosCtrl	100	100	100	100	100	100
umNegCtrl	0.07	0.72	0.86	0.13	0.75	0.51

The final standard curve dilution scheme was a 1:2 serial dilution using Millipore Methylated control subjected to bisulfite conversion. Standard curve included five dilution points for assay quantitation and provided linear regression for both ACTB and MGMT genes. This standard curve dilution scheme was modified to generate a standard curve that was reproducible with better linear regression metrics. 20µL starting from 12.5ng dilution point was transferred to the next dilution point in a series until ending at 0.78ng dilution point (Table 14).

Table 14. Final standard curve serial dilution scheme.

Sample Name	ng Total	Sample Volume (µL)	Diluent Volume (µL)
Millipore Methylated Control	12.5	40	NA
	6.25	20	20
	3.125	20	20
	1.56	20	20
	0.78	20	20

ACTB standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for ACTB shows a linear relationship indicated by the R^2 value ≥ 0.99 and efficiency $\geq$ 89% (Table 15).

Table 15. ACTB linear regression analysis with metrics over five replicates with final standard curve dilution scheme.

ACTB Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.60	-3.35	-3.42	-3.38	-3.51
Y-Inter	30.65	30.37	30.17	30.67	30.74
R^2	0.99	1.00	0.99	0.99	1.00
Eff%	89.72	98.68	95.93	97.72	92.58
Error	0.09	0.06	0.11	0.08	0.06

MGMT standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for MGMT did consistently show a linear relationship in four out of the five replicates. Rep-1 and Rep-4 indicated by the R^2 values ≤ 0.94 which were an improvement from the previous experiment. Rep-5 showed technical pipetting errors in the three CT replicates therefore, the PCR metrics were lower than expected. The efficiencies for Rep-1 through Rep-4 ranged from 89%-114%. As mentioned before, based on literature, the aim of this assay was to have a R^2 value ≥ 0.95 and PCR efficiency range between 80%-120% (Table 16).

Table 16. MGMT linear regression analysis with metrics consistent over four replicates with final standard curve dilution scheme.

MGMT Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.39	-3.45	-3.01	-3.62	-4.44
Y-Inter	33.65	33.89	32.62	33.38	34.40
R^2	0.94	0.98	0.96	0.92	0.89
Eff%	97.39	94.84	114.67	89.01	68.03
Error	0.24	0.15	0.18	0.29	0.44

Each of the standard curve dilution points tested showed reproducibility and a CV value ≤ 3% for both MGMT and ACTB. Each standard curve dilution point is an improvement based on previous standard curve results. This standard curve dilution scheme was modified to add more points between 12.5ng and 2.5ng to avoid dilution points on the lower range of the assay limit of detection. Each assay replicate for ACTB and MGMT have consistent Ct means and CV values which is indicative of an effective standard curve which can be used for assay quantification (Table 17).

Table 17. Results of final standard curve dilution scheme for each of the five standard dilution points.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
STD 1	1	26.75	0.15	0.56	30.00	0.17	0.56
	2	26.65	0.09	0.35	30.22	0.13	0.44
	3	26.38	0.15	0.56	29.27	0.09	0.30
	4	27.01	0.07	0.25	29.51	0.10	0.33
	5	26.94	0.08	0.31	29.95	0.10	0.34
STD 2	1	27.77	0.06	0.22	30.87	0.12	0.39
	2	27.67	0.08	0.28	31.15	0.16	0.52
	3	27.45	0.03	0.11	30.33	0.12	0.41
	4	27.85	0.07	0.26	30.63	0.32	1.04
	5	27.87	0.07	0.25	30.79	0.25	0.82
STD 3	1	28.83	0.05	0.19	31.95	0.14	0.44
	2	28.80	0.06	0.23	32.00	0.09	0.28
	3	28.49	0.06	0.22	30.97	0.56	1.79
	4	29.12	0.11	0.37	31.45	0.26	0.84
	5	29.01	0.03	0.11	31.87	0.34	1.07
STD 4	1	29.89	0.02	0.07	33.04	0.45	1.37
	2	29.75	0.16	0.54	33.08	0.15	0.47
	3	29.62	0.19	0.66	32.15	0.20	0.63
	4	30.00	0.03	0.09	32.16	0.75	2.34
	5	30.06	0.09	0.29	32.80	0.63	1.91
STD 5	1	31.11	0.30	0.98	34.01	0.83	2.45
	2	30.66	0.04	0.12	34.46	0.42	1.22
	3	30.44	0.35	1.16	32.90	0.34	1.04
	4	31.03	0.17	0.55	34.19	0.07	0.19
	5	31.14	0.14	0.46	35.63	0.86	2.40

