Increasing the selectivity of hypermethylated DNA enrichment by a two-step washing process

Bachelor thesis

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ABSTRACT

In the liquid biopsy urine the cancer biomarker hypermethylated (hmDNA) can be detected. Hypermethylated DNA (hmDNA) involves DNA methylation on certain parts of the genome. The methylation of specific genes can be associated with for example lung and cervical cancer. To determine methylation on a specific sequence, the hmDNA needs to be separated from the non-methylated DNA. Separation is done using the hmDNA enrichment method. This method involves flushing a DNA solution over a surface modified with methyl binding domain 2 (MBD2) proteins. These MBD2 proteins have affinity for the hmDNA, resulting into surface binding of hmDNA. After the enrichment method, the surface bound DNA is eluted by means of a solution of NH_4OH. The achieved DNA concentration is provided by AccuBlue. Using methyl sensitive restriction enzyme digest and quantitative polymerase chain reaction, the ratio of hmDNA/non-methylated DNA was determined after hmDNA enrichment. Currently, a ratio of 10:3 unmethylated DNA/hmDNA is achieved starting with a DNA solution containing 1% hmDNA. The aim of the research is to increase the selectivity of the hmDNA enrichment method further by applying a second washing step with a higher salt concentration. Compared to Ruben Kolkman's research, the achieved amount DNA was 10 times lower than planned, so it was necessary to investigate why. It was eventually found that the hmDNA was not properly methylated and therefore could not bind to the MBD2 proteins. It's important for the next time to use fresh reagents for the hmDNA enrichment experiment and check if the hmDNA is well methylated. Learned from these experiments is what chemistry toke place to enable the hmDNA enrichment experiment and how to determine the concentration from the captured DNA with AccuBlue. Future work should focus on whether the hmDNA enrichment selectivity could be increased by applying a second washing step with a higher salt concentration.

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1. Introduction

The number of cancer deaths in 2020 equals 10 million and is therefore one of the leading causes of mortality.[1] The mortality could be reduced if cancer is detected in an earlier stage. Early detection could prevent metastasis and thus makes treatment more feasible and thereby increase the survival rate. [2] There are already existing techniques for early stage cancer detection , for example, one could consider the detecting technique which is used for screening of HPV virus. HPV is the abbreviation for human papillomavirus, the virus which can cause cervical cancer. [3] The screening technique consists of a smear where a speculum is inserted into the vagina and cells are removed from the uterine wall with a scraper. These cells are examined for the presence of the HPV virus. Research has shown that 37% of women do not respond or do not want to participate in a screening study. This is precisely the most important group because it appears that 50% of cases of cervical cancer patients come from this group.[4] Taking a smear is experienced as very unpleasant, which makes women less likely to test than if this were not invasive.[5,6]

Liquid biopsy-based cancer diagnostics could be a good substitute for already existing cancer diagnostics[7–9]. A liquid biopsy sample is for example blood, urine or saliva and contains cell-free DNA (cfDNA).[7] The cfDNA is emitted by healthy cells and tumor cells and is released in the circulatory system by apoptosis, necrosis and secretion of the cells. CfDNA from tumor cells contains among others epigenetic information that can be used to detect cancer[10]. CfDNA derived from tumor cells has the great advantage of early detection of cancer biomarkers and good monitoring , because these genetic changes are already detectable in small numbers in the liquid biopsy. [7,8,10]

A biomarker for cancer is hypermethylated DNA (hmDNA).[8,11–13] Hypermethylated DNA (hmDNA) is a cancer biomarker which encompass DNA methylation at certain parts of the genome.[11,14,15] DNA methylation involves the binding of a methyl group to the cytosine base followed by a guanine base, the so called CpG complex. [11,12,14,15]The methylation of DNA is an epigenetic change, thus affecting the expression of genes without changing the DNA sequence. DNA methylation is a process that takes place in the body for gene regulation, but the methylation of tumor suppressor genes can attribute to tumor growth. [11,12,14,16,17]. hmDNA can be measured in urine. Obtaining urine is non-invasive, which is a major advantage. Urine is available in large quantities, which makes it also possible to monitor cancer disease progress in the future. The methylation of specific genes can be associated with certain types of cancer, for example, when there is methylation in the RARβ2 gene, this can be linked with colon, lung, lymphoma and breast cancer. Each type of cancer has its own methylation pattern, each a different sequence. [9,10,17,18] The detection of specific hmDNA sequences is used for cancer diagnostics by examining whether the DNA is methylated on a particular sequence.

DNA methylation is a process that takes place in the body and is present in 0.5% of the genome.[10,18,19] To finally determine whether a specific sequence is methylated, the hmDNA must be separated from the non-methylated DNA.[10,17,18,20] The method that can be used for this separation is the hmDNA enrichment method developed by Kolkman *et al*[10]. Upon hmDNA enrichment a DNA solution is flushed over a surface modified with methyl binding domain 2 (MBD2) proteins.[10,14,20,21] The MBD2 protein has 10-100 times more affinity for methylated CpG compared to a non-methylated CpG.[10,14] By flushing the DNA solution over the surface the methylated DNA binds to the MBD2-modified surface, after which the unmethylated DNA partially is flushed away. Because some of the non-methylated DNA remains on the surface, there is a washing step applied which causes removal of a part of surface bound non-methylated.[10]

The MBD2-coated surface is created by using a gold plate on which a thiol-based self-assembled monolayer (SAMs) is formed. The SAM will consist of an azide and hydroxy-functionalized thiol with a backbone of $(CH_2)_{11}(EG)_6$ for the azide-terminated group and $(CH_2)_{11}(EG)_5$ for the hydroxyl-terminated group.[14] The hydroxyl functionalized thiols gives anti-fouling properties. The azide-functionalized thiols makes immobilization of the MBD2 proteins possible.[14,22–25] The linker molecule is required for the immobilization of the MBD2 protein to the SAM on the gold layer. The linker molecule consists of EG_4 and has as its functional groups nitrilotriacetic acid (NTA) and the dibenzocyclooctyne (DBCO). DBCO can react with the azide groups and forms a bond between the SAM and the linker molecule. The NTA is flooded with nickel chloride, after which the NiNTA complex can bind to histidine 10-tag of MBD2. In this way, the MBD2 protein is bound to the gold surface as schematically illustrated in Figure 1.[10,14]

The ratio of azide to hydroxyl functionalized thiols is important because it has been shown in the research of Ruben Kolkman, that when the surface area has a ratio azide/hydroxyl of 15%/85% the highest hmDNA enrichment selectivity is achieved. [14]

