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Detection of *MGMT* methylation status using a Lab-on-Chip compatible isothermal amplification method

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***Abstract*— The growing cancer burden necessitates the development of cost-effective solutions that provide rapid, precise and personalised information to improve patient outcome. The aim of this study was to develop a novel, Lab-on-Chip compatible method for the detection and quantification of DNA methylation for *MGMT*, a well-established molecular biomarker for glioblastoma, with direct clinical translation as a predictive target. A Lab-on-Chip compatible isothermal amplification method (LAMP) was used to test its efficacy for detection of sequence-specific methylated regions of *MGMT*, with the method's specificity and sensitivity to have been compared against gold-standards (MethyLight, JumpStart). Our LAMP primer combinations were shown to be specific to the *MGMT* methylated region, while sensitivity assays determined that the amplification methods were capable of running at clinically relevant DNA concentrations of 0.2 – 20 ng/μL. For the first time, the ability to detect the presence of DNA methylation on bisulfite converted DNA was demonstrated on a Lab-on-Chip setup, laying the foundation for future applications of this platform to other epigenetic biomarkers in a point-of-care setting.**

I. INTRODUCTION

Clinical complications in cancer often arise due to multidrug resistance owing to the expression of genes resulting in poor patient outcomes for various high-grade, metastatic cancers. This phenomenon is very much highlighted in glioblastoma cases for instance, whereby the silencing of *MGMT* is associated with the prognosis of the disease and the prediction of response to temozolomide, a commonly used chemotherapy drug [1], [2]. *MGMT* codes for the enzyme O(6)-methylguanine methyltransferase reponsible for removing alkyl adducts from the genome. When it is epigenetically silenced by DNA methylation, patients have improved outcomes for therapies with alkylating agents [3]. In DNA methylation, a methyl group is covalently attached to cytosine by DNA methyltransferase enzymes (DNMTs), in certain regions within the genome also known as CpG islands. The resulted "hypermethylated" section within a promoter region then results in the prevention of transcription factors from binding to the site, thereby disrupting processes of gene expression leading to gene silencing.

There is a dearth of molecular tests that reveal these epigenetic signals in most clinical settings. Hence keeping this in mind, the aim of this project was to come up with a simple, cost-effective, point-of-care (PoC) assay using a microchip-based device and a Loop-mediated Isothermal Amplification (LAMP) method, first established by Notomi et al. [4]. A pH-sensitive version of this technique was first developed in our group for detection of DNA methylation in under-study gene targets using Ion-Sensitive Field-Effect Transistors (ISFETs) as the chemical sensors integrated on a Lab-on-Chip interface, combining molecular assays, circuit topologies, microfluidics and data processing algorithms in a small portable unit [5]–[11]. Furthermore, this approach has been adapted to detect *PIK3CA* mutation in breast cancer [12] and more recently for rapid COVID-19 detection [13].

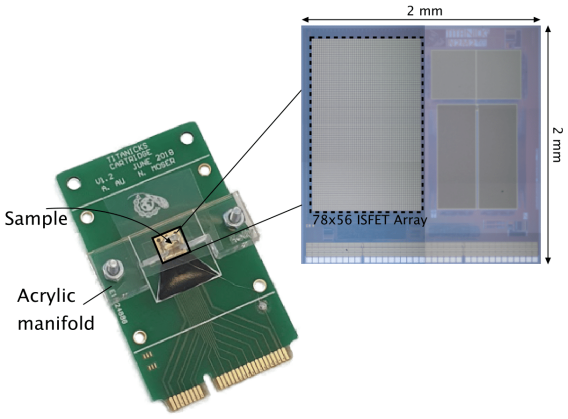

Fig. 1. A disposable Lab-on-Chip cartridge consisting of a ISFET-based sensor array for real-time detection of DNA methylation through ion imaging [14]