Millipore methylated and unmethylated controls were diluted in ratios to mimic patient samples with a specific MGMT methylated percentage to interrogate and apply the PMR calculation. Samples were diluted to the following MGMT methylated percentages: 100%M, 100%U, 50%M, 30%M, 20%M, 10%M, 5%M, 4%M, 3%M, 2%M and 1%M. This experiment attempted to re-test the PMR calculation of diluted controls at specific percentage ratios and the limit of detection of this assay. One finding to note is this experiment used Vial A Unmethylated Millipore control which was tested and proven to be unmethylated in MGMT in previous experiments. Vial B unmethylated Millipore control had low levels of MGMT methylation shown in previous experiments therefore, no further testing was conducted using this sample. Samples 100%U and umNegCtrl now show undetermined for each assay replicate which proves there is no MGMT methylation in this control sample. Since this was used as a diluent for the methylation percentage ratios the PMR calculations should be closer to the prepared percentages created. The Ct values for both MGMT an ACTB are consistent in each sample in each assay replicate. The CV values are $\leq 3\%$ for samples 100%M, 50%M, 30%M, 20%M, 10%M, 5%M and 3%M. Samples 4%M, 2%M and 1%M have CV values $\geq 3\%$ is some assay replicates. This data shows the limit of detection for this assay may be 10% methylation with the possibility to go as low as 5% if more data is generated. NTC was tested in this experiment and no amplification was indicated. (Table 18).

Table 18. Results for varying methylated samples for each assay replicate using Unmethylated Millipore control Vial A and final standard curve dilution scheme.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
100% M	1	26.72	0.00	0.01	29.99	0.05	0.15
	2	26.69	0.07	0.26	30.17	0.19	0.63
	3	26.19	0.04	0.17	29.20	0.14	0.48
	4	26.45	0.02	0.09	29.04	0.14	0.48
	5	26.59	0.07	0.28	29.50	0.17	0.56
100% U	1	27.12	0.07	0.28	Undetermined	Undetermined	Undetermined
	2	26.85	0.01	0.04	Undetermined	Undetermined	Undetermined
	3	26.31	0.06	0.23	Undetermined	Undetermined	Undetermined
	4	26.73	0.09	0.32	Undetermined	Undetermined	Undetermined
	5	27.05	0.06	0.21	Undetermined	Undetermined	Undetermined
50% M	1	26.87	0.01	0.05	31.09	0.10	0.31
	2	26.71	0.04	0.14	31.81	0.23	0.73
	3	26.30	0.06	0.23	30.19	0.22	0.73
	4	26.61	0.23	0.85	30.18	0.29	0.96
	5	26.82	0.06	0.24	30.73	0.36	1.19
30% M	1	26.90	0.05	0.19	32.02	0.42	1.30
	2	26.92	0.07	0.27	32.15	0.48	1.51
	3	26.30	0.03	0.13	31.00	0.07	0.24
	4	26.39	0.05	0.19	30.95	0.19	0.60
	5	26.75	0.05	0.17	31.48	0.24	0.77
20% M	1	26.99	0.08	0.29	32.74	0.50	1.53
	2	26.85	0.17	0.64	32.61	0.11	0.33
	3	26.47	0.07	0.26	31.93	0.15	0.46
	4	26.59	0.00	0.02	31.58	0.16	0.51
	5	26.79	0.04	0.15	32.18	0.20	0.62
10% M	1	26.93	0.05	0.20	33.15	0.65	1.96
	2	26.72	0.16	0.59	33.53	0.31	0.93
	3	26.28	0.09	0.36	32.63	0.29	0.89
	4	26.44	0.08	0.29	32.14	0.24	0.74
	5	26.80	0.11	0.40	32.59	0.35	1.08
5% M	1	26.98	0.07	0.25	34.00	0.57	1.68
	2	26.81	0.03	0.10	34.69	0.79	2.26
	3	26.33	0.04	0.15	33.88	0.31	0.90
	4	26.78	0.17	0.65	33.72	0.60	1.78
	5	26.91	0.11	0.40	34.26	0.64	1.86
4% M	1	27.04	0.09	0.32	34.12	0.46	1.36
	2	26.79	0.02	0.07	34.11	0.19	0.55
	3	26.51	0.15	0.57	34.79	0.40	1.14
	4	26.42	0.37	1.39	34.93	1.38	3.94
	5	27.10	0.08	0.28	33.97	0.44	1.31
3% M	1	27.24	0.10	0.37	36.00	0.32	0.90
	2	27.00	0.01	0.02	35.52	0.48	1.36
	3	26.68	0.12	0.45	34.12	0.27	0.78
	4	27.05	0.08	0.30	34.12	0.41	1.20
	5	27.10	0.02	0.08	34.88	0.04	0.11
2% M	1	26.96	0.04	0.16	36.25	0.57	1.56
	2	26.89	0.06	0.24	38.54	4.72	12.25
	3	26.42	0.20	0.75	35.32	2.08	5.89
	4	26.71	0.02	0.08	35.31	0.70	1.97
	5	26.96	0.08	0.29	35.27	0.51	1.46
1% M	1	26.98	0.09	0.34	35.86	1.35	3.75
	2	27.07	0.14	0.50	36.30	0.95	2.63
	3	26.44	0.25	0.95	35.39	0.68	1.91
	4	26.90	0.09	0.35	35.81	0.68	1.89
	5	27.04	0.07	0.26	36.91	0.39	1.05
mPosCtrl	1	26.54	0.10	0.37	29.68	0.07	0.24
	2	26.62	0.06	0.23	30.38	0.14	0.44
	3	26.61	0.32	1.21	29.66	0.26	0.88
	4	26.82	0.01	0.04	29.35	0.35	1.18
	5	26.55	0.04	0.15	29.68	0.21	0.70
umNegCtrl	1	27.23	0.14	0.52	Undetermined	Undetermined	Undetermined
	2	27.11	0.09	0.32	Undetermined	Undetermined	Undetermined
	3	26.32	0.05	0.19	Undetermined	Undetermined	Undetermined
	4	27.03	0.06	0.23	Undetermined	Undetermined	Undetermined
	5	27.07	0.10	0.36	Undetermined	Undetermined	Undetermined