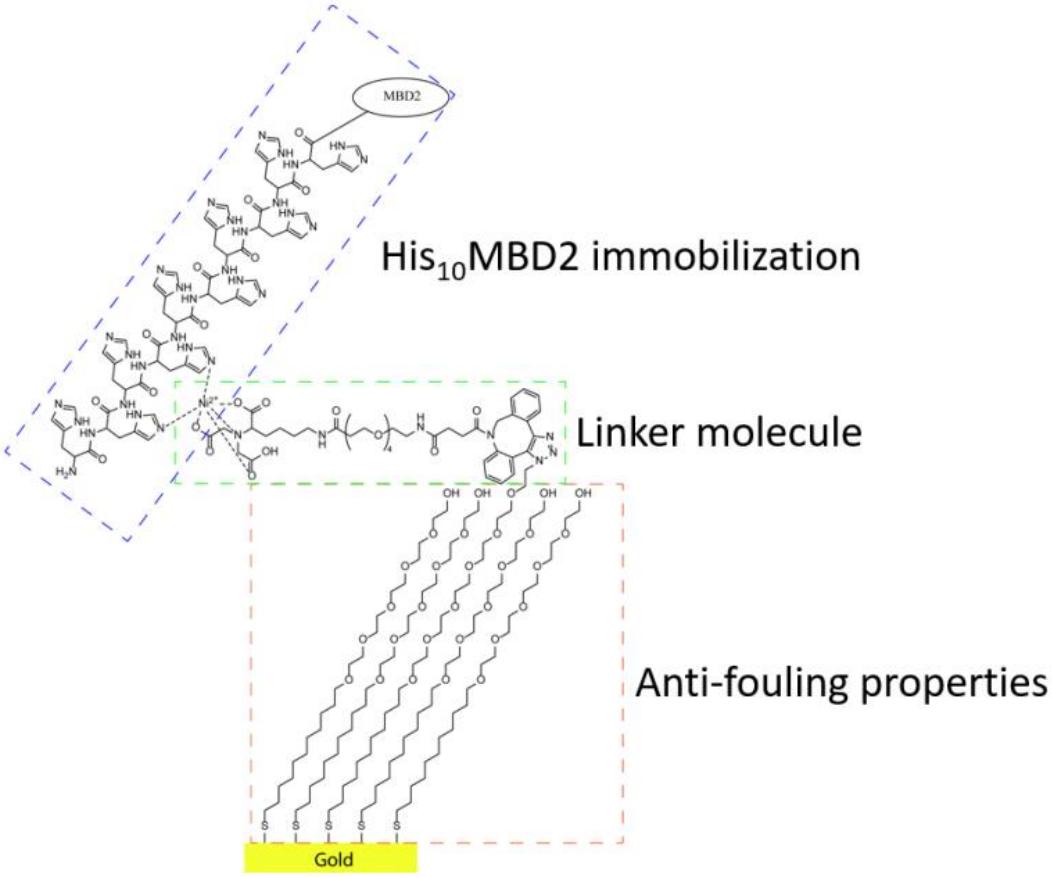


Figure 1. Final c*omposition after the surface chemistry. The SAM formation on the gold and Linker molecule to immobilize His10-tag MBD2 proteins.*

To determine the selectivity of the hmDNA enrichment method a model DNA sample was used to simulate urine with a percentage of 1% hmDNA by Kolkman *et al* [10]. The enriched DNA sample was subsequently characterized to determine the hmDNA level. First, the DNA sample was treated with methyl sensitive restriction enzymes. As a consequence, the non-methylated DNA in the enriched sample was digested. Subsequently, quantitative polymerase chain reaction (qPCR) was used to

determine the content of hmDNA in the sample.[10,26] After the hmDNA enrichment method, the hmDNA level was increased with approximately 30-fold. Controlling the density of the MBD2 proteins has a crucial role here.[10]

The aim of this research is to increase the selectivity of the hmDNA enrichment method by applying a second washing step with a higher salt concentration after the first elution step, making it a two-step washing process. Increasing salt concentrations (NaCl) ensure likely that less electrostatic interactions between the MBD2 protein and hmDNA/non-methylated DNA can be formed.[27,28] It is expected that this will cause more non-methylated DNA to come off from the MBD2-modified surface as the binding affinity is lower compared to hmDNA. The salt concentration is going to be varied between 0.2-2M.[29,30]

The research question of the thesis;

- By performing a washing step with higher salt concentrations, can the selectivity of the hmDNA enrichment method be increased?

To test this, the report is divided into specific objective parts;

- What surface chemistry takes place in the flow cell to enable hmDNA enrichment?
- How can AccuBlue be used to determine the DNA concentration after the enrichment method?
- How can the ratio of hmDNA/non-methylated DNA be determined by qPCR?
- At which salt concentration can the highest hmDNA enrichment selectivity be achieved?

2. Theory

2.1. The self-assembled monolayer

The SAM serves as a foundation for the MBD2 proteins by forming an interaction between alkanethiols and gold. A gold surface is known for how inert it is, which is important for not getting any other substances which can disturb the interaction between the SAM and the gold.[22,24] Self-assembly means a spontaneous formation of the thiols on the gold surface. This is done on the basis of immersing a gold surface in a thiol solution where the thiol heads want to reach a favorable kinetic state and in this way bind to the gold surface. In this way a thiol-gold interaction can be formed which forms the SAM.[22,23]

The SAM consists of three groups, the functional group, the molecular backbone and an end group. The head group provides the bond between the gold and the thiol group (chemosorption) through the formation of a covalent bond. The backbone consists of $(CH_2)_{11}(EG)_6$ for the azide-terminated group and $(CH_2)_{11}(PEG)_5$ for the hydroxyl-terminated group. The van der Waals and hydrophobic forces between backbone provides the stability of this structure as they interact between the hydrocarbons.[22,24] OH, ethylene glycol (EG) and carbon chain provide stability and has antifouling properties. Antifouling is used to prevent attachment of accumulating biomolecules. [25] The functional groups makes immobilization possible through azide and gives hydrophilic properties through the hydroxyl group. The azide hydroxyl ratio provides the density of the proteins on the surface.[10] The reaction for the formation of the SAM is shown in Figure 2A. The used composition of the alkanethiols with azide- and hydroxy-terminated thiols are $HS(CH_2)_{11}(EG)_6NS$ and $HS(CH_2)_{11}(PEG)_5$ OH.

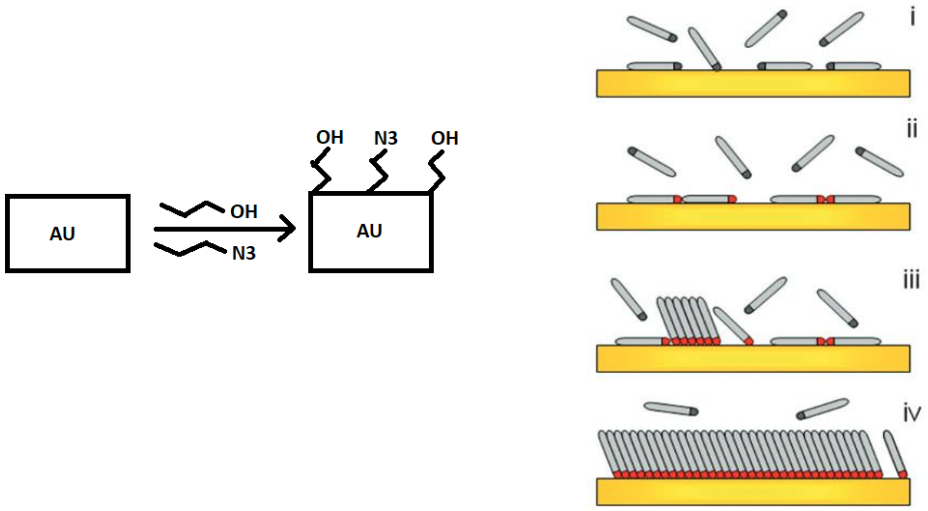
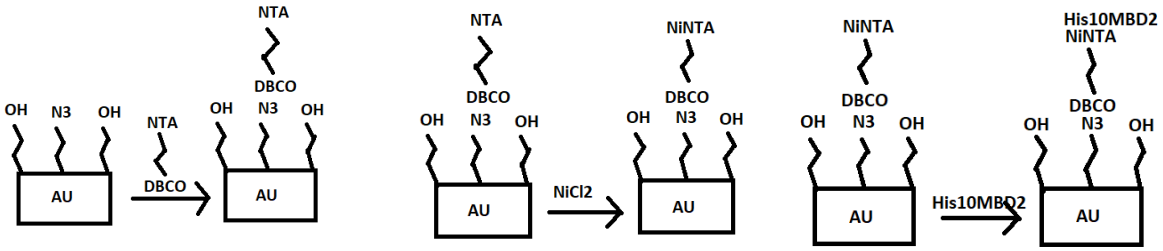


