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Extraction and Processing of Circulating DNA from Large Sample Volumes using Methylation on Beads for the Detection of Rare Epigenetic Events

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Abstract

The use of methylated tumor-specific circulating DNA has shown great promise as a potential cancer biomarker. Nonetheless, the relative scarcity of tumor-specific circulating DNA presents a challenge for traditional DNA extraction and processing techniques. Here we demonstrate a single tube extraction and processing technique dubbed “methylation on beads” that allows for DNA extraction and bisulfite conversion for up to 2 ml of plasma or serum. In comparison to traditional techniques including phenol chloroform and alcohol extraction, methylation on beads yields a 1.5 to 5-fold improvement in extraction efficiency. The technique results in far less carryover of PCR inhibitors yielding analytical sensitivity improvements of over 25-fold. The combination of improved recovery and sensitivity make possible the detection of rare epigenetic events and the development of high sensitivity epigenetic diagnostic assays.

Keywords

Circulating DNA; Cancer Epigenetics; Blood-Processing Protocols; Quantification; DNA Methylation; Methylation Specific PCR; Biomarkers; Plasma; Serum

1. Introduction

The presence of extracellular nucleic acids in the blood of healthy and diseased individuals was initially observed over 70 years ago [1]. While the particular mechanisms for release of DNA into the bloodstream under normal and pathological conditions have yet to be resolved, many paths have been hypothesized [2–4]. The phenomenon of circulating nucleic acids (CNA) has garnered particular interest as of late as both the amount of CNA and their specific characteristics have been shown to correlate with various disease states as well as

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tissue trauma [3, 5, 6]. Tumor specific circulating DNA has shown particular promise as a potential biomarker and has been detected and correlated with numerous cancer types including: lung, pancreatic, liver, prostate, and colorectal [2, 3, 7]. The analysis of circulating DNA may thus serve as a minimally invasive mode of diagnosis, prognosis and monitoring of cancer [5, 8].

DNA derived from cancerous tissue often contains abnormal genetic and/or epigenetic modifications [9]. Epigenetic modifications include heritable changes that occur within cells that do not result in alterations to the primary DNA sequence. Perhaps the most well known form of epigenetic modification, DNA methylation, has been found to play a key role in cancer initiation and progression, often through loss of expression of key tumor suppressor genes. Consequently, DNA methylation remains a potential marker for applications in cancer detection, diagnosis and prognosis [10].

The use of methylated tumor-specific circulating DNA has shown great promise as a cancer biomarker [11], but its use and reliability are often severely hampered by a number of issues, most notably its relative scarcity. While circulating DNA is found throughout the bloodstream, only a small fraction is likely to come from diseased or cancerous tissue. Furthermore, the even rarer population of cancer-specific genetically or epigenetically modified circulating DNA often places inordinate pressure on current DNA extraction and processing techniques [12]. This extreme rarity, coupled with high-loss processing techniques, results in both lower analytical and clinical sensitivity for diagnostic and prognostics tests. There is thus a clear need for improved techniques that allow for more efficacious extraction, processing and detection of rare circulating methylated DNA.

Traditionally, the methylation status of circulating DNA is determined by extracting the DNA from serum or plasma via phenol chloroform and ethanol precipitation (PC), bisulfite treatment of the extracted DNA, followed last by methylation-specific PCR (MSP) or quantitative MSP (qMSP) [13]. This process typically requires many labor-intensive steps as well as transfer between numerous reaction vessels, thus resulting in sample loss, long assay times, increased contamination, high rates of operator error and variable data and success rates. Furthermore, traditional DNA extraction methods often retain the PCR inhibitors found in blood that can significantly affect assay reliability [14]. Lastly, most commercial extraction techniques are not amenable to sample volumes larger than 500 μ l, as may be necessary in order to provide the clinical sensitivity required on rare genetic biomarkers, where concentrations may be as low as a few methylated gene copies per sample volume (or less). In these cases, the ability to extract DNA from larger volumes is particularly advantageous. While lower volume extraction methods can be used in parallel and pooled together, the effluent remains unconcentrated and provides little benefit in downstream reactions unless additional concentration methods are utilized. Likewise, concentration methods that are currently employed result in sample loss and deterioration due to exposure to elevated temperatures and the resulting concentration of PCR inhibitors and nucleases along with the DNA.