PMR was calculated for each sample tested in this cohort to determine if percent methylation could be calculated based on this PCR assay. PMR calculation uses the quantity values calculated on the QuantStudio software of the ViiA7 for MGMT and ACTB mPosCtrl against the quantity values of MGMT and ACTB of each sample tested multiplied by 100. Each percentage point for replicates 1-4 had a PMR value that was in range of the MGMT percentage that was prepared. The PMR values were lower and in range compared to the previous experiment since there was no MGMT methylation in the unmethylated control that was used as a diluent to prepare these samples. Replicate 5 PMR value were higher than expected but due to technician error mentioned previously in the standard curve the PMR results are skewed (Table 19).

Table 19. PMR calculations for methylated samples prepared at specific percentage ratios using Unmethylated Millipore control Vial A and final standard curve dilution scheme.

	PMR (%)					
Sample Name	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Mean of PMR
100M%	89.65	121.47	107.28	93.29	112.32	104.80
100U%	0.00	0.00	0.00	0.00	0.00	0.00
50%	46.85	41.52	54.36	50.28	69.84	52.57
30%	26.06	38.82	29.17	26.49	45.01	33.11
20%	17.07	26.36	15.03	20.45	31.94	22.17
10%	12.81	13.17	8.38	12.98	26.16	14.70
5%	7.29	7.01	3.34	6.20	12.14	7.20
4%	6.86	9.37	1.90	2.61	15.68	7.28
3%	2.14	4.37	3.51	5.63	9.64	5.06
2%	1.57	2.81	2.30	2.18	7.35	3.24
1%	2.63	2.96	1.22	1.82	3.27	2.38
mPosCtrl	100	100	100	100	100	100
umNegCtrl	0	0	0	0	0	0

ACTB standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls to test FFPE samples. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for ACTB shows a linear relationship indicated by the R^2 value ≥0.99 and efficiency ranging between 88%-101% (Table 20).

Table 20. ACTB linear regression analysis with metrics over five replicates to test FFPE samples.

ACTB Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.34	-3.36	-3.62	-3.30	-3.44
Y-Inter	30.68	30.61	30.78	30.61	30.79
R^2	1.00	1.00	0.99	1.00	1.00
Eff%	99.20	98.59	88.99	101.09	95.49
Error	0.06	0.04	0.09	0.05	0.06

MGMT standard curve results for five assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls to test FFPE samples. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for MGMT did consistently show a linear relationship in four out of the five replicates. Rep-2 through Rep-5 showed an R^2 values ≥ 0.95 which were consistent with the previous experiment. Rep-1 showed a lower R^2 value at 0.92 but still well within the range of acceptable PCR performance. The efficiencies for each assay replicate ranged from 90%-105%. As mentioned before, based on literature, the aim of this assay was to have a R^2 value ≥ 0.95 and PCR efficiency range between 80%-120% (Table 21).

Table 21. MGMT linear regression analysis with metrics over five replicates to test FFPE samples.

