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# Differential CpG DNA Methylation: qMSP a Promising New Approach towards Clinical Utility as Biomarkers

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## Abstract

Epigenetics modifications were shown to be directly related to disease pathogenesis as well as particular stages of their progression. Epigenomic profiles or specific epigenetic changes therefore have the potential to be used as biomarker for diagnosis, prognosis and prediction of response to treatment or stratification towards advancing personalised medicine. DNA-methylations on CpG-dinucleotide regulate the chromatin structure by modulating nucleosomes spatial distribution and controlling DNA packaging. Profiling DNA-methylation genome-wide has allowed for disease or event/outcome specific CpG to be identified. Quantitative Methylation Specific qPCR (qMSP) are assays targeting a specific change in the methylation status of a CpG-dinucleotide.

**Keywords:** Epigenetic; DNA-Methylation; qMSP; Biomarker

## Introduction

Over the past few years, epigenetics has become an important field of research. While epigenetic modifications do not change the DNA sequence, these alterations are critical for many processes of cell differentiation and to enable their functional capabilities [1-3]. Many diseases have been associated with such alterations [4-6]. Epigenetic modifications are now considered as important as mutations in tumorigenesis and re-labelled epimutations [7,8]. In autoimmune diseases which share immuno-genetic mechanisms, they are also important to the pathogenesis of such complex diseases [9-12].

Since epigenetic marks are believed to be directly related to the disease state, epigenomic profiles or specific epigenetic changes have the potential to be used as biomarker with clinical application for diagnosis, prognosis, prediction of response to treatment and stratification towards advancing personalised medicine. Much advances have taken place mainly in cancer field to date however, limitations were associated with the technologies used which were appropriate for research while being too laborious and time consuming for being applicable in clinical practice [13-18].

DNA-methylations regulate the chromatin structure and accessibility between 2 states by modulating nucleosomes spatial distribution and controlling DNA packaging **(Figure 1)**. Methylation of the DNA that occurs only on cytosines in CpG-dinucleotide (and regions rich in CpGs dinucleotide called CpG-islands) and characterises heterochromatin, a compacted or closed DNA state not allowing access to the DNA sequence. Euchromatin where cytosines are un-methylated, allows a relaxed DNA state, open to the transcription machinery.

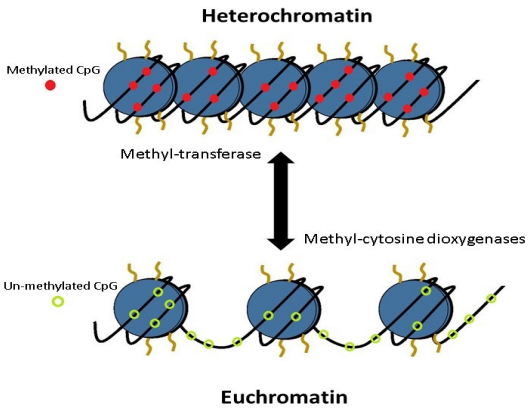


**Figure 1:** Schematic of hetero and Euchromatin. **Note:** ( • ) Methylated CpG ; ( ○ ) Un-methylated CpG. Heterochromatin is a tightly packed state of the DNA in nuclei of cells, where methylated cytosines in CpGs (red dots) are holding Histone closely. Euchromatin is a relaxed state and cytosines are unmethylated (green circles). In both states, histones can be heavily modified (brown symbols) with acetylation, methylation, phosphorylation and other post translational modifications, all also regulating the structure of the chromatin and its accessibility. Heterochromatin is though not to be accessible to the transcription machinery, while euchromatin is; although, according to recent literature, this is in fact a highly dynamic state that is continuously regulated notably by methyltransferase and 10- ten-eleven Translocation Methyl-Cytosine Dioxygenases (TET) enzymes.

Technological advances for the analysis of DNA methylation at the genome level (epigenome-wide EWAS) have provided more robust methods [19-21]. The array technology is still dependent on the identification of CpGs across the genome for the binding of CpG specific probes (for example the Human Methylation-450K or 850K CpG BeadChip) and remain useful to pilot large-scale experiments. Next generation sequencing technologies allows deeper details but are still expensive [22-25]. Data analytics and new tools have been designed to facilitate analysis and may example of DNA methylation profiles at genome level have been described [26-31]. Research then focused on how epigenetic modification could be used in clinical practice (as biomarkers) while the potential for therapeutic interventions focus on the machinery responsible for modification, with most advances in cancer again [32].