In order to address some of these issues, we previously reported the use of a single-tube method for the extraction and analysis of methylated DNA [15]. Here, we introduce an improved technique, an overview of which is shown in Figure 1, in order to further extend the original paradigm for detection and analysis of exceptionally rare epigenetically-modified circulating DNA in clinical samples. Dubbed “Methylation-on-Beads” (MOB), the process has been significantly amended for use with larger sample volumes (2 ml) and incorporates key improvements in order to retain and process circulating DNA from plasma with 25-fold more analytical sensitivity than current standard techniques, thereby greatly

enhancing the clinical sensitivity of circulating DNA-based diagnostics and clearing the way for the detection of rare epigenetic events.

2. Material and Methods

2.1. Genomic DNA Samples

CpG methylated HeLa genomic DNA was obtained from New England Biolabs. All samples using genomic DNA were diluted to their respective concentrations using RNase and DNase free water.

2.2. Plasma Sample Preparation

Patient blood samples were obtained from a previous study [8] conducted according to the Declaration of Helsinki and with Institutional Review Board approval. While the original study contained a patient population of 45 (Average Age: 64, 23 Male/22 Female, 39/8/1 White/Black/Asian, 89% current or former smokers), adequate remaining sample volume for this study was obtained from 25 of the 45 patients. All patients had been diagnosed with Stage IV or unresectable metastatic non small cell lung cancer (NSCLC) and previously already received at least one form of chemotherapy, had measurable disease per RECIST 1.0, Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, life expectancy > 3 months and adequate liver, renal and bone marrow function. All participants provided written informed consent before participating. Plasma was extracted using standard Ficoll preparation. Briefly, blood samples were immediately placed on ice after draw and, within 60 min, ~10 ml of each blood sample was gently poured onto 3 ml of Ficoll (Sigma-Aldrich) and spun at 1000g for 10 min. The translucent layer on top was then removed and stored at -80°C until use.

2.3. Large Volume Methylation on Beads Process

A 2 ml sample of plasma was digested with the addition of 3 ml of Buffer AL (Qiagen 19075) and 1 ml of Proteinase K (10mg/ml, Invitrogen) at 50°C for 2–4 hours (alternatively, methylated genomic DNA was dissolved in water). Following digestion, 3 ml of 100% IPA and 150 µl of SSBs (Promega Magnesil KF - MD1471) were added. The lysate was incubated at room temperature for 10 min to allow for DNA precipitation and binding to the surface of the SSBs. 10 µl of carrier RNA (1 µg/µl) was subsequently added to facilitate DNA binding by co-precipitation, and the lysate was again incubated at room temperature for an additional 5 min.

Next, the SSBs containing DNA bound to their surface were isolated and purified from the remaining plasma via magnetic decantation. While the tube remained within the magnetic field, the supernatant was carefully removed without disturbing the isolated SSBs. After discarding the supernatant, the tube was removed from the magnetic holder, and 800 µl of Buffer AW1 (Qiagen 19081) was added to the SSBs. The solution was gently vortexed, and transferred by pipette to a 1.5 ml micro-centrifuge tube (for ease of processing). The DNA bound to the SSBs was purified by repeating the steps of SSB isolation within a magnetic field, discarding the supernatant, and removing the tube from the magnetic holder. This process was repeated twice with 500 µl of Buffer AW2 (Qiagen 19072). Once the final supernatant was discarded, the remaining supernatant was evaporated off by air-drying within a 70°C heat block for approximately 10 min to remove residual liquid.