MGMT Standard Curve Metrics					
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
Slope	-3.58	-3.31	-3.24	-3.20	-3.35
Y-Inter	34.05	33.78	33.96	33.12	33.77
R^2	0.92	0.98	0.97	0.98	0.97
Eff%	90.25	100.32	103.58	105.25	99.00
Error	0.29	0.14	0.17	0.13	0.17

Each of the standard curve dilution points tested showed reproducibility and a CV value ≤ 3% for both MGMT and ACTB. Each standard curve dilution point is an improvement based on previous standard curve results. This standard curve dilution scheme was modified to add more points between 12.5ng and 2.5ng to avoid dilution points on the lower range of the assay limit of detection. Each assay replicate for ACTB and MGMT have consistent Ct means and CV values which is indicative of an effective standard curve which can be used for assay quantification (Table 22).

Table 22. Results of standard curve for each of the five standard dilution points to test FFPE samples.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
STD 1	1	27.00	0.05	0.20	30.24	0.23	0.75
	2	26.91	0.04	0.13	30.24	0.17	0.58
	3	26.87	0.03	0.10	30.49	0.16	0.51
	4	26.99	0.02	0.07	29.68	0.10	0.34
	5	26.98	0.05	0.19	30.16	0.10	0.33
STD 2	1	28.09	0.06	0.22	30.95	0.24	0.78
	2	27.98	0.00	0.01	31.07	0.15	0.47
	3	27.85	0.09	0.32	31.19	0.07	0.23
	4	28.03	0.08	0.28	30.55	0.06	0.18
	5	28.09	0.02	0.09	31.06	0.10	0.34
STD 3	1	29.01	0.06	0.22	32.28	0.12	0.36
	2	28.92	0.08	0.26	32.22	0.22	0.67
	3	29.00	0.10	0.35	32.58	0.25	0.77
	4	28.94	0.09	0.30	31.46	0.41	1.29
	5	29.16	0.08	0.26	32.05	0.46	1.44
STD 4	1	29.95	0.13	0.45	33.63	0.61	1.82
	2	29.92	0.08	0.26	32.86	0.20	0.59
	3	30.04	0.27	0.89	33.11	0.11	0.33
	4	29.91	0.05	0.16	32.38	0.15	0.47
	5	30.05	0.08	0.26	33.22	0.41	1.23
STD 5	1	31.10	0.07	0.24	34.30	0.83	2.43
	2	31.00	0.12	0.38	34.33	0.16	0.45
	3	31.22	0.15	0.48	34.41	0.39	1.13
	4	31.01	0.07	0.24	33.59	0.11	0.34
	5	31.17	0.14	0.46	34.11	0.32	0.95

MGMT methylated cell line K562 and MGMT unmethylated cell line RKO were formalin and paraffin embedded into cell pellets in our histology department, extracted, then diluted in ratios to mimic FFPE patient samples with specific MGMT methylated percentages to interrogate and apply the PMR calculation. Samples were diluted to the following MGMT methylated percentages using RKO as a diluent: 100%M, 100%U, 50%M, 10%M, 5%M, 4%M, 3%M, 2%M and 1%M. This experiment attempted to test the PMR calculation of diluted FFPE cell lines, limit of detection, and to determine does adding FFPE samples introduce artifacts in this PCR assay. FFPE clinical samples will be received for this study therefore, this PCR assay needed to be tested to ensure FPPE samples would perform as well as commercial controls tested up until this point. The Ct values for both MGMT and ACTB are consistent in each sample and in each assay replicate. The CV values are $\leq$ 3% for samples 100%M, 100%U, 50%M, 10%M, 5%M and 2%M. Samples 4%M, 3%M and 1%M have CV values $\geq$ 3% is some of their assay replicates. This data shows the limit of detection for this assay may be 10% methylation with the possibility to go as low as 5% if more data is generated. NTC was tested in this experiment and no amplification was indicated. (Table 23).