The combination of novel amplification chemistry and CMOS sensing is the core working principle of the Lab-on-chip platform used within this report [14]. ISFETs here operate on the basis of ion sensitive gates that have threshold voltages supplied by a Ag/AgCl reference electrode, and are sensitive to electrolytic solutions in which they are found. Figure 1 illustrates the microchip that consists of a 78x56 pixel CMOS ISFET array, mounted on a disposable PCB cartridge, to carry out ion imaging. A Peltier module beneath the cartridge heats up the reaction chambers to carry the LAMP amplification. The gates are coated with an insulating membrane such as silicon (IV) nitride (Si_3N_4) which can bind to protons. The release of protons from the isothermal LAMP reaction causes a shift in the voltage which

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is proportionally traced as alterations in the ISFET sensor's threshold voltage. The underlying premise of our work is the ability to distinguish between methylated and unmethylated forms of DNA. Key to this ability to discriminate between a methylated or unmethylated gene, is the overlapping of primers on CpG dinucleotides, which in the unmethylated form, are converted to TpGs on amplification after a bisulfite conversion reaction [15]. In this case, LAMP primers are designed to specifically bind to methylated CpGs and amplify the region, with no amplification to occur if the CpGs are unmethylated, allowing us to trace the amplification-specific pH change of the methylated region of interest using ISFET sensors.

For the purpose of detecting *MGMT* methylation, LAMP primers were designed to bind to specific sites, from previously published studies, within the CpG rich promoter regions of the *MGMT* gene [16]. Our methylated primers exhibit high specificity for the selected methylated region, and detect as low as 1.0×10^3 copy numbers of synthetic DNA fragments in less than 15 minutes. Lastly, we report a successful detection of the *MGMT* methylated sequence with the methylated primers in a subsequent pH-LAMP reaction and on the Lab-on-Chip device, serving as a layout for future work using this platform.

II. Materials and Methods

A. GeneArt Fragments, Primer Design and MS-PCR

420 bp methylated and 312 bp unmethylated *MGMT* synthetic DNA fragments (Integrated DNA Technologies Inc.), with sequences representing the state after bisulfite conversion, were used, in conjunction with *MGMT* methylation specific LAMP primers (Thermo Fisher Scientific), designed for methylated/unmethylated specific assays using the PrimerExplorer.v4 web tool, as seen in Table I. MethyLight reactions were carried out using the MethyLight PCR + ROX Vial Kit (Epitect) and Jumpstart (Sigma-Aldrich) as per kit's instructions. The primers and probes for MethyLight and Jumpstart were designed based on previously reported studies by Fiegl *et al.* [16] and Eads *et al.* [17]. Human DNA (5 µg/µl) (Cambridge Biosciences) was used to yield bisulfite converted DNA (BSC-DNA) with the EZ DNA-methylation Gold kit (Zymo Research). BSC-DNA concentration was measured by means of a Qubit ss-DNA Assay kit on a Qubit Fluorometer. The reactions were carried out on a LightCycler 96 instrument (Roche).

B. LAMP and pH-LAMP

A 5.00 µL mixture of the LAMP reaction was comprised of 1x IAB, 6 mM $MgSO_4$, 1.4 mM dNTPs, 0.5 mg/mL BSA, 0.80 M Betaine, 0.5 µM SYTO™ 9, 320 U/mL Bst 2.0 DNA polymerase, 1x primer mix, 0.50 µL DNA solution and 1.25 µL nuclease-free water. The LAMP protocol consisted of 50 cycles of 60 seconds isothermal heating at 64 °C, followed by a melting analysis comprised of 95 °C for 10 seconds, 65 °C for 60 seconds and 97 °C for 1 second. The pH-LAMP method was adapted from previously reported studies, conducted within our group [12], [18].