In preparation for bisulfite conversion, 45 µl of water and 5 µl of M-Dilution Buffer (Zymo D5001-2) were added to the SSPs. The solution was incubated at 37°C for 15 min, then 100 µl of CT Conversion Reagent (Zymo D5001-1, prepared according to protocol instructions by adding 750 µl of water and 210 µl of M-Dilution Buffer) was added, and the solution

incubated in the dark for 12–14 hours. The sample was later cooled down in an ice water bath for 10 min. This was followed by adding 400 μ l of M-Binding Buffer (Zymo D5001-3) and incubating at room temperature for 10 min. The next step was to add 5 μ l of Carrier RNA (Qiagen 1017647) and wait for another 5 min at room temperature. After this step, the tube was placed on the magnetic holder and once the SSB were bound to the wall of the tube, the liquid phase was removed and discarded. The particles were then resuspended by adding 400 μ l of M-Wash Buffer (Zymo D5001-4). The tube was once again placed in the magnetic holder and the liquid phase removed. After this wash step, 200 μ l of M-Desulphonation Buffer (Zymo D5001-5) was added and the sample was incubated at room temperature for 13 min. At the end of this incubation period, an additional 5 μ l of Carrier RNA were added and the sample was incubated for an additional 3 min. At the end of this step, the tube was placed in the magnetic holder and the liquid phase again removed. Two subsequent wash steps were performed by adding the M-Wash Buffer, placing the tube in the magnetic holder to remove the liquid phase, and repeating. After the liquid was removed for the second time, the tubes were spun down in order to bring the SSB to the bottom as well as release some of the liquid out of them. The tubes were placed on the magnetic holder again to remove this excess liquid. The tubes were then transferred to a hot plate at 90°C for the ethanol from the wash buffer to evaporate. Once the SSB were dry, 62 μ l of M-Elution Buffer were added (D5001-6). The SSB were then incubated at 90°C for 10 min. The tube was placed on the magnetic holder and the liquid transferred to a new tube. Then an additional 50 μ l were added to the tube containing the SSB and it was incubated at 90°C for 10 min. The tube was placed on the magnetic holder and the liquid transferred to the same tube containing the 62 μ l transferred previously. Due to evaporation and the liquid absorbed by the SSB, the final volume yield is ~100 μ l.

2.4. Phenol Chloroform and Alcohol Extraction

500 μ l of methylated genomic DNA or processed plasma samples (note that for 2ml samples all volumes were scaled up by a factor of four) were transferred into microcentrifuge tubes containing 0.5 mL of DNA Extraction Buffer and 100 μ L of Proteinase K (Sigma Aldrich). The tubes were mixed and incubated at 55°C overnight. One 2 ml MaXtract gel tube (Qiagen) per sample was spun for 3 min at 15,000 rpm. To each gel tube, an equal volume of Phenol/Chloroform (pH 8.0) and digested sample was added. The gel tubes were then spun for 5 min at 15,000 rpm, separating the phases into the aqueous (above the gel matrix) and the organic (below the gel matrix). Using a pipette, the aqueous (top) layer of each sample was then transferred to a fresh microcentrifuge tube. For each sample, 650 μ l of 100% EtOH, 200 μ L of 7.5 M Ammonium Acetate (NH_4Ac), and 2 μ L of GlycoBlue was added to each microcentrifuge tube and vortexed. The tubes were then placed in a –20°C freezer overnight to precipitate for up to 3 days. The microcentrifuge tubes were next spun for 45 min at 15,000 rpm and the precipitate mixture decanted. The resulting pellets were washed with 1 mL of 70% EtOH and spun for 15 min at 15,000 rpm. The supernatant was then discarded, being careful not to dislodge the pellet. Each sample was then air dried in a chemical hood until all the EtOH was evaporated. Lastly, the pellets were resuspended in 100 μ L of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.5. Qiagen Commercial Kit Extraction

For the MOB comparison to commercial kits, the QIAmp Circulating Nucleic Acid kit (Qiagen) was used according to the manufacturer's instructions prior to bisulfite conversion.

2.6. Standard Bisulfite Conversion

DNA recovered by the PC or the Qiagen kit was subject to bisulfite conversion using EZ DNA Methylation™ Kit (Zymo) according to the manufacturer's instructions. The bisulfite conversion buffers used in the single-tube MOB process are the same as those used in the

Zymo EZ DNA Methylation Kit. In order to provide a consistent comparison, the DNA extracted using the Qiagen kit and the PC method were bisulfite converted by using the silica matrix spin-columns included in the Zymo kit according to the manufacturer's protocol. The final elution volume was adjusted to 100 μ l in all cases.