Table 23. Results for varying FFPE methylated samples for each assay replicate.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
100% M	1	27.34	0.15	0.56	29.50	0.09	0.29
	2	27.13	0.03	0.12	29.28	0.16	0.56
	3	27.02	0.07	0.24	29.63	0.05	0.16
	4	27.56	0.08	0.28	29.23	0.05	0.16
	5	27.43	0.06	0.21	29.41	0.08	0.26
100% U	1	27.64	0.19	0.67	Undetermined	Undetermined	Undetermined
	2	27.28	0.06	0.22	Undetermined	Undetermined	Undetermined
	3	27.14	0.04	0.13	Undetermined	Undetermined	Undetermined
	4	27.53	0.01	0.04	Undetermined	Undetermined	Undetermined
	5	27.43	0.03	0.12	Undetermined	Undetermined	Undetermined
50% M	1	27.74	0.05	0.20	30.71	0.32	1.05
	2	27.52	0.10	0.38	30.53	0.09	0.29
	3	27.32	0.02	0.06	30.76	0.19	0.62
	4	27.76	0.05	0.17	30.37	0.04	0.13
	5	27.77	0.04	0.16	30.52	0.13	0.42
10% M	1	28.30	0.09	0.33	33.07	0.27	0.81
	2	27.92	0.05	0.18	33.43	0.50	1.49
	3	27.78	0.02	0.06	33.63	0.19	0.57
	4	28.29	0.14	0.51	33.48	0.05	0.16
	5	28.13	0.02	0.07	33.04	0.63	1.91
5% M	1	27.87	0.05	0.18	34.01	0.56	1.64
	2	27.61	0.15	0.56	33.84	0.69	2.05
	3	27.52	0.07	0.24	34.00	0.23	0.67
	4	27.42	0.03	0.11	32.78	0.13	0.39
	5	27.90	0.08	0.28	34.02	0.23	0.67
4% M	1	27.78	0.21	0.77	34.21	1.03	3.03
	2	27.73	0.06	0.20	35.03	0.21	0.60
	3	27.50	0.08	0.28	35.18	0.22	0.64
	4	27.66	0.03	0.10	34.29	0.46	1.33
	5	27.86	0.02	0.07	34.64	1.15	3.33
3% M	1	27.28	0.08	0.29	34.23	0.25	0.74
	2	27.05	0.04	0.16	33.78	0.56	1.65
	3	27.06	0.05	0.20	34.85	0.55	1.58
	4	27.38	0.02	0.07	34.93	0.97	2.76
	5	27.29	0.03	0.12	34.17	1.03	3.01
2% M	1	27.40	0.12	0.42	34.41	0.60	1.75
	2	27.21	0.07	0.27	35.48	0.95	2.68
	3	27.38	0.10	0.36	36.72	0.20	0.53
	4	27.44	0.20	0.74	35.07	0.47	1.33
	5	27.29	0.03	0.12	34.84	0.49	1.42
1% M	1	27.50	0.11	0.41	39.51	4.23	10.70
	2	27.45	0.02	0.08	36.92	0.06	0.15
	3	27.24	0.03	0.11	36.39	1.23	3.39
	4	27.44	0.20	0.74	35.07	0.47	1.33
	5	27.46	0.05	0.19	37.27	0.61	1.64
mK562 PosCtrl	1	27.32	0.02	0.08	29.31	0.09	0.31
	2	27.25	0.09	0.35	29.51	0.06	0.20
	3	27.14	0.08	0.28	29.51	0.07	0.23
	4	27.12	0.08	0.29	28.86	0.10	0.36
	5	27.24	0.07	0.26	29.19	0.04	0.13
umRKO NegCtrl	1	27.55	0.04	0.15	Undetermined	Undetermined	Undetermined
	2	27.48	0.02	0.07	Undetermined	Undetermined	Undetermined
	3	27.57	0.05	0.17	Undetermined	Undetermined	Undetermined
	4	27.41	0.08	0.30	Undetermined	Undetermined	Undetermined
	5	27.40	0.19	0.69	Undetermined	Undetermined	Undetermined

PMR was calculated for each FFPE sample tested in this cohort to determine if percent methylation could be calculated based on this PCR assay using FFPE cell line samples. PMR calculation uses the quantity values calculated on the QuantStudio software of the ViiA7 for MGMT and ACTB mK562 PosCtrl against the quantity values of MGMT and ACTB of each FFPE sample tested multiplied by 100. Each percentage point for each assay replicate had a PMR value that was in range of the MGMT percentage that was prepared. The PMR values were tighter and more consistent than using the commercial Millipore controls shown in previous experiments. The PMR values were in range and comparable to previous experiments (Table 24).

Table 24. PMR calculations for FFPE methylated samples prepared at specific percentage ratios.

	PMR (%)					
Sample Name	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Mean of PMR
100% M	91.54	108.50	84.63	112.56	97.51	98.95
100% U	0.00	0.00	0.00	0.00	0.00	0.00
50%	54.86	59.41	46.18	57.22	57.31	55.00
10%	17.56	10.89	8.06	8.80	13.62	11.79
5%	7.44	6.80	5.26	7.97	5.63	6.62
4%	6.67	3.04	2.24	3.32	4.22	3.90
3%	4.12	4.73	2.24	1.90	3.95	3.39
2%	4.11	1.74	0.69	1.61	2.56	2.14
1%	0.56	0.67	0.98	0.85	0.47	0.71
mK562 PosCtrl	100.00	100.00	100.00	100.00	100.00	100.00
umRKO NegCtrl	0.00	0.00	0.00	0.00	0.00	0.00

ACTB standard curve results for three assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for ACTB shows a linear relationship indicated by the R^2 value equal to 1.00 and efficiency ≥ 96% (Table 25).