Figure 2. *A) visualization SAM formation using the chemical structures $HS(CH_2)_{11}(EG)_6NS$ and $HS(CH_2)_{11}(PEG)_5OH$ B) process during SAM formation with i physisorption ii laying down parallel iii standing up fase iV SAM formation complete.* [22]

The formation of the thiol-based SAM shown in Figure 2B occurs through a process that begins with physisorption, this process occurs through van der Waals interactions. Then the thiol molecules lie parallel on the gold surface which is followed by chemosorption. Chemisorption makes the sulfur headgroup being adsorbed by the gold. Finally, a complete packed structure is formed where they increasingly cover the surface of the gold with thiol bonds. Due to the interactions between the backbone chains, this molecule series becomes increasingly stable and a SAM is created. [22,24]

2.2. Linker Molecule and His10-tag MBD2

The linker molecule is required for the immobilization of the MBD2 protein. The linker molecule consists of EG_4 which has as its functional group nitrilotriacetic acid (NTA) and the group dibenzocyclooctyne (DBCO).[14] The DBCO group of the linker molecule interacts with the azide group located on the SAM. This binding occurs through the Strain Promoted Alkyne-Azide Cycloaddition (SPAAC) reaction (Figure 3A). This reaction provides stable products, is fast, and is selective. [31] The NTA is flooded with nickel chloride ($NiCl_2$) solution (Figure 3B). As a result, nickel ions are complexed by NTA, thus forming NiNTA. The NiNTA interacts with the His-10 tag attached to the MDB2 protein (Figure 3C). This allows the MDB2 protein to immobilize.[10,14,32]



A) ***B)*** ***C)***

Figure 3. *A) linker molecule NTA-DBCO flushed over the SAM forming an reaction between N_3 and DBCO B) $NiCl_2$ is flooded over NTA to form NiNTA C) immobilization between the MBD2 proteins and His10-Tag takes place.*

2.3. Characterization techniques

A model DNA solution which is flushed through a Teflon flow-cell consist of 90 bp long DNA in which 1% is methylated (1:100).[10,18,19] The model sequence to be used is that of the MAL gene which consist of 3 CpGs, MAL3CpG. The MAL sequence is hypermethylated in cancer cells.[10,14] The Teflon cell consists of two layers, a glass plate and the gold plate where the surface chemistry takes place. After the DNA mixture is flushed through the Teflon cell (Figure 4A), the surface-bound DNA can then be removed by an elution step. This can be done using ammonium hydroxide (NH_4OH) with a pH of 11.3 (Figure 4B). The pH is so high that the MBD2 proteins release the bound DNA and this ends up in the NH_4OH solution.[10] This ends up in an amount of 1200 µl. The enriched DNA solution must be concentrated because the quantity of the DNA is too low for further characterization. Care is taken to reduce this 1200 µl solution to 30 µl solution using NucleoSpin Gel and PCR clean-up column. Here, the 1200 µl solution is passed through a column with different buffers in order to concentrate the DNA solution to 30 µl. Next, the DNA concentration is determined by AccuBlue. AccuBlue is fluorescent upon binding to double-stranded DNA and can therefore be used to determine the total DNA concentration. If different DNA concentrations were used during qPCR it affects the Ct value, which is used to determine the ratio of unmethylated to methylated as was shown by Kolkman *et al*.[10]

To find out what the hmDNA level is in a hmDNA enriched DNA sample, the DNA must be cut with methyl-sensitive restriction enzymes (Figure 4C). The function of these enzymes is to cleave DNA at specific DNA sequences, in this case non-methylated MAL3CpG. The enzymes used are HhaI and HpaII, and cut at 5'-GCGC-3' and 5'-CCGG-3'. They become active if there is an absence of methylation at MAL3CpG.[10,14,33] When unmethylated DNA is present, the enzymes cuts the piece of DNA to small fragments. [10,14] When the HpaI and HpaII enzymes have cut, the primer cannot bind for amplification with qPCR, therefore, preventing amplification. The more non-methylated-DNA is present after the enrichment method, the more digestion takes places. As a consequence, a higher C_t value in qPCR will be achieved.

Copying of a sequence occurs in repeating cycles with qPCR. During each cycle, a piece of sequence is duplicated by means of the primer. This is repeated so many times in the PCR apparatus until the C_t is reached. The C_t value is the amount of cycles required to make the sample detectable. When the CpG is methylated, it can be seen that the cycle of the qPCR rises earlier than when it is not methylated. The C_t threshold is thus reached earlier for hmDNA. By measuring different mixtures of hmDNA/non-methylated DNA in qPCR, the the C_t of DNA ratios can be determined. This serves as a reference to measure the ratio of hmDNA/ non-methylated DNA of the enriched DNA mixtures (Figure 4D).[10,14]

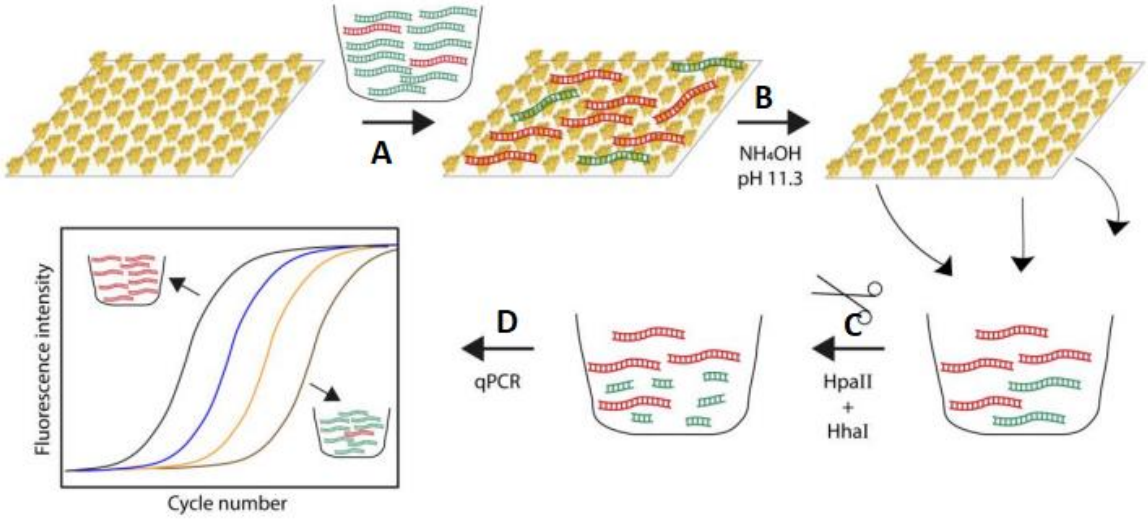


Figure 4. *Schematic overview process with A) DNA binding to MBD2-modified surface B) elution with NH_4OH to concentrate DNA solution C) methyl-sensitive restriction enzymes HpaI and HpaII cut the non-methylated DNA D) ratio of hmDNA/non-methylated DNA determination with the use of qPCR.* [14]

The MAL3CpG DNA is methylated by the CpG Methyltransferase M.SssI, which methylates all cytosine with 5'CG 3' as the recognition point. It can be checked that the reaction for the methylation of the 90 bp long MAL3CpG went well by Gel Electrophoresis. Gel Electrophoresis is a separation technique in DNA of different sizes can be separated. The negatively charged DNA passes through an electric field of the Electrophoresis, which consists of a positive pole and a negative pole. The negative DNA is guided through the gel to the positive pole. The DNA is stained with a fluorescence dye which bind with the DNA. The detector sense the fluorescence intensity of the DNA. Small DNA molecules move through the gel faster than larger DNA molecules and thus are registered earlier by the detector. Over time, this is visible in a band pattern on the gel. By usage of a ladder containing DNA with known fragment sizes through Gel Electrophoresis, it can be compared to the sample. By comparing the bands, the length of the analyzed DNA samples can be determined.

