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An Examination of Critical Parameters in Hybridization-Based Epigenotyping using Magnetic Microparticles

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Abstract
Gene-specific promoter methylation is involved in gene silencing and is an important cancer biomarker. Cancer-specific methylation patterns have been observed and clinically validated for numerous gene promoters, but the knowledge gleaned from this large body of work is currently under-utilized in the clinic. Methylation-specific PCR is currently the gold standard method for clinical methylation assessment, but several research groups have proposed hybridization-based techniques which could be simpler to implement and provide more accurate results. However, the sensitivity of this easier alternative must be improved dramatically in order to compete with methylation-specific PCR. Efficient sample capture is a key step in maximizing sensitivity, so here we investigate the key parameters involved in i.) maximizing the capture of gene-specific target DNA molecules at the surfaces of functionalized, magnetic microparticles and ii.) recognizing DNA methylation using an engineered methyl-CpG-binding domain (MBD) protein. The magnetic bead density, the probe concentration, and the MBD concentration were very important for maximizing detection, and other variables such as the hybridization time also impacted the target capture efficiency but had a smaller effect on the overall methylation assay. The effect of genomic DNA on the capture of the target sequence was also investigated, and model methylated versus unmethylated target sequences could be distinguished in the presence of 1 ng/μL genomic DNA.

The findings we report related to the underlying binding events involved in hybridization-based epigenotyping can be leveraged in combination with the many signal amplification and detection approaches that are currently being developed.

Introduction
Epigenetic modifications, including DNA methylation, are among the earliest modifications in cancer.\(^1\) Because of this, DNA methylation shows great promise in diagnosing cancer and guiding treatment plans. Two examples of promoter methylation assessment are currently used in cancer clinics: promoter methylation of the \(O^6\)-methylguanine DNA methyltransferase (\(MGMT\)) gene for the treatment of glioblastoma\(^2\) and human mutL homolog 1 (\(hMLH1\)) promoter methylation analysis to determine whether patients with certain colorectal cancers have a hereditary form of the disease.\(^3\)\(^-\)\(^5\) In addition to the examples where promoter methylation is currently assessed in cancer hospitals, there are many other genes and types of cancer where determination of the methylation status could provide valuable information.\(^6\)
Currently, all clinical methylation testing uses bisulfite conversion of DNA followed by PCR or sequencing. In bisulfite conversion, unmethylated cytosines are chemically converted to uracil while methylated cytosines remain unchanged. This results in two different DNA sequences depending on whether the region had unmethylated or methylated CpG dinucleotides, and therefore the methylation status can be determined by performing PCR with primers designed for each of the two cases, a technique called methylation-specific PCR (MS-PCR). While this is the method of choice in the clinic at this time, inconsistent results between multiple tests performed on the same sample are fairly common, observed 12% of the time in a recent study by Xia et al. This highlights the need for methods that provide greater accuracy.

Several alternatives to MS-PCR have been proposed. Some of these methods rely on bisulfite conversion but offer alternatives to PCR and gel electrophoresis. One such example uses ligase chain reaction coupled with gold nanoparticles to give a colorimetric readout. Other techniques have been developed to differentiate methylated and unmethylated CpGs without chemical conversion using a nanopore or antibodies on the surface of an optofluidic ring resonator. Numerous studies have used affinity agents, such as antibodies or methyl-CpG-binding domain (MBD) proteins, to distinguish methylated and unmethylated CpG dinucleotides without requiring chemical conversion. Many of these methods use affinity agents to enrich for densely methylated regions of the genome prior to sequencing or analysis via microarray, and more recently, MBD capture techniques have been used with isothermal recombinase polymerase amplification in a bridging flocculation assay for a simple and sensitive readout method.

Another approach, hybridization-based epigenotyping, proposes to avoid amplification of the target sequences of interest. Methods have been developed to capture a DNA sequence of interest by hybridization with a complementary capture probe, recognize methylated sequences, if present, with a MBD, and determine the methylation status by quantifying MBD bound using SPR, fluorescence, or polymerization-based amplification. The simplicity of this approach, which envisions use of methodology analogous to that used in enzyme-linked immunosorbent assays, and the accuracy of hybridization-based techniques make this class of methods well suited for clinical use. However, most hybridization-based methods currently lack the sensitivity required for detecting gene-specific promoter methylation in clinical samples, and almost all studies have used model systems of synthetic DNA oligos, exclusively, in the absence of genomic DNA. A previous study demonstrated profiling of repetitive sequences in blood or plasma samples using a hybridization-based approach, but methylation assessment of specific gene promoters from genomic DNA using similar methodology remains as a challenge due to the much lower abundance of individual gene promoters as compared to repetitive sequences within the genome.