Table 25. ACTB linear regression analysis with metrics over three assay replicates to test PDX samples.

ACTB Standard Curve Metrics			
	Rep-1	Rep-2	Rep-3
Slope	-3.40	-3.40	-3.39
Y-Inter	30.27	30.80	30.71
R2	1.00	1.00	1.00
Eff%	96.76	97.00	97.39
Error	0.05	0.04	0.05

MGMT standard curve results for three assay replicates were generated using the listed assay setup and appropriate bisulfite converted methylated controls. Linear regression statistical analysis was performed to evaluate the PCR efficiency and assay quantitation. The assay for MGMT did consistently show a linear relationship in all three replicates. The efficiencies for each assay replicate ranged from 86%-107%. The R^2 for each assay replicate were ≥ 0.98. As mentioned before, based on literature, the aim of this assay was to have a R^2 value ≥ 0.95 and PCR efficiency range between 80%-120% (Table 26).

Table 26. MGMT linear regression analysis with metrics over three assay replicates to test PDX samples.

MGMT Standard Curve Metrics			
	Rep-1	Rep-2	Rep-3
Slope	-3.57	-3.15	-3.70
Y-Inter	34.00	34.06	33.80
R2	0.98	0.97	0.99
Eff%	90.50	107.87	86.38
Error	0.16	0.16	0.12

Each of the standard curve dilution points tested shows reproducibility and a CV value ≤ 3% for both MGMT and ACTB. Each assay replicate for ACTB and MGMT have consistent Ct means and CV values which is indicative of an effective standard curve which can be used for assay quantification (Table 27).

Table 27. Results of standard curve for each of the three standard dilution points for PDX samples.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
STD 1	1	26.56	0.09	0.35	30.18	0.14	0.46
	2	27.05	0.05	0.18	30.56	0.18	0.58
	3	27.04	0.03	0.11	29.80	0.05	0.16
STD 2	1	27.52	0.01	0.04	31.14	0.14	0.44
	2	28.12	0.05	0.18	31.55	0.29	0.91
	3	27.99	0.01	0.02	30.82	0.19	0.62
STD 3	1	28.60	0.03	0.11	32.07	0.09	0.27
	2	29.10	0.01	0.04	32.61	0.23	0.69
	3	29.03	0.01	0.04	31.90	0.29	0.89
STD 4	1	29.59	0.13	0.42	33.30	0.19	0.56
	2	30.20	0.03	0.10	33.47	0.39	1.17
	3	29.98	0.04	0.12	33.11	0.07	0.22
STD 5	1	30.64	0.12	0.39	34.48	0.53	1.54
	2	31.13	0.11	0.35	34.34	0.29	0.83
	3	31.14	0.16	0.52	34.22	0.35	1.01

Based on previous experiments the data showed this PCR assay could be used on commercial MGMT methylated and unmethylated control samples and FFPE MGMT methylated and unmethylated cell lines. This experiment tested if this assay would be able to test Patient Derived Xenografts (PDX) mouse models with human tumor tissue implanted. First question was to interrogate if the ACTB and MGMT primers were specific to human DNA alone and not mouse DNA. This was accomplished by using Basic Local Alignment Search Tool (BLAST) to interrogate if the primer sequences overlapped with mouse sequence. The online BLAST search indicated there should be no overlap between ACTB and MGMT primer design with mouse DNA. The next question was to interrogate if the ACTB and MGMT primers are specific for human MGMT methylated DNA only and is the assay able to detect then amplify it. The following samples were tested in this cohort: mouse only DNA (mammary gland, liver, and lymph node), Millipore methylated control DNA diluted in mouse DNA at 20% (mammary gland+hMDNA, liver+hMDNA and lymph node+hMDNA), human only DNA at 20% with Methylated Millipore control, and positive and negative controls. Mouse only DNA samples (mammary gland, liver, and lymph node) showed no ACTB or MGMT amplification except for the ACTB in the mouse DNA liver sample. Each of the other samples (mammary gland and lymph node) assay replicates showed no amplification in either target. The amplification in the liver mouse DNA sample for ACTB was determined to be possible contamination of the mouse DNA sample provided. Initial thoughts were there could be possible contamination during the bisulfite conversion and/or PCR but was ruled out when amplification occurred in each of the three replicates tested on different days and with different operators. Mammary gland mouse samples

only had enough material for two out of three replicates. Based on this information it was concluded the ACTB and MGMT primer design were specific to only human DNA but further PDX testing would need to be conducted based on the liver ACTB amplification observed.