3. Experimental procedures

3.1. Materials

Teflon flow cell Gold coated chips, His10MBD2, IB (50 mM Tris HCL, 300 mM NaCl, 0.1% mercaptoethanol) , BB (50 mM Tris HCL, 350 mM NaCl, 0.1% Trition X-100), BB+(350 mM NaCl + extra salt used to achieve 50 mM Tris HCL, 350 mM NaCl, 0.1% tritionX-100), milliQ, DBCO-PEG_4-NHS, all solution are filtered.

Elution of DNA bound to MBD2-coated surfaces Ammonium hydroxide, Nucleospin gel and PCR clean-up kit.

DNA concentration determination Ammonium hydroxide, 96 well Microplate, AccuBlue Broad Range dsDNA Quantitation Kit, SpectraMax® iD3 Molecular Device.

DNA hybridization Mal FWD 90 bp 3 Me, Mal Rev 90 bp 3 Me, T100 thermocycler (BioRad), milliQ.

DNA methylation Mal3CpG DNA, rCutsmart bufferTM, M.SssI enzyme, S-adenosylmethionine, T100 thermocycler , nuclease free water.

Methyl-sensitive restriction enzyme hmDNA, DNA, rCutSmart®, HpaI, HpaII, NFW, T100 thermocycler (BioRad).

Gel electrophoresis Experion DNA 1K Analysis Kit, stain, loading buffer, Ladder, gel, samples, Experion automated electrophoresis station (BioRad).

DNA Concentration determination NanoDrop NanoDrop 2000b, NanoDrop One, DNA sample.

3.2. Methods

Teflon flow cell

Gold coated chips immersed in thiol solutions after UV-ozone activation for SAM formation overnight. The gold surface is rinsed with milli-Q water and is dried by air. The chip is placed in the flow cell. The chamber of the Teflon flow cell is filled with water, then the linker molecules was flushed through the flow cell. In the meantime, the His10-MBD2 proteins should be centrifuged at 11 RCF for 30 minutes. The MBD2 proteins solution can then be made and consists of 48.8 µl of MBD2 proteins and 1076.2 µl of IB. The hmDNA concentration consists 0.277 ng/µl in a 1800 µl solution of BB+ and NFW. After surface chemistry and during flowing of the DNA solution, the Teflon flow cell looks like Figure 5. On the gold plate are the MBD2 proteins and at the top of the cell is a glass plate that can used to observe the presence of air bubbles. The flow cell protocol is performed with a flow rate of 30 µl/min in the manner of Table 1.

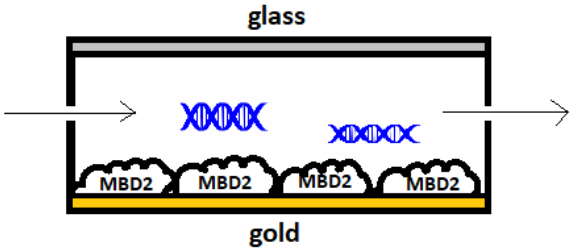


Figure 5. *Schematic illustration of the Teflon flow cell.*

Table 1. *Flow cell protocol*

Linker molecule	1,5 h
NiCl2	10 min
MilliQwater	5 min
MBD2 protein mix	17 min
IB	1.5 h
DNA solution	1h
BB	1h

Elution of DNA bound to MBD2-coated surfaces

The gold chip is removed from the Teflon flow cell and flooded with 1200 µl of ammonium hydroxide with pH 11.3. Due to the high pH, the hmDNA elutes from the MBD2 proteins and the hmDNA is collected in a 1200 µl ammonium hydroxide solution.

DNA concentration determination

After the elution step, the concentration of the eluted DNA was determined by AccuBlue Next generation. A 200 µl buffer mix consisting of dye, enhancer and buffer is pipetted into a 96 well Microplate F-Bottom. DNA sample can be added and then the AccuBlue signal is observed in the fluorescence microplate reader. This signal can be compared with the made calibration line, and in this way the concentration can be determined.

DNA hybridization

Single strand DNA is hybridized in which MAL FWD 90 bp 3 Me is mixed with MAL Rev 90 bp 3 Me. First, a concentration of 100 mM FDW/REV was used. 20 µl of both these solutions is added in a vial supplemented with 4 µl of water. After this the vial is placed into the T100 thermocycler (BioRad). Temperature steps used; 95 °C 2 minutes, 45 °C 48 minutes and then kept instant at 4 °C for unlimited time. The final concentration of the hybridized DNA is 45 mM, 160 ng/ µl. After this the samples are stored in the freezer.

DNA methylation

The double-stranded DNA is used for methylation. This involves mixing 291.2 ng DNA with 2.55 µl of rCutsmart bufferTM, 2.30 µl of M.SssI enzyme, 0.54 µl of S-adenosylmethionine and 21.21 µl of NTW, end concentration 600 µM. The reaction was performed with the settings of the T100 thermocycler (BioRad), 37 °C for 15 h, 65 °C for 20 min and then it is kept at 4 °C instantaneously. After this, the samples are stored in the freezer.

Methyl-sensitive restriction enzyme digest

hmDNA and non-methylated DNA are processed with Methyl-sensitive restriction enzymes HpaI and HpaII. First, both the hmDNA and non-methylated DNA solutions are diluted towards a concentration of 3.0 ng/µl, an amount of 160 ng is in both solutions. From these solutions, 3.125 µl of both are mixed with 4.875 µl of water. Thereby, 4.8 µl of rCutSmart® , 2.4 µl of HpaI and 2.4 µl of Hpa II are added in both. The reaction was performed with the settings of the T100 thermocycler (BioRad), 37 °C for 15 h, 65 °C for 20 min and then it is kept at 4 °C.

Gel Electrophoresis

The Experion DNA 1K Analysis Kit (BioRad) was used. Equilibrate Kit Reagents (stain, loading buffer, Ladder and gel) were used for the Gel Electrophoresis. Experiment was performed according to the description of the manufacturer.

DNA concentration determination

The NandoDrop 2000b can be used to measure the concentration of DNA by absorbing light with a wavelength of 260 nm. There is a relationship between the concentration and absorbance of a solution according to Beer-Lambert's law. The light shines through the sample, and the amount of absorbed light gives the concentration. A 1 µl MilliQ is used as a blank, after which 1 µl of the DNA sample is analyzed. The NanoDrop One and the NanoDrop 2000b are both measured at a pathlength of 1 mm.