One specific area of interest is to perform hybridization-based epigenotyping of individual promoters on magnetic beads. Bead-based assays have grown into a multibillion-dollar industry with the advent of widespread nucleic acid sequencing and analysis. Illumina’s BeadArray technology is used in both focused and whole-genome genotyping, and xTAG® Technology by Luminex is used for diagnosing diseases ranging from cystic fibrosis to gastrointestinal infections. Illumina® also offers an Infinium® Methylation
Assay, which uses bisulfite conversion together with their genotyping technology to determine the methylation status of CpG sites.\textsuperscript{26} Hybridization-based epigenotyping without bisulfite conversion followed by PCR, however, has unique challenges. Unconverted promoter CpG islands are extremely GC rich, which makes secondary structures that could inhibit probe/target hybridization common and high temperatures for hybridization reactions necessary. Furthermore, unconverted genomic DNA cannot be amplified prior to target capture and methylation detection as this would cause the methylation information to be lost. Therefore, maximizing the target capture is extremely important in developing a hybridization-based methylation assay without bisulfite conversion and PCR.

Here, we evaluate key parameters that impact the capture efficiency of target sequences and thus the sensitivity of methylation profiling with a MBD. Target DNA is captured on the surface of the magnetic microparticles using an unmethylated DNA probe such that an unmethylated duplex is formed if the target sequence is unmethylated or a hemi-methylated duplex if the target sequence is methylated, as shown in Figure 1a. Then, a MBD engineered to bind hemi-methylated DNA is used to determine the methylation status of the captured DNA. The effects of bead, probe, and MBD concentrations were evaluated along with binding reaction times. Target capture was performed in the presence of genomic DNA to assess the ability to capture the sequence of interest from a complex sample. Considering the system in terms of reaction stoichiometry and molar concentrations of reactive sites proved instructive, and the understanding developed here of how to optimize DNA hybridization followed by protein-based recognition of duplex DNA bearing epigenetic marks is likely to be useful in combination with a wide variety of signal amplification techniques that can be added to the underlying binding events that are the focus of this work.

**Materials and Methods**

**Isolation of Genomic DNA**

HT-29 cells (ATCC) were grown in McCoy’s 5A Medium (Lonza). Genomic DNA was isolated from the cells using the QIAamp® DNA Mini Kit (Qiagen). The genomic DNA was sheared using an E220evolution™ focused-ultrasonicator (Covaris) to give a target fragment size of 100 bp. The resulting fragments ranged from 25 to 500 bp in length with the largest proportion of DNA fragments between 50 and 300 bp in length, as shown in Figure S1 in the Supplementary Information.

**Probe/Target Hybridization and Capture on Magnetic Beads**

Dilutions of the probe and target oligos were prepared in DNA hybridization buffer\textsuperscript{20} (6xSSC, 5xDenhardt’s solution). For each sample, a total volume of 500 μL was used. This volume was chosen for convenience in studying the binding events using flow cytometry but easily could be scaled down to 10-50 μL, as would be necessary in a clinical setting. Since the kinetics and thermodynamics of the hybridization reaction are governed by concentration and not copy number, our findings can be generalized to other sample volumes. For experiments where the probe was attached to the Sera-Mag™ Oligo(dT)-Coated Magnetic Particles (GE) prior to target capture, 100 nM DNA probe was used to fully saturate all capture sites and then target capture was performed at room temperature overnight. For
experiments where the probe and target DNA were pre-hybridized, 50 pM probe was used for all samples (except for the probe concentration optimization experiment) while the target and genomic DNA concentrations were varied. The DNA mixture was denatured at 95°C with 700 rpm shaking for 5 min using an Eppendorf Thermomixer® R (Eppendorf AG). To begin annealing the DNA, the temperature of the thermomixer was decreased by 5°C every 5 min until it reached 60°C, then the samples were incubated at 60°C for 2 hours to allow the hybridization reaction to reach equilibrium. After hybridization, the probe/target duplex was incubated with the SeraMag™ Oligo(dT)-Coated Magnetic Particles (GE) on a rotator for three hours at room temperature.