Millipore methylated control diluted in mouse DNA at 20% samples (mammary gland+hMDNA, liver+hMDNA and lymph node+hMDNA) showed ACTB and MGMT amplification and based on the undetermined results of the mouse DNA the data showed what was amplifying was human DNA within the mouse model. The Ct values for both MGMT and ACTB are consistent in each sample in each assay replicate. The CV values are $\leq 3\%$ for all samples tested in this cohort. The hMDNA, mPosCtrl, and umNegCtrl all performed as expected and reproducible between all assay replicates (Table 28).

Table 28. Results for PDX samples for each assay replicate.

Sample Name	Assay Replicate	ACTB			MGMT		
		CT Mean	CT SD	CV (%)	CT Mean	CT SD	CV (%)
Mammary Gland	1	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
	2	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
	3	NA	NA	NA	NA	NA	NA
Liver	1	32.47	0.20	0.63	Undetermined	Undetermined	Undetermined
	2	32.50	0.29	0.90	Undetermined	Undetermined	Undetermined
	3	32.55	0.10	0.32	Undetermined	Undetermined	Undetermined
Lymph Node	1	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
	2	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
	3	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Mammary Gland+hMDNA	1	28.93	0.06	0.22	33.07	0.24	0.72
	2	28.91	0.07	0.24	32.34	0.14	0.43
	3	NA	NA	NA	NA	NA	NA
Liver+hMDNA	1	28.89	0.08	0.29	32.76	0.31	0.94
	2	28.95	0.04	0.13	32.45	0.22	0.68
	3	29.06	0.08	0.27	32.51	0.07	0.21
Lymph Node+hMDNA	1	28.86	0.10	0.35	32.71	0.22	0.66
	2	29.13	0.03	0.12	32.63	0.31	0.95
	3	29.20	0.04	0.14	32.21	0.18	0.56
hMDNA (20%)	1	26.76	0.08	0.29	32.54	0.20	0.62
	2	27.00	0.06	0.24	32.93	0.02	0.05
	3	27.18	0.07	0.28	32.35	0.16	0.49
mPosCtrl	1	26.43	0.06	0.24	30.32	0.09	0.30
	2	26.84	0.13	0.50	30.49	0.26	0.84
	3	26.71	0.09	0.35	29.60	0.12	0.42
umNegCtrl	1	26.94	0.05	0.18	Undetermined	Undetermined	Undetermined
	2	27.06	0.07	0.26	Undetermined	Undetermined	Undetermined
	3	27.23	0.02	0.07	Undetermined	Undetermined	Undetermined

PMR calculation was tested in this sample cohort to interrogate if MGMT methylated percentage could be determined. Mouse DNA samples (Mammary gland, Liver, and Lymph Node) indicate no MGMT percent methylation since the Ct values were undetermined. Although ACTB amplified in the mouse liver DNA sample due to possible contamination there was no MGMT methylation indicated therefore, the percent methylation is zero. The initial thought for the Millipore methylated control diluted in mouse DNA at 20% (mammary gland+hMDNA, liver+hMDNA and lymph node+hMDNA) would indicate a 20% methylation percentage but the methylation percentage calculated was between 80%-111% for all samples. After further review this

methylation percentage range is appropriate because the Millipore methylated control used was diluted in mouse is at 100% methylation. Even though the Millipore control was diluted in mouse at 20% the ACTB and MGMT primers/probes do not recognize mouse DNA therefore, it will calculate the methylation percentage of the human sample which is 100% for this control. To interrogate different MGMT methylation percentages in mouse models we would have to dilute the Millipore Methylated Control in the Millipore Unmethylated Control at varying ratios then dilute into DNA This data further confirms the primer/probe design for MGMT and ACTB are specific for human DNA only and this assay is sensitive to MGMT methylation at the appropriate percentage in mouse models. For confirmation, the sample hMDNA diluted at 20% with unmethylated human DNA indicates a PMR percentage ranging between 18%-30% therefore, we concluded PMR can be calculated appropriately for PDX mouse samples. The hMDNA, mPosCtrl, and umNegCtrl all performed as expected and reproducible between all assay replicates. NTC was tested in this experiment and no amplification was indicated. (Table 29).

Table 29. PMR calculations for PDX mouse samples.