Profiling DNA-methylation with high accuracy is now relatively "easy" following recent advances in Quantitative Methylation Specific qPCR (or qMSP). This assay target a specific change in the methylation status of a particular CpG dinucleotide hence is designed for a specific single base-pair CpG target. This new technology is highly dependent on the sequence to be analysed but where possible the PCR technology allows for robust assay design. Epigenetic modifications are cell-type specific (cancer cells versus non-cancer cells or specific to one lymphocyte subset in AIDs for example). The targeted CpG would have therefore been identified in blood, purified cells, tissue or body fluids/sources. In a PCR context, DNA from any contaminating cells will dilute the signal. The selection of a CpG of interest is therefore critical and should consider the type of samples that will be used, the amplitude of the differential methylation between the cells of interest and any other (contaminating) cells present in the samples as well as the amplitude of the difference and distribution of data between the groups to be tested. Therefore, this is a technique that can be used for a biomarker assay as opposed to the discovery/ screening of changes in DNA-methylation.

To differentiate between methylated cytosine (m-C) and un-methylated cytosine (um-C) by PCR, bisulfite conversion if the DNA is necessary to provide a DNA-template where these can be differentiated by changing all um-C into a Uracil but keeping the m-C untouched **(Figure 2)**. Designing the qPCR assay then relies on the same principles as other fluorescent-probe based assay, using primers to ensure specificity of the PRC product and the probe to quantify it. Both the sense and the antisense DNA-strands can be used for designing primers and probes.

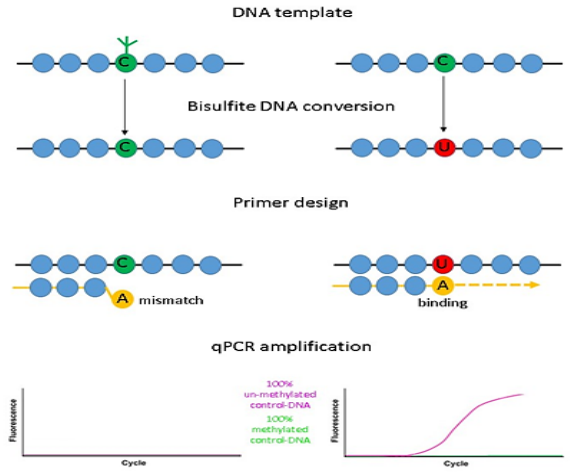


**Figure 2:** qMSP assay principle.

The genomic DNA sequence of the regions surrounding the CpG of interest (2-300 pb before and after) needs to obtained (for example from UCSC or NCBI database) and then bisulfite converted in silico (using sequence manipulating function in online platforms such as MethPrimer). The qPCR design can then use general guidelines [33,34]. Due to the lower complexity of bisulfite converted DNA, a higher GC content in methylated DNA sometimes facilitates aiming for the high primer Tm optimal for qPCR at 59-60 ºC. For many candidates, the DNA sequence may not allow for primers design and each assay design will therefore be highly dependent on the sequence surrounding the CpG. Often, the primer sequences have to be manually edited to obtain the best Tm (59-60ºC), positioning the candidate CpG site at 3' end of one of the primers (**figure 2**) aiming for the length of PCR product to be about ~100 pb, and avoiding self-dimers/hairpins or primer-dimer as for any qPCR. The probe can also be designed to contain CpGs (as sometime difficult to avoid in a CpG-island) in their methylated or unmethylated version depending on which type of the DNA is quantified. The PCR product also need to be checked in silico to ensure the specificity to the sequence targeted for the absence of homology with bisulfite-converted genomic DNA, using a blasting function (Bi-search primer-design for example). Both the TaqMan® probe and primer should be designed using qPCR specific Software.

Two types of reactions are needed for a quantitative MSP assay; one for the methylation-dependent CpG of interest and one for a methylation-independent CpG for a control gene used for normalization (as in any qPCR using a relative quantification

method). The development of a qMSP assay starts with reactions optimisation. qPCR conditions need to be optimised manually like for any qPCR, to ensure both the specificity using 100% methylated versus 100% un-methylated control-DNAs and most importantly here, the equal efficiency (same yield=same Ct) for both PCR products : gene of interest and normalisation target from the same input of target DNA. Primers being the main factor affecting the specificity and efficiency of the qPCR reaction optimisation start with a primer concentration matrix optimisation usually between 50-900 nM. Both control DNA should be used and no amplification should be seen for the template not targeted for the gene of interest while both should be equally amplified for the normalisation target. Reactions are compared directly using Cts, choosing the primer concentrations that allow the closest Ct for both assays. Some assay will be extremely sensitive to primers concentration and others will not all directly relate on the sequence surrounding the CpG of interest. A dilution series of both templates DNA (0.2 to 50 ng) is then used to compare efficiencies of the assays. Linearity should be achieved (plotting Ct against the Log (DNA concentration ng/ uL) fitting a linear curve and a 2-fold increase in DNA input should be matched by only 1-cycle difference in Ct. A regression model then provide the slope used to calculate qPCR efficiency which should be no less than 95%, while an efficiency above 100% suggest >2-fold amplification/cycle and should be discarded.