**Methylation Profiling with MBD-GFP**

hMBD2 Variant H4 was expressed as a GFP fusion protein and purified using immobilized metal affinity chromatography (IMAC) purification, as shown in Figure 1. 150 nM hMBD2 Variant H4 was prepared in MBD binding buffer\(^{18}\) (20 mM HEPES, pH 7.9, 3 mM MgCl\(_2\), 100 mM KCl, 10% v/v Glycerol, 0.01% v/v Tween-20, 1 mM dithiothreitol and 0.1% w/v bovine serum albumin). The magnetic beads bound with the probe/target duplex were washed once with 1 mL of PBSA (1xPBS with 0.1% w/v bovine serum albumin) before they were resuspended in 500 μL of the 150 nM MBD solution. Samples were incubated with the MBD at room temperature on a rotator for 45 min. After the MBD binding step, all samples were washed with 1 mL PBSA. All of the wash buffer was removed and the samples were stored on ice. Immediately before analysis, the samples were resuspended in 200 μL PBSA. Samples were analyzed on a BD LSRFortessa™ flow cytometer.

**Results and Discussion**

Our experimental system consisted of Sera-Mag™ Oligo(dT)-Coated Magnetic Particles (GE) functionalized with DNA probes designed to be complementary to the target DNA sequence of interest. In order to investigate the key variables involved in maximizing the capture efficiency of the target sequence, a model biotinylated target DNA sequence was used in order to quantify the amount of target DNA on the magnetic beads under varying conditions. The binding scheme is depicted in Figure 2a. The first important variable was the bead density in the capture reaction. If too many beads are used, then the concentration of capture sites is much greater than the concentration of target molecules, giving a low number of target molecules per beads. However, if the bead concentration and target concentration are both low, transport limitations could prevent efficient capture of the target DNA. Based on the Gibbs free energy of the probe/target hybridization reaction estimated using nearest neighbor thermodynamics,\(^{28}\) the equilibrium constant is 3.4×10\(^{30}\) M\(^{-1}\) for the biomolecular association reaction Probe + Target ⇔ Duplex. This suggests that the amount of DNA captured per bead should be maximized when the concentration of capture sites and target molecules is approximately equal. We tested this for two DNA concentrations, 1 nM and 100 pM. Based on the reported binding capacity of the Sera-Mag™ Oligo(dT)-Coated Magnetic Particles, the overall concentration of capture sites on the bead surface would be 1 nM when the bead density is 3.33 μg/mL. The experimental data shown in Figure 2b confirm the expected binding capacity, as decreasing the bead concentration from 5 μg/mL to 2 μg/mL (and the concentration of capture sites from 1.5 to 0.6 nM) increased the amount of target DNA captured.
DNA captured per bead while further decreases in the bead concentration had no effect. Similar results were found with the 100 pM target DNA concentration, where the amount of target captured per bead was maximized when the bead concentration was between 0.2 μg/mL and 0.5 μg/mL, as shown in Figure 2c.

While capture of single-stranded target DNA via hybridization with probe-functionalized beads at room temperature was possible, this protocol would not work for clinical samples where the double stranded DNA needs to be denatured and higher hybridization temperatures are required to give specificity for one DNA sequence in the presence of genomic DNA. Furthermore, the kinetics of the target capture would be improved by increasing the temperature of the hybridization reaction to eliminate hairpin structures formed by the probe.

However, the Sera-Mag™ Oligo(dT)-Coated Magnetic Particles were not designed for use at temperatures above 40°C. To allow for the use of high temperatures for denaturing and hybridizing the DNA, we modified our protocol to allow for pre-hybridization of the probe and target DNA followed by capture of the probe/target duplex on the surface of the magnetic beads, as shown in Figure 3a. In order to do this, it is important to use a probe concentration that is high enough to minimize transport limitations in the probe/target hybridization as well as the duplex/bead hybridization but also low enough to maximize the ratio of probe/target duplex to unhybridized probe DNA when the target is dilute. To investigate this, we varied the probe concentration (and the bead density proportionally, based on equal number of probes and capture sites) while keeping the target concentration constant. Shown in Figure 3b, we found that when a 10 pM target DNA concentration is used, the amount of target DNA captured per bead was maximized at a probe concentration of 50 pM. Therefore, this concentration was used for all future experiments. Additional variables, such as the hybridization time for both the probe/target hybridization reaction and the duplex/bead hybridization reaction, were also investigated. As shown in the Supplemental Information, 90 minutes at 60°C was required to maximize the probe/target hybridization step and 2-3 hours at room temperature was required to maximize the capture on the surface of the beads. However, in our study, these variables appeared to be secondary to those discussed previously, as implementing the longer incubation times improved target capture somewhat but had minimal effect on the limit of detection of the full methylation assay.