	PMR (%)			
Sample Name	Rep-1	Rep-2	Rep-3	Mean of PMR
Mammary gland	0.00	0.00	NA	0.00
Liver	0.00	0.00	0.00	0.00
Lymph Node	0.00	0.00	0.00	0.00
Mammary gland+hMDNA	92.69	104.37	NA	98.53
Liver+hMDNA	111.83	99.31	80.57	97.24
Lymph Node+hMDNA	111.45	99.22	107.84	106.17
hMDNA (20%M)	30.12	18.47	24.87	24.48
mPosCtrl	100.00	100.00	100.00	100.00
umNegCtrl	0.00	0.00	0.00	0.00

DISCUSSION

MGMT promoter methylation is known to be associated with prolonged survival rates in patients with glioblastoma multiforme undergoing clinical therapy with alkylating agent TMZ. Determining the MGMT methylation status of patients diagnosed with glioblastoma is crucial to triage therapies. A real-time PCR assay was developed to detect the PMR using designed primers and probes specific to methylated MGMT. This assay used methylated and unmethylated commercial controls and cell lines to test specificity, sensitivity and limit of detection to assess the robustness, efficacy and efficiency of the assay. The results of this study showed this MGMT methylation PCR assay is an effective and robust method to determine the MGMT methylation status of patient samples that will aid physicians in their decision making for clinical therapies.

Millipore Methylated DNA Controls were optimized and used for the generation of a quantitative standard curve. The initial standard curve used methylated controls bisulfite converted and eluted at 2.5ng/µL at 20µL then serially diluted 1:5 with nuclease free water generating four standard curve points. Based on these results it was concluded that more dilution points needed to be added between 12.5ng and 2.5ng. The data was not reproducible at the lower ends of this dilution series which would affect downstream data analysis of PMR values and linear regression calculations. The final optimized standard curve used methylated controls bisulfite converted and eluted at 2.5ng/µL at 20µL then serially diluted 1:2 with nuclease free water generating five standard curve points to input into the Applied Biosystems ViiA7 QuantStudio software including a no template control. Each standard curve dilution point was an improvement and ACTB and MGMT

had consistent Ct means and CV values which was indicative of an effective standard curve which can be used for assay quantification.

The initial primer and probe design for MGMT and ACTB at varying DNA inputs were tested to interrogate the following question: determine if primer and probe design is specific to methylated MGMT DNA. After reviewing the initial results, the MGMT primers and probe were specific for methylated MGMT and the unmethylated MGMT performed as expected. Further review of the results, the primers and probes for ACTB were efficient but the PCR amplification curves for MGMT primers and probe did not indicate a sigmoidal curve indicative of an efficient PCR reaction. Based on the Tm recommendations from IDT the MGMT primers/probe was redesigned and interrogated the PCR amplification curve of MGMT did improve to indicate a sigmoidal curve indicative of an efficient PCR reaction.

Reproducibility of this assay was affirmed using triplicate samples on different days and with different reagents with Millipore methylated and unmethylated sample ratios and with FFPE methylated and unmethylated cell lines. These tests confirmed that the assay designed is reproducible over time. Based on the literature, the aim of this assay was to achieve an R^2 value ≥ 0.95 and PCR efficiency range between 80%-120% for each standard curve, no MGMT amplification in the Millipore Unmethylated negative control and to have MGMT amplification in the Millipore Methylate positive control. The PMR was calculated for each sample tested in each replicate of the assay to determine if percent methylation could be calculated based on the PCR assay designed. PMR calculation used the quantity values calculated on the QuantStudio software of the ViiA7 for MGMT and ACTB methylated positive control against the quantity values of MGMT

and ACTB of each sample tested multiplied by 100. Based on the data generated it was established that the limit of detection for this assay is 5% methylation but the limit of reporting for a clinical report is predicted to be 10% methylation.

PDX mouse models with human tumor tissue implanted was tested to question the following: if ACTB and MGMT primers were specific to human DNA sequence and not mouse DNA and would we be able to use PDX mouse models for this assay for animal research. BLAST was used to interrogate if the primer sequence overlapped with mouse sequence. The online BLAST search indicated there should be no overlap between ACTB and MGMT primer design with mouse DNA. Experiments were run to interrogate if the ACTB and MGMT primers are specific for human MGMT methylated DNA only and is the assay able to detect then amplify it. The experiment showed amplification in the liver mouse DNA sample for ACTB but was determined to be possible contamination of the mouse DNA sample provided. Based on this information it was concluded the ACTB and MGMT primer design was specific for just human DNA but further PDX testing would need to be conducted based on the liver ACTB amplification observed. PMR can be calculated appropriately for PDX mouse samples using this assay. The primers and probes are specific to interrogate human DNA without interference from mouse DNA providing the opportunity to calculate MGMT methylation percentage of PDX mouse samples which is important for clinical animal research.

Future work for the implementation of this assay is to establish appropriate QC metrics and perform a clinical validation using clinical glioblastoma samples and possible colorectal samples. The clinical validation will include testing reproducibility, accuracy,

sensitivity and limit of detection in preparation to test patients in a prospective clinical study.

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