2.7. Methylation-Specific PCR and Cycle Threshold Calculation

2 μ l of bisulfite converted DNA target (or equivalent plasmid DNA) was added to 23 μ l of quantitative PCR reaction mixture. Final reaction conditions were as follows: (10x buffer), 300 nM sense primer, 300 nM anti-sense primer, 100 nM probe, 10 nM fluorescein reference dye (Life Technologies), 200 μ M dNTPs (Denville Scientific), and a single unit of Platinum Taq® DNA Polymerase (Life Technologies). Thermocycling was controlled as follows: 95°C for 5 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec within the MyIQ thermocycler (Bio-Rad Laboratories).

The Cycle Threshold (Ct) value is defined as the PCR cycle number that the fluorescence signal surpasses a threshold level. The threshold level was typically calculated by using the computer software provided with the qPCR thermocycler. However, when the computer algorithms were visibly unable to determine the accurate Ct Value, manual and comprehensive alterations were made for the selection of the background fluorescence and threshold level for the comparative samples, as permitted within the software provided by Bio-Rad Laboratories.

2.8. Determination of “Positive” Clinical Samples

Three individual qPCR reactions were performed for each plasma sample. One of the 25 patient plasma samples contained insufficient volume for triplicate measurement, leaving a total of 24 samples for RASSF1A analysis. Due to the relative scarcity of methylated DNA, the patient was considered to be positive for the Ras association domain family 1 isoform A (RASSF1A) gene if at least two of the three qPCR reactions demonstrated DNA amplification with the methylation specific primer set for the RASSF1A gene.

3. Results

3.1. Methylation on Beads Extraction and Processing of Genomic DNA

We first tested the ability of the MOB process to extract and process genomic DNA dissolved in water as an idealized model system. As DNA concentrations in the plasma and serum of humans typically range from 1 to 1000 ng/ml, depending on the individual and burden of disease [3], we demonstrated the linearity of DNA recovery for the streamlined MOB process using DNA concentrations within this range, as shown in Figure 2. Here, fully methylated genomic DNA was diluted in 2 ml of water to final concentrations of 1, 10, 100, and 1000 ng/ml. The samples were then subject to the entire MOB process, and qPCR was performed for the β -actin gene as a means of determining the amount of DNA recovered [16]. In qPCR, the cycle threshold (Ct) value is the fractional cycle number at which the number of amplified copies reaches a fixed threshold. The Ct value is thus typically used as a means to assess the quantity, either absolute or relative, of DNA present in a sample. From the dilution series, we used the Ct values of each dilution in order to demonstrate that the MOB process provides a linear rate of DNA recovery ($R^2 > 0.99$) and should, in the absence of interfering substances, ostensibly allow for quantification of DNA concentration within this range.

3.2. Quantification of Methylated Genes Using MOB

In order to assess the ability of the MOB process to be used for direct quantification of circulating DNA on concentrations as low as 10 copies per reaction volume or fewer, we

proceeded to compare the recovery rate and qPCR quantification of genomic DNA against an in-house plasmid standard control equivalent to a set number of bisulfite converted adenomatous polyposis coli (APC) gene copies. Thus by comparing the Ct values for the APC gene from MOB processed *genomic* DNA with standards from known copy numbers of the plasmid DNA, we can compare the [approximate] copy numbers of the input genomic DNA with values back calculated from the plasmid standards. Here we assume 6 pg of diploid genomic DNA per full genome copy. The data is plotted in Figure 3. Overall, the results between the plasmid APC standard and MOB-processed DNA display a high level of correlation, particularly at lower copy numbers (the region of interest). With a genomic DNA input concentration of 2 ng/ml (corresponding to approximately six gene copies per qPCR reaction), the MOB process yields approximately a 90% recovery in comparison to the expected Ct values based on the plasmid DNA dilution series.