4. Results and discussion

The hmDNA enrichment experiment is performed by means of a Teflon flow cell in the sequence shown in Figure 6. First, the SAM was formed overnight (Figure 6A). This SAM consists of azide and hydroxyl-functionalized thiols. The ratio between the azide and hydroxyl is 3:17 for optimal hmDNA enrichment selectivity. For the immobilization of the MBD2 proteins, the linker molecule DBCO-NTA was flushed through the Teflon cell (Figure 6B). By flushing with $NiCl_2$, NiNTA is formed and to this the His10-tag MBD2 proteins was bound (Figure 6C). After this, the model DNA solution methylated MAL3CpG (Figure 6D) passes through the Teflon cell which consists of only pure hmDNA. NH_4OH (Figure 6E) is used to elute the DNA from the surface followed by determination of the DNA concentration in the eluted DNA sample (Figure 6F). The collected DNA concentration is determined with AccuBlue (Figure 6G).

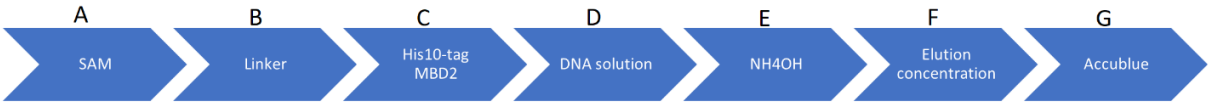


Figure 6. *Schematic representation of the steps required for the enrichment method with A) SAM formation B) flushing of Linker molecule C) immobilization of His10-Tag MBD2 D) flushing of the DNA solution over the formed MBD2 surface sheet E) DNA elution from the MBD2 surface F) elution concentration by the PCR clean up column G) DNA concentration determination with AccuBlue.*

4.1. AccuBlue and Teflon cell

A calibration line was made using dilution series of hmDNA with AccuBlue. AccuBlue is a fluorescent dye that binds on double stranded DNA. A concentration of 210 pg is diluted 4 times in a dilution series. This means that each solution contains 210 pg, 105 pg, 52.5 pg and 26.25 pg of DNA. From these concentrations, the fluorescence intensity is determined using a plate reader. The calibration line can be plotted on a graph as shown in Figure 7. In this way, the concentration of an unknown sample obtained by AccuBlue can be determined by relating the measured fluorescence intensity to the DNA concentration displayed on the x-axis.

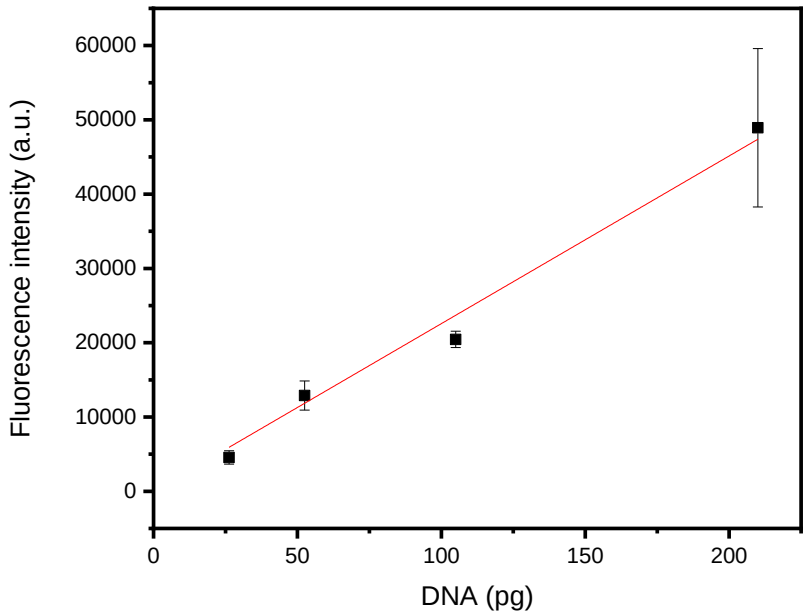


Figure 7. *AccuBlue calibration line with a slope of y=225,7x and R-square of 0,99.*

After MBD2 immobilization, the hmDNA solution was flushed through the Teflon flow cell, followed by elution and determination of the DNA concentration. 0.127 ng hmDNA was obtained in the eluted DNA sample. When this is compared with Ruben Kolkman's results, it is almost 100 x less.[10] In fact, about 30 ng of DNA is expected. The obtained DNA concentration is too low to perform methyl-sensitive restriction enzyme digest and qPCR analysis. Therefore, in one of the 7 steps of Figure 6 there is an experimental error which causes the collection of less hmDNA then expected. It was found that here too little buffer was used to make the DNA solution solution. The whole solution was not 1800 µl but 1400 µl. Therefore, the solution was already over the surface within 40 minutes instead of an hour. As a result, the DNA may not have enough time to bind properly to the MBD2 proteins.

The experiment was repeated, and care was taken that all steps were performed according to the protocol. This time, enough buffer was added for the DNA solution and therefore allowed for 10 minute longer flushing over the surface. 50 minutes is still less than 1 h which typically is performed. So, there is an increased flow rate, which may have an effect on the binding of the hmDNA to the MBD2 proteins. Thereafter, the DNA was eluted from the surface. The golden chip was shaken slowly in the NH_4OH, this is done to ensure that all the hmDNA releases from the MBD2 proteins. In the previous experiment this was not done so there may have been less hmDNA in the NH_4OH solution. After the experiment, an amount of 0.915 ng was measured in the eluted DNA sample. The DNA concentration is 9x more than the previous experiment, but still not enough. It may be that the 2nd experiment was performed better due to experience of the 1st experiment and, therefore, more hmDNA was detected with AccuBlue. The experiment has not been repeated often enough to say it is a significant difference.

Because of the low amount of hmDNA obtained in both experiments, we examined which step in the process causes the shortcoming. It has been looked at the concentration step (Figure 6F). In this step, the 1200 µl of NH_4OH solution is concentrated to a 30 µl solution through the Nucleospin gel & PCR clean-up column. To validate this process, a DNA amount of 30 ng is mixed in 1200 µl of NH_4OH, and

processed with the column. The concentration is afterwards determined by AccuBlue. 18.4 ng was obtained. Indeed, the Nucleospin gel & PCR clean-up column consists of a column with three different buffers to concentrate the solution. NT3, NTI and NE. Because of the different steps, losses of the DNA occur. 18.4 ng is therefore a logical quantity. As a result, it can be concluded that the little DNA uptake of both hmDNA enrichment experiments is not due to the concentration step.

4.2. Gel Electrophoresis and NanoDrop 2000

The hmDNA is stored in the freezer until use. However, freezing can compromise the quality and structure of the DNA.[34] The hmDNA used has been in the freezer for 9 months so it could be that the structure is affected and as a result the hmDNA is less well methylated. This could be the possible reason why so little hmDNA was obtained during the enrichment method. With less methylation, there is less binding to the MBD2 proteins, and thus a lower yield. Because of this, there is need to test if the hmDNA is still properly methylated. MAL3C*pG (methylated) and MAL3CpG are mixed with the HpaI and HpaII enzymes which can cut for the non-methylated sequences GCGC and CCGG into small fragments. Also two controls were measured, hmDNA and DNA without enzymes. Then the DNA samples were measured with Gel Electrophoresis and compared with a DNA Ladder as reference. If 90 bp is retained for the Mal3C*pG it means that the methylation is good and it can be used for the hmDNA enrichment experiment. [14]

Figure 8 shows the fluorescence intensity over time. The fluorescence intensity indicates how much light is emitted, the higher the peak the more DNA is in the sample. As can be seen in Figure 8, there is no peak at 44 seconds for the methylated DNA which means that the methylated DNA is well methylated. If this were not the case, a peak would also have been observed at this time point. The unmethylated DNA was cut to 60-70 bp and the methylated DNA remained 90 bp which was measured using known DNA fragments from the DNA. The hmDNA and DNA without enzymes are not cut and remain 90 bp seen in Figure 8. It would be expected that the hmDNA and controls would appear at longer migration times because they are longer, and this is indeed the case.