In the full assay, the methylation status was then profiled using a MBD variant engineered to bind hemi-methylated DNA. By selecting a protein that binds hemi-methylated DNA but not unmethylated DNA, methylated target sequences bound to an unmethylated capture probe can be detected. This will simplify further development by allowing simple, easily available capture probes to be used instead of probes with specific methylation patterns that must be synthesized for every sequence studied. The engineered MBD was also expressed as a GFP fusion protein, allowing protein binding to be detected based on GFP fluorescence so we can study the binding event directly without any additional labelling steps to eliminate potential lurking variables. After MBD binding, GFP fluorescence was measured using flow cytometry. Methylated and unmethylated target DNA were distinguished based on differences in fluorescence from MBD binding. This assay is depicted in Figure 4a.
most important variable in the MBD binding step was the MBD concentration. As shown in Figure 4b, MBD concentrations below 100 nM showed very little binding to the unmethylated DNA while concentrations above 200 nM yielded high levels of non-specific binding. A MBD concentration of 150 nM was chosen for further experiments.

After studying the concentrations and incubation times required to maximize the signal, a titration of the target DNA concentration during the hybridization step was performed in order to evaluate the limit of detection of our simple assay. The results are shown in Figure 4c, and the limit of detection for distinguishing methylated and unmethylated target DNA was determined to be 5 pM target DNA based on a two-sample Wilcoxon test at a significance level of 0.01. This gives a 60-fold improvement in sensitivity as compared to a previously developed assay, and no signal amplification techniques were used. All of the key parameters studied here in order to obtain this improvement of sensitivity can be generalized to other systems with signal amplification techniques, as will be necessary to achieve the clinically relevant limit of detection for many sample types.

In clinical assays, it is also necessary to capture the correct sequence from genomic DNA. While our model system is not sensitive enough to capture a single-copy number target from genomic DNA directly, we analyzed the effect of including genomic DNA in the hybridization mixture along with our probe and target oligo. For this, 10 pM methylated or unmethylated target DNA (just above the limit of detection from Figure 4c) was hybridized with 50 pM probe in the presence of varying amounts of genomic DNA, the hybridization mixture was incubated with magnetic beads to capture the probe on the bead surface, and the human MBD2 Variant H4 protein was added to bind the hemi-methylated DNA sequences, as shown in Figure 5a. The results of this experiments are presented in Figure 5b. At the highest genomic DNA concentration tested, 1 ng/μL, a statistically significant difference between the unmethylated and methylated samples was retained at the 0.01 level based on a two-sample Wilcoxon test.

To further verify the limit of detection in the presence of genomic DNA, a titration of the target DNA concentration was performed with 100 pg/μL genomic DNA in the hybridization mixture. The results of this study are shown in Figure 5c. The titration curve in the presence of genomic DNA looks very similar to that without genomic DNA, and the limit of detection in the presence of genomic DNA was determined to be 5 pM target DNA based on a two-sample Wilcoxon test at a significance level of 0.01. However, further improvement in sensitivity is still required in order for the assay to be useful for clinical samples, where only 10^6 copies of the genome may be available and sub-pM sensitivity is required. Such improvements could potentially be made by either using a system with improved transport properties to concentrate the target DNA on a smaller number of beads, thus increasing the amount of target captured and MBD bound per bead, or by using more sensitive detection mechanisms.

The format of this assay, however, is quite reasonable for implementation in a clinical setting. Equipment requirements consist of a magnet, temperature control, a mixer or rotator, and a flow cytometer – all things that are commonly used in clinical settings. Furthermore, the assay can easily be adapted to examine different gene promoters of interest simply by
using a different capture probe, and magnetic bead-based assays can be used with numerous readout methods and signal amplification techniques, which this investigation establishes as necessary to arrive at a simple but also highly sensitive and specific assay that can be used with patient samples.