3.3. Recovery of DNA via MOB vs. Other Standard DNA Processing Techniques

We sought to compare the recovery rate of the improved MOB technique to traditional laboratory techniques (phenol chloroform extraction) and a commercially available kit (Qiagen QIAmp Circulating Nucleic Acid kit). Each extraction and processing technique was tested using methylated genomic DNA diluted into 2 ml of water with final concentrations ranging from 1 ng/ml to 1 µg/ml, corresponding to a total DNA input range from 2 ng to 2 µg. Following bisulfite conversion, the recovered DNA was then quantified using qPCR for β-Actin and the Ct values for each technique compared. Figure 4 shows the percent recovery using various standard extraction techniques, as compared to the MOB technique. As can be seen from the graph, the MOB technique showed superior recovery at all concentrations tested particularly at the low input levels (2 ng).

3.4. MOB vs. Traditional Phenol Chloroform Extraction in Human Plasma Samples

We next compared the performance of the improved MOB technique to traditional PC in the processing of clinical samples. A library of plasma samples from 24 patients diagnosed with Stage IV lung cancer was used for assessment. Using identical starting material, circulating DNA was extracted from 2 ml plasma samples using the improved MOB technique and compared with DNA previously extracted using standard PC from corresponding 500 µl plasma samples. After processing, the samples were quantified, as previously, using qPCR for β-Actin. Figure 5 shows the results of the two methods in terms of total DNA recovery. The MOB technique shows far lower Ct values (more DNA recovery) than the phenol chloroform method. The difference in average Ct value of 6.8 accounts for a recovery rate of $\sim 2^{6.8} = 111$ -fold more analytic sensitivity (Paired two-tail t-test, $p = 1.4 \times 10^{-5}$) when using the 2 ml MOB process as compared to the traditional PC technique. Furthermore, the precision of the MOB technique far outperformed phenol chloroform extraction, yielding an average Ct standard deviation of 0.3 cycles, as compared with 1.9 cycles for phenol chloroform.

Lastly, in order to demonstrate the potential for clinical significance of the improved MOB technique, we performed qMSP on the processed DNA from 24 plasma samples for the Ras association domain family 1 isoform A (RASSF1A), a normally unmethylated tumor suppressor gene whose methylation is known to be associated with lung and various other cancers [17, 18]. The results of MSP for the RASSF1A gene are shown in Table 1. The samples processed using traditional phenol chloroform extraction and bisulfite conversion methods showed RASSF1A methylation in only 3 of 24 (12.5%) samples, while those samples processed with the MOB technique demonstrated a methylation rate of 42% (10 of 24), a rate that falls within the upper end of the range reported for tissue in lung cancer patients [19]. Improvements such as this may significantly improve both cancer screening, as well as provide increased sensitivity for the monitoring of epigenetic therapies [8].

4. Discussion

The relative scarcity of tumor-specific methylated circulating DNA in the bloodstream exerts heavy demands on techniques for the extraction and processing of this DNA for detection and quantification. Likewise, there is a consistent need for new and improved methods that will allow for detection of rare epigenetic events. Here, we sought to introduce and characterize an improved MOB technique for the extraction and processing of methylated DNA. We also compared the MOB technique to other standard methods of DNA processing in terms of extraction efficiency and clinical sensitivity. Overall, the improved MOB technique performed superiorly, compared to both traditional phenol chloroform and alcohol methods, as well as a commonly employed commercial extraction kit.

The streamlined Methylation-on-Beads (MOB) process utilizes silica superparamagnetic beads (SSBs) as the DNA carrier to integrate DNA extraction and bisulfite conversion into a single platform. SSBs are micro/nanoparticles that are frequently used for solid phase nucleic acid extraction, and commercially available SSB vary in size from 5 nm to 400 μm [20–22]. The silica surface provides a solid substrate for nucleic acid adsorption. The superparamagnetic property allows SSB to be easily manipulated remotely with an external magnetic field, thereby greatly simplifying sample processing.

The general principle of the updated MOB process is illustrated in Figure 1. In short, the process allows for the ultra-high efficiency extraction of circulating DNA from up to 2 ml of serum or plasma, followed by bisulfite conversion of the cell-free DNA and 20-fold (or more) concentration of the sample to reach a final volume of 100 μl (or less). The step-by-step procedure follows the simple process of adding the consecutive reagent(s), placing the tube in a magnetic holder to isolate the magnetic particles, removing the supernatant, and repeating the process. This facile method allows for easy implementation within the laboratory setting, and personnel do not need to be extensively trained in order to perform it. In addition, the process utilizes commercially available buffers and reagents in order to increase reproducibility and uniformity across samples and between laboratories.