Interesting is that the fluorescence intensity of the unmethylated DNA with the restriction enzymes is twice as high compared to hmDNA. This could mean that there is a different concentration in the hmDNA than in the unmethylated DNA. If the hmDNA were lower concentrated, a lower amount than the 160 ng/µl would have passed through the Teflon cell. This would result in a lower concentration having passed through the Teflon cell, and therefore a lower concentration is also expected in AccuBlue. Because of this, it is necessary to see what concentration the hmDNA possesses.

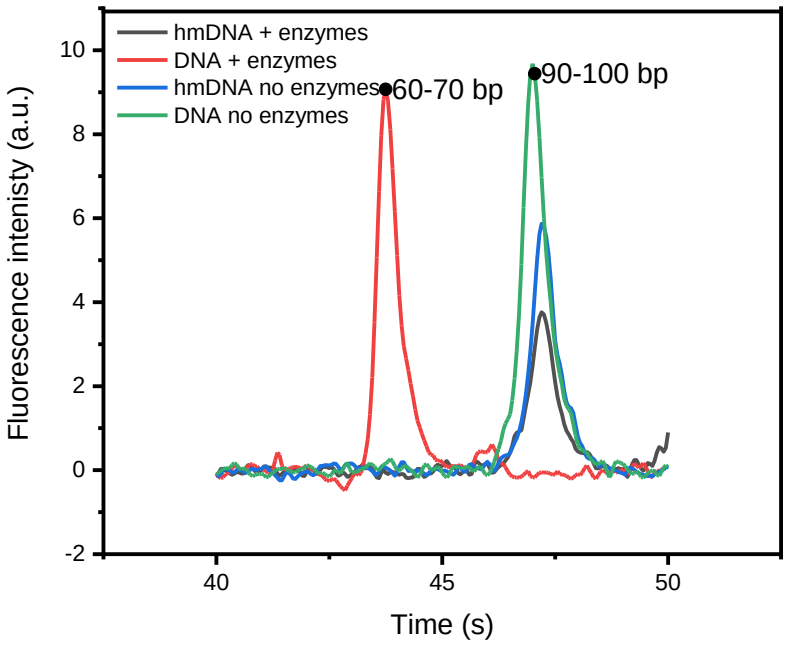


Figure 8. *Gel Electrophoresis with the black line indicating the hmDNA mixed with the Hpa I and Hpa II enzymes which peaks for 90-100 bp in length. The red line is the unmethylated DNA mixed with the Hpa I and Hpa II enzymes which peaks for 60-70 bp in length. The blue line which possesses only hmDNA without enzymes and peaks at length 90-100 bp. And finally the green line which possesses unmethylated DNA without the enzymes and peaks at length 90-100 bp.*

Electrophoresis showed that the unmethylated DNA had a higher fluorescence intensity than the hmDNA. This is further investigated using NanoDrop 2000. The UV-Vis NandoDrop 2000 can measure the concentration of DNA by absorbing light with a wavelength of 260 nm. The amount of absorbed light gives the concentration according to Beer–Lambert law. The result is shown in Figure 9. It can be seen that the unmethylated DNA has almost 3 times less absorbance than the methylated DNA. This is not as expected, because Figure 8 shows that the unmethylated DNA has a higher concentration.

1 optical density (OD) is equivalent to 500 ng/µl of DNA using a path length of 1 mm. This means that according to the NanoDrop spectrum, there would be a concentration of 718.5 ng/µl of hmDNA and 249.5 ng/µl of non-methylated DNA. This is a big difference from the known concentration of 160 ng/µl. These are not values expected when compared to the 160 ng/µl listed on the manufacturer's packaging. Previously, the concentration elution step was tested for by passing an amount of 30 ng through the column. If the concentration was actually that high, a value of 18.4 ng could never have been obtained. The high value could be due to a wrongly used blanko. MilliQ was used as a blank except that the DNA solution contains rCutsmart. Furthermore, DNA is going to be re-methylated and it is going to be re-analyzed in the NanoDrop 2000 with the rCutsmart blanko.

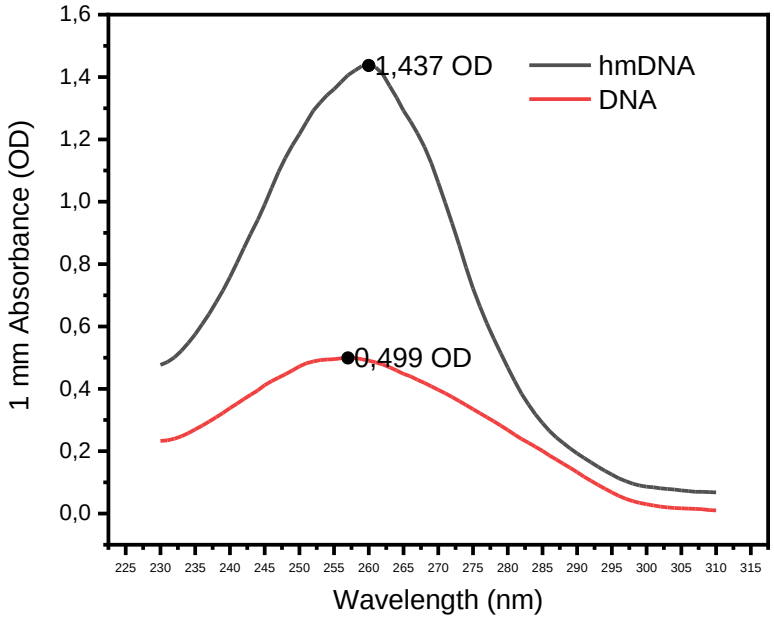
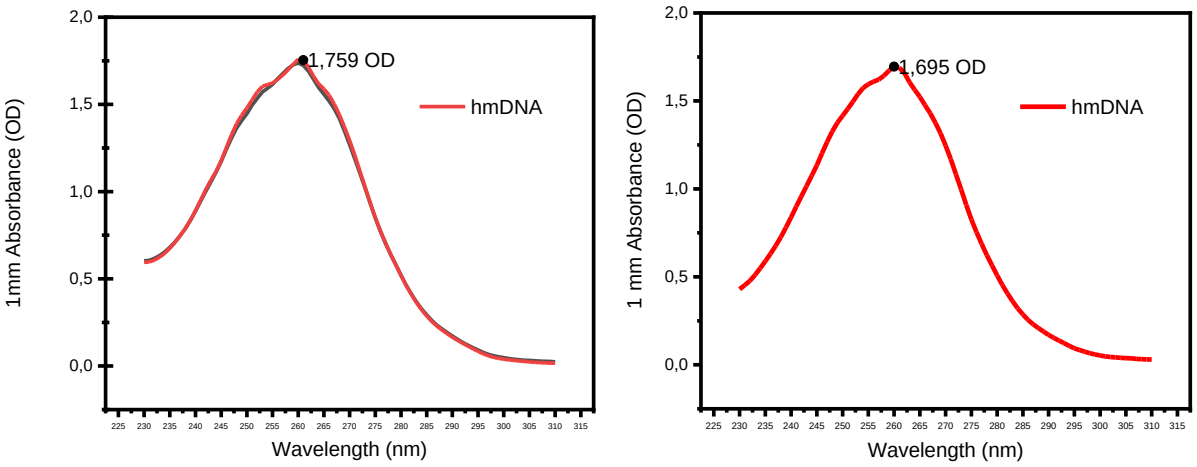