Conclusions

Hybridization-based methods that use affinity agents to determine the methylation status of a gene promoter of interest offer a simple, reliable way to obtain important information about which genes are silenced; however, these methods are currently not sensitive enough for clinical use. In this work, we investigated key parameters in maximizing the target capture efficiency and the limit of detection in gene-specific, affinity agent-based methylation assays by systematically studying the key variables in each binding event. In doing so, we improved the sensitivity of the assay from 0.3 nM, as reported previously,\textsuperscript{20} to 5 pM using an engineered methyl binding protein in a magnetic bead-based system with no signal amplification methods. The steps taken to improve the target capture and affinity agent binding are generalizable beyond this specific assay and can be used in combination with various signal amplification strategies and readout methods. Furthermore, we evaluated the performance of the assay in the presence of human genomic DNA, which is important for verifying the suitability of the assay for use with realistic sample types. This information about the key variables in optimizing hybridization-based assays should prove useful as future work focuses on developing highly sensitive, gene-specific methylation assays. As this future work is completed, it is important to remain focused on simple, cost-effective assays that fit into realistic clinical workflows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Hemi-methylated DNA is created by hybridizing a methylated target sequence to an unmethylated probe sequence. b) A map of the hMBD2 Variant H4-GFP-B protein. A His$_6$ tag was used to facilitate immobilized metal affinity chromatography (IMAC) purification. The eGFP protein was used for fluorescent detection of the protein as well as to improve soluble protein yield during bacterial expression. The HA tag and biotin acceptor sequence were not used in this assay but offer ways to potentially incorporate signal amplification techniques in future work. c) An SDS-PAGE gel showing the clarified cell lysate containing the hMBD2 Variant H4-GFP-B protein before purification and the purified protein. MW = 44 kD
Figure 2.

a) Sera-Mag™ Oligo(dT) Magnetic Microparticles were coated with the DNA probe, then the biotinylated target DNA was hybridized to the probe on the surface of the beads and labelled with a streptavidin-conjugated fluorophore. b) 1 nM target DNA was hybridized with varying concentrations of magnetic beads. The amount of target captured per bead reached a maximum when the bead concentration was between 2 μg/mL and 5 μg/mL. c) 100 pM target DNA was hybridized with varying concentrations of magnetic beads. The amount of target captured per bead was maximized when the bead concentration was between 0.25 μg/mL and 0.5 μg/mL. For the graphs in (b) and (c), each point is the average of three replicates and the error bars show the standard deviation.
Figure 3.
a) The probe and target DNA sequences were hybridized prior to incubation with the SeraMag™ Oligo(dT) Magnetic Microparticles. b) 10 pM target DNA was hybridized with varying concentrations of DNA probe. The amount of target DNA captured per bead was maximized when a 50 pM probe concentration was used. Each point is the average of two replicates and the error bars show the standard deviation.
Figure 4.
a) The probe and target DNA sequences were hybridized prior to incubation with the SeraMag™ Oligo(dT) Magnetic Microparticles. The beads were then incubated with the engineered MBD-GFP fusion protein and flow cytometry was used to quantify GFP fluorescence from MBD binding. b) The MBD concentration was varied to determine what range of concentrations give good distinction between methylated and unmethylated target DNA. Each point is the average of two replicates, and the error bars show the standard deviation. c) A titration of the target DNA concentration was performed with 50 pM probe and 150 nM MBD. Each point is the average of a minimum of three replicates for methylated samples and a minimum of two replicates for unmethylated samples. Error bars show the standard deviation.
Figure 5.
(a) The DNA hybridization step was performed in the presence of genomic DNA. MBD-GFP was then used to determine the methylation status of the captured target. b) 10 pM target DNA was used for all samples. As the genomic DNA concentration was increased up to 1 ng/μL, the methylated DNA was still distinguished from the unmethylated DNA. All points represent the average of a minimum of three replicates and error bars show the standard deviation. Lines representing the assay without genomic DNA show the average of at least five replicates. c) A titration of the target DNA concentration was performed in the presence of 100 pg/μL genomic DNA. The results match the titration performed without genomic DNA. All points for methylated samples represent the average of a minimum of three replicates and all points for unmethylated samples represent the average of a minimum of two replicates. The error bars show the standard deviation.