The next step is then dependent on the template that needs to be used in the assays. The need for robustness in biomarker assay design therefore favours the less processed samples type while the utility of the biomarker may resides in a rare population of cells (for example tracking cancer cells) hence the high specificity of the CpG selection criteria described above.

qMSP assays were successfully designed and used in several studies in cancer [35-39]. Several assays are available to quantify exhaustion molecules such as LAG3, PD1 or CTLA4 in relation with the recent development of immune checkpoint inhibitors [40-48]. qMSP were also designed in non-cancer conditions, targeting tissue or disease specific pathways notably in AIDs [49-52].

In the context of AIDs, several blood cell subsets have shown potential biomarkers value. Multiple lymphocyte subset enumeration in frozen blood samples is an important outcome for certain clinical studies targeting these cells. qMSP assays were developed (commercially) to enumerate most blood cell types (total CD3+T-cells, CD4+T-cells, CD8+T-cells, B-cells, NK-cells, monocytes, eosinophil, neutrophils, basophils etc…), which helps substituting for other techniques or standardizing the data in multicentre trials for example [53-56]. Subset of a specific lymphocyte type (naïve, memory for example) is also offered. Regulatory T-cells were shown to predict various outcomes [57]. These are usually assessed with flowcytometry by routine clinical services. In particular situation where flow services are not accessible, a qMSP assay is available that can quantify Treg (FoxP3 gene) in whole blood, fresh or retrospectively (frozen).This is currently expensive and used mainly in clinical trials but demonstrates the usefulness and feasibility of the concept. A role for Th17 cells in autoimmune diseases has also been established. Quantifying these cells in the blood of patients is also possible by flowcytometry in research settings but it is less practical in clinical services due to the need for activating the cells (PBMC isolation, CD4+T-cell separation and activation for 3-5 days under sterile conditions). An alternative 5 hours protocol only quantify activated-Th17 (actively secreting IL17) which is less relevant in the blood compared to the disease site. Using a qMSP assay detecting an epigenetic mark on the IL-17A gene provides a good biomarker alternative, particularly as it allows quantifying both resting and activated Th17 cells [58].

Based on our recent work in rheumatoid arthritis (RA), several of the differentially methylated (DM) CpG identified could serve as candidate for qMSP assay design. These were identified in purified naïve CD4+T-cells comparing RA and healthy controls. Most of this DM-CpG were not differentially methylated in memory CD4+T-cells as well as in monocytes. To select a candidate to be tested in blood for diagnosis, prognostic or stratification purposes, criteria for an ideal candidate in the context of RA would therefore be:

Significantly demethylated in RA naïve CD4+T-cells for disease specificity. Prioritising large amplitude of the delta between patients and controls. Same methylation profile of the region surrounding the CpG in memory CD4+T-cells, not to interfere with the assay. Eventually with DM between RA and controls. Base on the source for DNA template available for clinical samples to develop the qMSP assay. Refining the rules based on access to PBMC or WB, filtering for fully methylated CpG candidates in all other cell types (not to contribute to the signal) and with no DM between RA and controls. CD8, B and NK cells as well as monocytes for a PBMC derived DNA template. All lymphocytes, monocytes and eosinophil, basophils and neutrophils for a whole blood (WB) revived DNA template. Availability of large cohort of patients related to the outcome to be tested. Diagnosis/classification comparing early inflammatory arthritis patient with/developing RA versus other conditions. Stratification for treatment. Use well established statistical methodologies for biomarker pipelines using data acquired in cohorts of patients with clearly defined outcomes to. Establish the biomarker value of the qMSP assay. Test the utility of the qMSP assay over current practice.

Many CpGs will likely fulfilled these criteria and further prioritising them would need to take place, selecting genes with multiple DM-CpG for instance, or genes with a relevant role in arthritis, but ultimately, the DNA sequence surrounding the CpG candidates will determine whether an assays can be designed successfully. qMSP assays were successfully designed and used in various cancers, to quantify exhaustion molecules in relation with immune checkpoint inhibitors, to target tissue or disease specific pathways in autoimmune diseases as well as for multiple lymphocyte subset enumeration. These assays are easy to

design and are robust enough to become a reliable biomarker technology.

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