Figure 9. *NanoDrop 2000 concentration determination, the red line indicates the unmethylated DNA peaking at 0.499 OD. the black line indicates the hmDNA which peaks optimally at 1.437 OD.*

4.3. New DNA methylation and concentration determination

It is best to work with "freshly" made DNA samples.[35] Therefore, DNA is going to be remethylated again. The new double-stranded DNA is methylated by the CpG Methyltransferase M.SssI, which methylates all cytosine with 5'CG 3' as the recognition point. After the DNA is methylated, it is measured again by the NanoDrop 2000 shown in Figure 10A. The hmDNA concentration is 880 ng/µl. To see if this high concentration is due to non-matching blanko, the blanko of MilliQ is replaced by a blanko with rCutsmart and MilliQ. The graph in Figure 10B is the new NanoDrop measurement with the new methylated DNA and blanko rCutsmart/MilliQ, no difference can be seen compared to Graph 10A. The suspicion is that there is contamination and therefore such a high concentration is obtained. This is being investigated through the NanoDrop One.



A) ***B)***

Figure 10. *NanoDrop 2000 new methylated DNA concentration determination with A) MilliQ water used as blanko giving a peak at 1.759 OD and B) rCutsmart and MilliQ used as blanko giving a peak at 1.695 OD.*

Due the thought that there is contamination in the NanoDrop 2000, the DNA concentration was controlled with the NanoDrop One. The result shown in Figure 11 reveals that the concentration of the old and new hmDNA are 614.2 ng/µl and 924.8 ng/µl. However, the concentration of the old unmethylated DNA, 240.0 ng/µl, is close to the expected DNA concentration of 160 ng/µl. Through literature research, it was found that the S-adenosylmethionine combined with M.SssI increases light absorption at 260 nm. As a consequence, a higher concentration of hmDNA was determined than is actually the concentration. [36] This also allows the conclusion that the high concentration of hmDNA in the Nanodrop 2000 measurement was not due to contamination. However, it is striking that the new hmDNA has a concentration of 924.8 ng/µl and the old 614.2 ng/µl. So despite the fact that the S-adenosylmethionine combined with M.SssI increase the absorption of both, the concentration of the new hmDNA is higher. This could be a good explanation of the little DNA uptake from the previous hmDNA enrichment experiments.

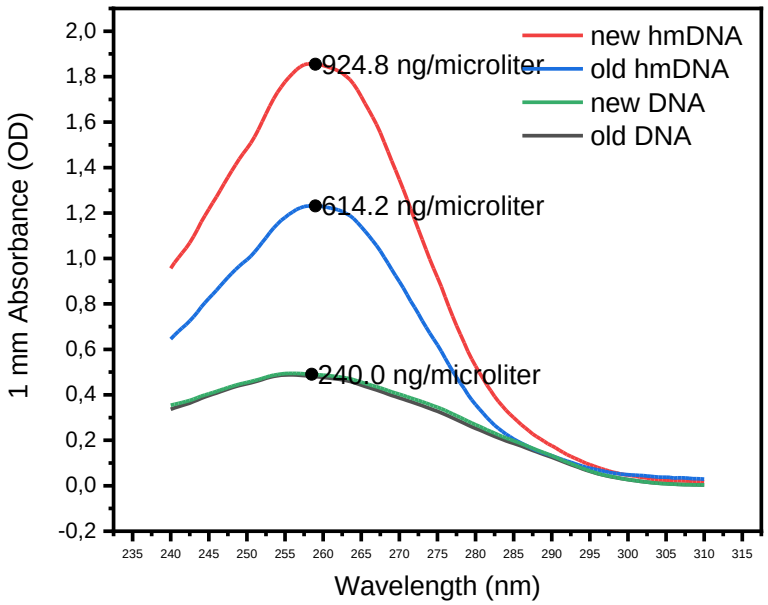


Figure 11. *New concentration measurements using NanoDrop One. The red line is the newly methylated DNA which has a concentration of 924.8 ng/µl. The blue line is the old methylated DNA which has a concentration of 614.2 ng/µl. The old and new unmethylated DNA are indicated by the black and green lines and have a concentration of 240 ng/µl.*

The enrichment method using the Teflon flow cell was performed with the newly methylated DNA. A total of 0.28 ng was collected. This time a tube was placed at the end of Teflon cell to collect the DNA solution mixture to see if the DNA can be detected here. In this tube an amount of 900 ng came out after AccuBlue. This is much more than what was in the DNA solution (500 ng). This captured DNA solution did not pass through the elution column and thus consists of BB+ and DNA. The other blanko caused such a high signal to be obtained in AccuBlue. Indeed, it cannot be that more comes out that was put in.

The Teflon DNA enrichment experiment took place again. All components used for hmDNA enrichment were used fresh prior to start of the experiment. Nevertheless, only 0.15 ng was obtained. New DNA is methylated, but not verified that the newly methylated DNA is properly methylated. It is rechecked by HpaI and HpaII restriction enzymes to see if this is the case. For this purpose, these enzymes are added to unmethylated DNA and the hmDNA, then characterized with Gel Electrophoresis (Figure 12). It can be seen that the enzymes HpaI and HpaII cut unmethylated DNA and the hmDNA used for the previous two Teflon experiments to 60-70 bp. This explain the low yield of the previous enrichment experiments. There was no methylation on the DNA, so the DNA was never able to bind to the MBD2-modified surface. Because there is no methylation it can be concluded that the M.SssI enzymes used for DNA methylation were not functioning anymore, thus, preventing DNA methylation.

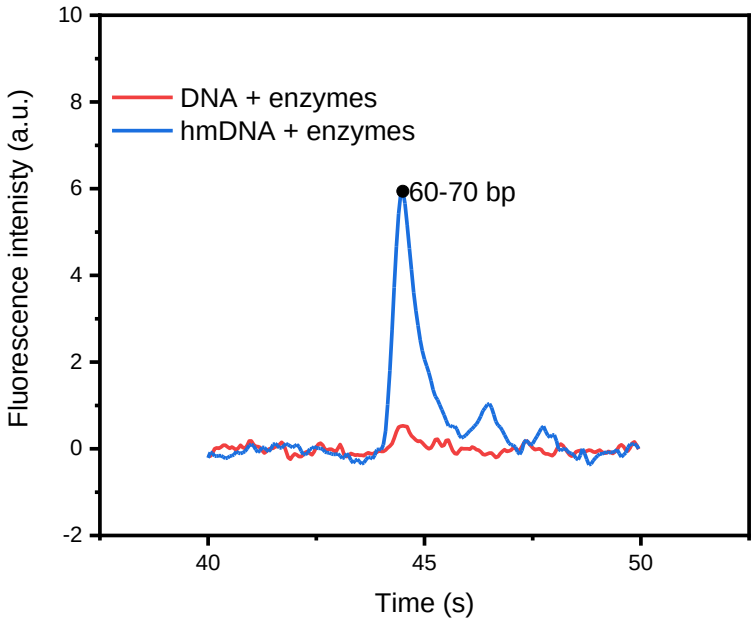


Figure 12. *Gel Electrophoresis, the red line presents the unmethylated DNA with the restriction enzymes. the blue line presents the hmDNA with restriction enzymes used for the last two hmDNA enrichment experiments. Both conditions were cut to 60-70 bp.*

DNA is remethylated with new and fresh bought M.SssI enzymes. The methylated DNA was then treated with the restriction enzymes and analyzed using Gel Electrophoresis (Figure 13). The methylated DNA was not cut and remains 90-100 bp in size while the DNA without methylation was cut to 60-70 bp. From this it can be concluded that it was indeed due to the M.SssI enzymes that no methylation occurred on the DNA. Because of this, the MBD2 proteins could never bind with the DNA, and no/little DNA was present in the enriched sample.