TABLE I
LAMP Primers for *MGMT* Methylation Specific LAMP Assay

Primer Name	Sequence
	Methylated
F3	TTTTCGGTTTCGTTTCGTT
B3	TCGAAACTACCACCGTCC
FIP	CGAACTATCCCAACATATCCGAAAC
BIP	TTTACGTCGTTATTTTCGTGT GTAGGTTTTCGCGGTGCGTA
LF	AAAACTCCGCACTCTTCC GCGAAAACGAAACCGAAAA
LB	TCGTTTGCGATTTGGTGAGTGTT
	Unmethylated
F3	GGTTTTGTTTTTGTGTTTTGGAT
B3	CAACAACAAAATAAAAACACCTAC
FIP	ACAATACACACCACAAAAACCTACA
BIP	GGGATAGTTTGTGTTTTTAGAAT TTGTGATTTGGTGAGTGTTTGGG
LF	ACTCAAAACTACCACCATCC AACATCAAAACACAAA
LB	GTTTTGTTTTTGGAAGAGTGTGGAG

C. Chip Set-up and Analysis

A handheld, microchip-based Lab-on-Chip device, known as 'Lacewing', was used as the detection platform, following techniques described in [14], and was further miniaturised and designed to interface with a mini Peripheral Component Interconnect express (PCIe) connector. The microchip was encapsulated as a disposable cartridge (Fig. 1) assembled on a standard printed circuit board, a manifold with a 3-D printed integrated microfluidic chamber, hosting the understudy sample, and an ISFET-based microchip bonded on the board. The device was designed to i) provide power from a battery, ii) perform data acquisition from the microchip, iii) achieve temperature regulation using a PID controller, on-chip temperature sensor and a Peltier module and iv) stream real-time sensor data to a smartphone via Bluetooth to allow visualisation of the signal from all the sensors. In unison with the chip reaction, a separate pH-LAMP was conducted on the LightCycler for direct comparison. pH values of both pH-LAMP and Lab-on-Chip based pH-LAMP were measured using a pH-meter, pre- and post-amplification.

The Lab-on-Chip derived output curves were smoothened using a microchip integrated algorithm. From the smoothened-out plots, the max derivative was identified to determine the amplification peaks. Next, the amplification's lower and upper bounds were identified using a thresholding method and a compensation for drift was then carried out by a subtraction of the microchip's smoothened averaged output and the drift only signal to produce the resulting amplification curve. The data were then fit into a sigmoid curve and normalised to produce the final graphical output.

III. Results and Discussion

A. Specificity Assays

Discrimination between methylated and unmethylated genes was facilitated by methylation specific primers. The results shown in Fig. 2, indicate that our methylated primers are specific to the methylated gBlocks and DNA, with no amplification occurring on the unmethylated strands. In

contrast and as expected, the β-actin primers showed the same amplification propensity independent of the methylation content.

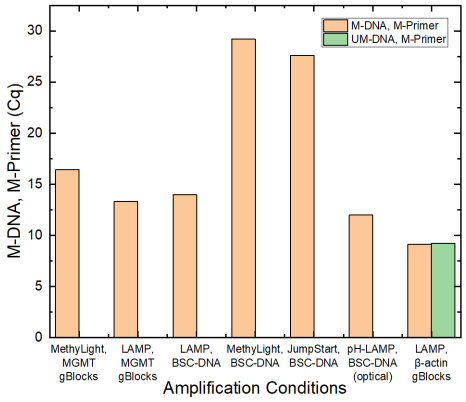

Fig. 2. Comparative studies of specificity of primers on methylated and unmethylated *MGMT* and β-actin using different amplification techniques. gBlocks at 10^6 copies/μL, BSC-DNA at an estimated 17 ng/μL (5000 copies/μL).

B. Sensitivity Assays

The sensitivity of our assays was investigated by the preparation of serial dilutions of synthetic DNA, ranging from 10^7 copies/μL to 10^2 copies/μL of *MGMT* (gBlocks) in solution (Fig. 3). The analytical sensitivity was then determined by the slope of the plots. The LAMP assay showed an analytical sensitivity of –3.53 Cq per $\log_{10}$ copies/μL and a limit of detection of approximately 10^2 copies/μL. MethyLight and JumpStart showed higher sensitivities (–2.90 Cq per $\log_{10}$ copies/μL and –2.97 Cq per $\log_{10}$ copies/μL) and lower limit of detection values (10^0 copies/μL). However, LAMP was consistently faster at detecting the genetic material, with Cq values being recorded on an average of 9 values before the qPCR alternatives.