The entire MOB process takes approximately 16 hours to complete, only 4 of which require hands-on benchwork and the remaining 12 are for sample incubation. This processing time is significantly shorter than the widely used PE process that includes lengthy precipitation waiting times and requires at least two days to complete. Furthermore, we have internally verified that the entire MOB process can be reliably performed in as few as five hours through incorporation of rapid bisulfite conversion reagents such as those found in the EZ-DNA Methylation-Lightning Kit (Zymo Research; data not shown). This will enable the user to complete the entire sample to analysis process within a single working day. In terms of cost, if the reagents are purchased at medium volume, the price per extraction by the MOB process is approximately ten dollars. The main expense is Proteinase K, which represents 80% of the cost and would ostensibly be utilized by any DNA extraction method. The overall cost of the MOB process is thus comparable between all the presented methods, including phenol chloroform, which requires a similar amount of Proteinase K for the initial digestion and approximately one to three dollars in additional reagents. A phase lock gel tube for extraction such as the Qiagen Maxtract costs approximately 70 cents per unit and is commonly used since it simplifies the process and minimizes contamination, but additionally requires the use of a centrifuge.

Overall, the MOB technique shows a drastically improved recovery rate as compared to traditional PC methods. Even if one accounts for the four-fold increase in starting material (2 ml vs. 500 μl), there still remains an over 25-fold increase in signal from the recovered circulating DNA. This can likely be accounted for by at least two advantages of the MOB

technique: (1) improved recovery as shown in Figure 4 and, notably, (2) significantly reduced carryover of PCR inhibitors. The improved DNA recovery of the MOB technique is likely in part due to incorporation of carrier RNA in several key processing steps. The rationale for this inclusion is that while silica exhibits a relatively high affinity for DNA, recovery can sometimes prove problematic in low DNA solutions such as found circulating within the bloodstream. The use of carrier RNA helps facilitate the precipitation of DNA so that it can be more readily captured onto the silica surface of the SSB, resulting in significantly higher yields [23].

A particular advantage of SSB-based DNA processing is the ability to extract and concentrate the DNA with little carryover of PCR inhibitors [24]. Silica-coated beads have a specifically high affinity for the adsorption of nucleic acids, thereby providing the ability to readily aspirate away contaminants and inhibitors, particularly when used in a single concentrating step. Thus, by lowering the concentration of PCR inhibition, more DNA can be incorporated into each reaction volume thereby proportionately increasing the detection sensitivity. This is particularly relevant in the case of circulating DNA, as plasma and serum samples are known to contain high levels of numerous PCR inhibitors, thus resulting in false negatives and reduced PCR efficiency [14]. Alternative solutions for DNA concentration have included dehydration using a vacuum manifold, heating, or a combination of both. While these methods do increase the concentration of DNA, they concomitantly increase the concentration of contaminants and PCR inhibitors, a problem that is exacerbated in concentrating larger sample volumes required to detect rare events. This leads to a lower PCR efficiency and higher CT values for a given amount of DNA, which may be misinterpreted as a lower DNA quantity. We independently confirmed this by performing an internal comparison between the concentrated output of ten MOB-processed 200 μ l samples and one MOB-processed 2 ml sample following the protocol presented in this manuscript. Our results indicated that the latter showed both significantly better PCR efficiency and consistency across all samples tested (data not shown).

Comparison of the RASSF1A qMSP results between the traditionally-processed and MOB-processed NSCLC samples shows a significantly higher positive rate using the MOB-process. While the MOB-processed RASSF1A positivity rate does indeed fall within upper end of the traditionally reported range for NSCLC tissue samples, implying improved clinical sensitivity [25], the positive predictive value (PPV) could not be confirmed in these studies due to a lack of matching tissue samples to act as a gold standard. Furthermore, the results shown in Table 1 indicate that two of the three samples that were positive for RASSF1A methylation using the traditional processing techniques were not positive when processed via MOB. Thus, while this study does demonstrate improved clinical sensitivity, further studies, particularly using matching tissue samples, will be required to verify the utility of the MOB process for improved PPV. Likewise, while the genes tested in this study, RASSF1A and APC, are rarely methylated in healthy individuals [18, 26], future studies that include healthy samples will be required in order to investigate the effect of the MOB process on negative predictive value (NPV).