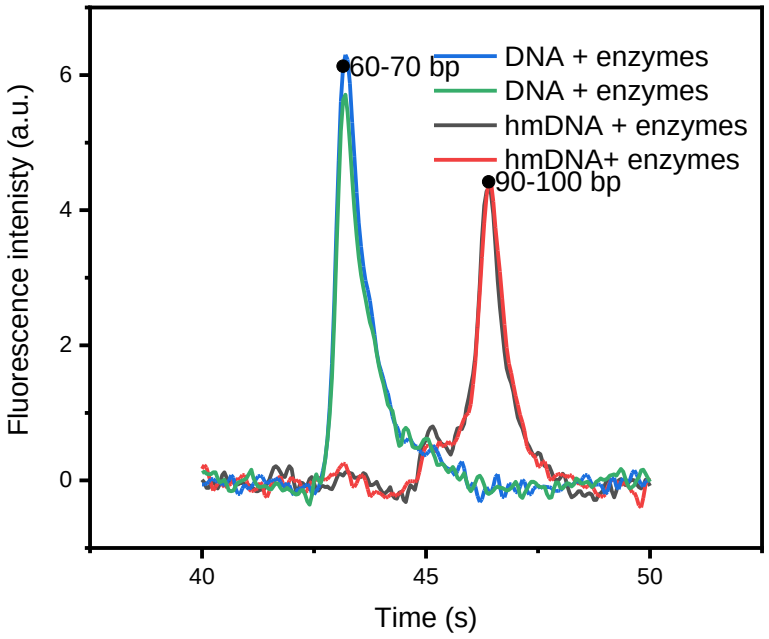


Figure 13. *Gel Electrophoresis. The green and blue line indicate the unmethylated DNA with the restriction enzymes, and are cut to 60-70 bp. The red and black line represent the hmDNA with the restriction enzymes, which are not cut and thus are 90-100 bp long.*

5. Conclusion

In this work, the intention was to add an additional wash step to increase the selectivity of the hmDNA enrichment method. A model hmDNA solution was used with DNA of 90 bp in length which was methylated by M.SssI enzymes.

After all steps of the enrichment method were performed without the additional washing step, it became clear that significant less DNA was present in the hmDNA enriched sample that compared to Ruben Kolkman *et al* [10]. This made it necessary to investigate what was the reason for the low DNA quantity. Because the DNA had been in the freezer for almost a year, degradation could have occurred, resulting in less methylated DNA. Because of this, restriction enzymes HpaI and HpaII were used prior to the Gel Electrophorese of the DNA samples to see if they would cut the methylated DNA. This was not the case, so the DNA was methylated well. A low fluorescence intensity could be seen, which could indicate a reduced concentration. As a result, the DNA was re-methylated and then after doing the hmDNA enrichment experiments again, it was again found that the DNA quantity obtained was still too little. The new hmDNA was rechecked for proper methylation, and it was found that the newly methylated DNA was not methylated because the M.SssI enzyme that was used was past its expiration date. After working with new M.SssI enzymes, Electrophoresis made it clear that the DNA was now methylated. It could be confirmed that the M.SssI enzymes was not active anymore, and therefore no DNA was bound to the MBD2 proteins in the hmDNA enrichment experiments.

In the future, the hmDNA enrichment experiment should be done using fresh reagents. After all, if even one element is incorrect, it is like looking for a needle in a haystack to find the error. The methylated DNA must be checked for methylation before passing through the Teflon cell. This way, enough DNA would indeed likely be obtained in hmDNA enriched DNA samples. Then, the effect of an extra washing step to increase hmDNA enrichment selectivity can be studied.

6. Acknowledgements

I would like to thank the BIOS and MNF group for allowing me to be a small part of this mega promising new diagnostic method to diagnose cancer in a non-invasive way. In particular, I would like to thank Ruben Kolkman as my daily supervisor for all his help, time and guidance in my Bachelor thesis. I would thank Loes Segerink and Jurriaan Huskens for the biweekly meeting where I could present my results and get useful feedback out of it. I hope that in the future this concept will be further developed and the selectivity will be high enough to make this a real screening method.

7. Recommendation

In this study, the intention was to add an additional wash step with NaCl to the hmDNA enrichment method. Although there was no conclusion from this study, due to errors in the system that prevented the hmDNA from binding to the MBD2 proteins, various methods can be used to obtain higher selectivity.

Salt concentration ensure likely that non-specific bonds caused by the electrostatic interactions between the protein and non-methylated DNA are broken.[27,28] People's eating habits make it that everyone has different salt concentration in the urine.[37] When there is a lot of salt in the urine, it may affect the selectivity. Because of this, the ideal salt concentration should first be determined by adding the extra wash step of 0.2-2 M NaCl. Once the ideal selectivity concentration is determined, the salt concentration in patients' urine should be determined. When the concentration of NaCl is too high, dilute the DNA sample, and when the concentration is too low add NaCl.

Besides using different concentrations of salt solutions, there are other purification methods that can be used to get more unmethylated DNA off the surface after the first elution step. Adding denaturing conditions allows proteins to unfold by decreasing hydrophobic interactions. Urea is an important denaturing agent. Urea makes that the protein-protein contact is less stable than the protein-urea contact. By deteriorating the hydrophobic effect, any bonds are also deteriorated. [38–40] The presence of Urea might therefore be detrimental to enrichment selectivity. Because when the proteins denature, they no longer do anything and thus no binding takes place. HmDNA is extracted from urine by passing urine over the enrichment column. Urine contains Urea. The effect of Urea on selectivity should therefore be considered. In the morning the amount of Urea is more concentrated than in the evening. One can see what difference morning urine has on selectivity compared to evening urine.

In Ruben Kolkman's research, it was assumed that flow rate of 30 µl/min is the ideal rate to pass the DNA solution through the Teflon cell. This gives the hmDNA enough time to be able to bind with the MBD2 proteins without being flushed from the surface. It could be that if the flow rate is increased, this will have an effect on DNA binding to MBD2-coated surfaces. Because the MBD2 proteins have a higher affinity for the hmDNA than for the non-methylated DNA, a higher flow rate could cause the non-methylated DNA to bind even worse on the surface and thus be flushed away. [10,14] Here it is important that not also the hmDNA is washed away. It can be examined at which flow rate the ideal selectivity is obtained.

Finally, temperature is an important variable for the structure of proteins. A small difference in temperature can already have major consequences for the proteins. Temperature change causes the solubility of the proteins to change. The MBD2 proteins are also found in the body and thus work optimally at a temperature of 37 degrees. By heating the elution step to a temperature higher than 37 degrees, the solubility could be shifted, causing the MBD2 proteins to have even less affinity for the DNA and thus detach from the surface.[41] Temperature increase may be good to get more non-methylated DNA off the surface and thereby increase selectivity.

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