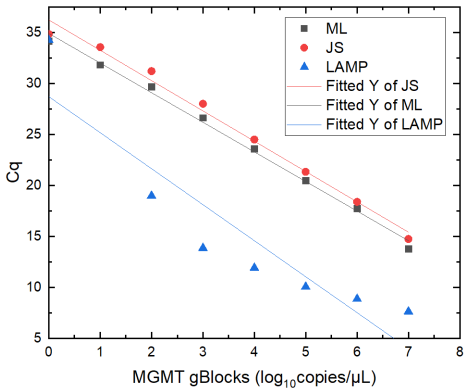

Fig. 3. Comparison of *MGMT*-M gBlocks using the qPCR techniques (MethyLight and JumpStart) and LAMP. Standard deviation values are depicted as error bars.

C. Percentage Methylation References

The percentage of methylated reference (PMR) is a quotient used to quantify and compare the % methylation content at a locus across a range of samples [19]. As shown in Eq. 1, the quotient of the concentration of *MGMT* to β-actin in an unknown sample is divided by the quotient of the concentration of *MGMT* to β-actin in a reference sample of known concentration.

$$\text{PMR} = \frac{\left(\frac{[MGMT]_{\text{Unknown Sample}}}{[\beta - actin]_{\text{Unknown Sample}}}\right)}{\left(\frac{[MGMT]_{\text{Reference Sample}}}{[\beta - actin]_{\text{Reference Sample}}}\right)} \quad (1)$$

The comparison of the 'unknown' samples to the controls of known concentration, convert the data from absolute figures to relative figures. The concentration of each component can be extrapolated from the regression equation of previously prepared standardisation plots at known different concentration points. For example, the concentration of DNA (x) in the LAMP PMR was calculated by inputting the Cq value (y) of the β-actin gene into the regression equation of the standard plot (y = – 0.0327 x + 16.973). One of the benefits of using a PMR methodology, in place of directly extrapolating the quantity of methylation from the standardisation plots, is that it has methylated controls in place to account for sample-independent sources of error, such as experimental and instrumental variation [20]. Additionally, analysis of β-actin acts as a control for false negative results that may arise due to sample wells being void of genetic material.

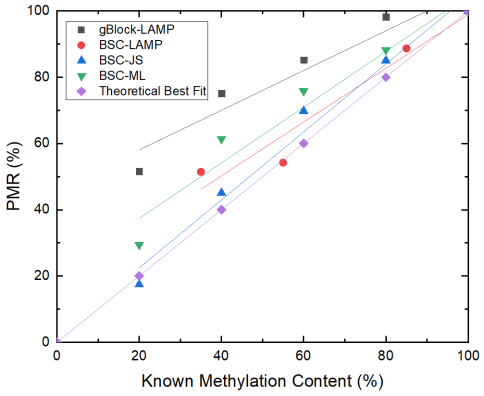

Fig. 4. Data plots for calculated PMR values using LAMP, MethyLight and JumpStart. A gBlock-LAMP model was run at 40,000 copies/μL. LAMP, MethyLight and JumpStart were run on BSC-DNA, at an estimated 17 ng/μL (5000 copies/μL).

The PMR values in Fig. 4 provide an interesting avenue for our future work. The results indicate that LAMP can serve as a comparable methodology to the standard ML when utilised within a PMR setup. In general, the four different assays showed a pre-disposition to overestimate the percentage methylation of the content, with deviation from the expected value increasing as the methylation content decreased. Initial readings were taken on a gBlock mimic of BSC-DNA, composed of a mix of β-actin, *MGMT*-M, and *MGMT*-UM. Each of the gBlocks was present in solution at a

concentration of 40,000 copies/µL. Unexpectedly, the mimic had the biggest discrepancy between the relative ratios of DNA found in solution, and the PMR values generated. The PMR values on the BSC-DNA however, were much closer to the anticipated amounts. On our samples, LAMP and JumpStart appeared to show the best correlation. Importantly however, these results indicate for the first time the potential suitability of PMR values to be generated from LAMP BSC-DNA. The importance of LAMP for PMR generation is underscored by the ease by which LAMP can be switched over to pH-LAMP and thus allow for analysis within low-cost, non-optical devices. Future work should primarily envisage to generate PMR values using pH-LAMP.