Sometimes taken for granted, improved sample processing techniques can dramatically improve assay sensitivity, both analytical, as demonstrated in Figure 4, and clinical, as demonstrated in Table 1. These improvements may appreciably impact clinical care through reliable detection of epigenetic events, such as methylation, at earlier stages, thus allowing for prophylactic measures to be undertaken in order to avoid cancer initiation and/or progression.

5. Conclusions

Methylation-on-Beads represents a simple, but efficacious method for the extraction and processing of circulating DNA in preparation for methylation specific PCR. Its numerous advantages include: simplicity, use of commercial off-the-shelf reagents, high DNA retention and little carryover of PCR inhibitors resulting in significantly improved sensitivity for the detection of rare epigenetic events.

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Highlights

- Methylation on Beads is a high yield DNA extraction and processing technique
- The MOB process allows for processing of methylated DNA from samples up to 2 ml
- MOB results in up to a five-fold increase in DNA yield compared to other techniques
- MOB results in lower PCR inhibition, increasing analytical sensitivity by 25-fold
- MOB can provide improvement to the clinical sensitivity of methylation-based assay

Figure 1.

Overview of the Methylation-on-Beads (MOB) Process. Circulating DNA from up to 2 ml of plasma is extracted and purified via SSBs. The purified DNA is then subject to bisulfite conversion and analyzed via methylation specific PCR (MSP). The entire sample preparation process can be performed in a single tube and consists of an iterative process of adding reagents, magnetic decantation, and removal of supernatant.

Figure 2. β-Actin Cycle Threshold (Ct) values of MOB processed DNA versus initial DNA concentration. The Ct value shows an inverse correlation with respect to starting DNA concentrations, thus demonstrating the linearity of the MOB process, from sample preparation to methylation specific PCR, of over 4 orders of magnitude.

Figure 3.

APC Gene Ct Values vs. Gene Copy Number for MOB-processed DNA. Ct values of MOB-processed genomic DNA were compared with known copy numbers of APC plasmid DNA. The Ct values show excellent rate of recovery and correlation with respect to the plasmid DNA standard.

Figure 4. Normalized DNA recovery, as quantified by β -Actin qPCR, of MOB compared with traditional phenol chloroform and alcohol (PC) extraction and a Qiagen Extraction Kit. The MOB technique exhibits superior recovery rates at all DNA concentrations tested.

Figure 5.

β -Actin Ct values for MOB processed vs. Phenol Chloroform extracted and traditionally processed plasma samples from 24 patients diagnosed with metastatic non small cell lung cancer. The MOB technique demonstrates consistently higher and less variable recovery, as demonstrated by the lower average Ct value (33.8 vs. 40.6 cycles) and Ct standard deviation (0.3 vs. 1.9 cycles), respectively.

Table 1

Detection of methylation of the RASSF1A tumor suppressor gene in circulating DNA; MOB vs. Traditional Phenol Chloroform and Alcohol Extracted and processed plasma samples from 24 patients diagnosed with metastatic non small cell lung cancer. The MOB technique identified 7 more methylation positive samples corresponding to the potential for over 3-fold higher clinical sensitivity than traditional phenol chloroform methods.

Sample #	Positive by Phenol Chloroform	Positive by MOB Technique
8	N	N
11	Y	N
12	N	Y
13	N	Y
16	N	Y
19	N	Y
20	N	N
21	N	Y
22	N	N
26	N	N
28	N	N
29	N	N
32	N	Y
34	N	N
36	N	N
37	Y	Y
38	Y	N
39	N	Y
40	N	N
41	N	N
42	N	Y
43	N	Y
44	N	N
45	N	N
Total Positives	3/24 (13%)	10/24 (42%)