D. pH-LAMP and Chip Data

Having established the applicability of LAMP for PMR studies, we finally investigated BSC-DNA's suitability in a PoC pH-LAMP analysis. In a typical pH-LAMP setup, reaction vessels are prepared and run within a real-time PCR setup, in order to validate the Cq values optically. During the experimental validation, one of the replicates was not loaded for analysis but was rather used to standardise the pre-amplification pH value, measured by a handheld pH meter (Sentron). At the end of amplification, the pH of the assay mix that amplified using the Lab-on-Chip interface, was measured using the same pH meter. The Lab-on-Chip set-up provides pH measurements across the surface of arrays of ISFET sensors, based on the same detection principle, while monitoring the electrochemical changes taking place during amplification in situ. Within each chip setup, the cartridge's chamber is loaded with a single analyte solution. The pH data are then compared to the Cq values on the qPCR device, in order to establish whether any pH changes correlate with the observed amplification. During an optical based amplification reaction, the generated fluorescence is automatically converted into an amplification curve by the software used. Cq values are then determined by assigning the correct threshold value. Analysis of chip results was conducted using a Lacewing-integrated signal processing algorithm, which extracted the amplification curves with the inherent chemical drift and generated the output plots as shown in Fig. 5.

TABLE II
pH LAMP RESULTS USING OPTICAL AND CHIP BASED DETECTION

Sample	Cq	pH (before)	pH (after)
Optical	15.43	8.44	7.82
Chip	15.27	8.41	7.79
Optical (NC)	-	8.47	8.38
Chip (NC)	-	8.47	8.32

Using both optical and chip based analysis, methylated DNA was selectively amplified using pH-LAMP (Table II), as demonstrated by the decrease in pH of 0.62 units for 5000 copies of *MGMT*-M/ul observed, while the negative control (NC) underwent a change in pH of $\leq$0.15. These are the first reported instances of BSC-DNA being analysed on a portable, rapid ISFET based Lab-on-Chip setup via pH-LAMP.

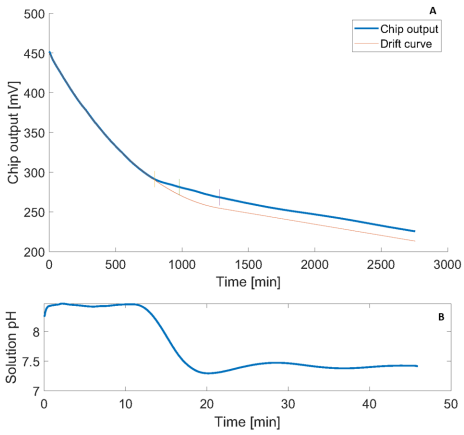

Fig. 5. A) The blue and orange curves indicating the output data and drift. B) Subtraction of the blue and orange curves was carried out in order to generated a graph of solution pH with time.

IV. CONCLUSION

In this paper, we have demonstrated a pH-sensitive assay for the detection of sequence-specific methylated regions of *MGMT* using an ISFET based Lab-on-Chip platform, demonstrating the potential for a future clinical application of detecting the DNA methylation status of epigenetic biomarkers for monitoring of cancer treatment response. Given the significant differences in Cq values from conventional qPCR methods, this demonstrates a quick, feasible way to be adopted as a low-cost alternative for locus-specific methylation analysis. This method can be validated further with DNA isolated from FFPE samples and then with circulating tumour DNA, in conjunction with the investigation of alternative to bisulfite conversion methods, for better DNA preservation and analysis on small amounts of starting genetic material. The next phase of this project should also focus on the quantification of methylation levels which will lead to greater precision in assessing the methylation status of the epigenetic biomarkers.

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