Preserving Methylation in DNA Amplification: Using Helicase-Dependent Amplification and DNA Methyltransferase One

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DNA methylation is one of several epigenetic mechanisms that can affect an organism's operation. The purpose of this research was to discover and refine a method to amplify DNA while conserving its methylation pattern. This amplified and methylated DNA could then be transformed into organisms to explore how methylated DNA segments operate and affect gene expression. In order to preserve methylation sites during Polymerase Chain Reaction (PCR), we plan to use DNA methyltransferase 1 (DNMT1), the enzyme responsible for conserving methylation in living cells. However, the temperature at which DNMT1 falls apart is lower than the temperature that is needed for standard PCR. For this reason, we have decided to utilize helicase-dependent amplification (HDA), an alternative DNA amplification procedure. HDA operates at lower temperatures than standard PCR, making it suitable for use with DNMT1. We are going to combine DNMT1 with HDA so that the DNA can be amplified and methylated at the same time. We designed a DNA strand that includes primers suitable for HDA and two restriction enzyme cut sites that are sensitive to methylation. One of these cut sites will be methylated and one will not. Should the DNA methyltransferase not maintain the pattern, it will not produce the right number of fragments after the strand is digested. If amplification of methylated DNA segments is successful, this opens the door to research possibilities by enabling the insertion of methylated genes into plasmids as a way to observe the effects of methylation of a particular gene on the organism as a whole.

Keywords: DNA methylation, DNA amplification, DNMT1

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Watch a video introduction by the authors at https://youtu.be/l91pNLQJzPM

DNA methylation is one of the chief mechanisms of gene control in eukaryotic cells. Along with a number of other cellular processes, it is classified as an epigenetic factor—that is, it alters expression of traits by changing the structure of the DNA molecule itself, rather than rearranging sequences of nucleotides (Dupont et al. 2009). Methylation occurs when a methyl group is bound to the DNA; this regulates gene expression by preventing transcription of certain genes (Bird 2002). Methylation is important in many cellular processes, including embryonic development, chromosome stability, genomic imprinting, and X-chromosome inactivation, among others. Although normal methylation is necessary for an organism to function properly, errors in methylation can arise. Such errors have been linked to an array of severe consequences (Phillips 2008). A well-known example of such consequences is tumor growth: genes that normally suppress the growth of tumors

can be inactivated by abundant methylation, or hypermethylation. The absence of these tumor-suppressing genes creates an environment in which tumors can grow more easily (Kulis and Esteller 2010).

Methylation, in general, occurs when a methyl group (CH_3) is transferred from a methyl donor molecule to an acceptor (Vrana et al. 2015). The enzyme responsible for this is DNA methyltransferase, which both maintains the existing pattern of methylation and creates new patterns (Phillips 2008). The enzyme attaches a methyl group to the C5 end of a cytosine base (the 5′ end of the deoxyribose "backbone"), converting cytosine to 5-methylcytosine. A pair of methylated cytosine nucleotides, called "cytosine residues," positioned diagonally from each other across the DNA strand, constitute a common pattern of cytosine methylation. These cytosine residues are often followed by a guanine nucleotide in the DNA sequence, giving rise to the term "CpG sequence" (Moore et al. 2013).

While human cells contain many varieties of DNA methyltransferase, the most important for our purposes is DNA methyltransferase 1 (DNMT1). This enzyme is responsible for replicating existing patterns onto newly synthesized DNA strands after replication. This is called in vivo methylation, since it does not involve the creation of new patterns. Other types of DNA methyltransferase perform de novo methylation—the creation of new patterns (Moore et al. 2013).

Under normal cellular conditions, sequences of methylated DNA (CpG sequences) are distributed sparsely throughout the mammalian genome (Song et al. 2010). However, errors in methylation can result in "CpG islands"—sections of a DNA strand, approximately 1 kb long, which contain unusually large quantities of CpG sequences. CpG islands can trigger gene silencing in the wrong places—for example, the silencing of tumor suppressor genes in cancer cells (Phillips 2008).

Thanks to its significance in epigenetics, DNA methylation is a crucial stage in the process of DNA replication for its regulation of gene expression. The most useful tool for analyzing a DNA sequence is amplification by means of Polymerase Chain Reaction (PCR), but conventional PCR does not maintain DNA methylation patterns. DNMT1 is not present in the conventional PCR reaction because it cannot withstand the high temperatures necessary for the procedure (Walker et al. 1991). While alternative methods exist that can indicate where methylation has occurred within the DNA strand, these methods cannot produce a usable amplified DNA end product. Such a product would be very useful for many applications, such as transforming methylated DNA sequences into bacterial DNA to analyze the long-term effects of methylation on specific gene functions.

In order to amplify usable methylated sequences, we plan to use helicase-dependent amplification (HDA), a process that avoids the thermocycling necessary for PCR by employing a helicase enzyme to unzip DNA strands (Vincent et al. 2004). However, one-step HDA requires a temperature of 60°C, and normal DNMT1 is not stable at this temperature. The 2013 Heidelberg iGEM team developed a version of this enzyme called "PCR 2.0." This enzyme is stable at temperatures up to 65°C due to the flexible linker that makes the enzyme circular and thus, able to withstand higher temperatures without degrading. By using HDA in conjunction with Heidelberg's PCR 2.0, we believe we will be able to amplify a methylated DNA product that can then be transformed into bacterial cells for use in the applications described above (Tuteja and Tuteja 2004).

Materials and Methods

Competent Cell Transformation. The first step of this project was to test the competency of the cells provided by Open Bio Labs (Charlottesville VA), because the cells were kept in a refrigerator that did not reach the desired temperature of -80°C. The competency of the cells was tested by conducting a transformation that included the use of the pVib gene. Two 250 mL tubes were used, both with a concentration of cells at 250 µL of cells in 250 mL of nutrient broth. One tube of the nutrient broth with the supposed competent cells was transformed with the pViB gene and one was not. Next, six plates were used: two inoculated with ampicillin and two with chloramphenicol (both of which were mixed with nutrient broth and agar), and two with only nutrient broth. Each set of three plates were plated with 20 µL of the transformed cells and another with 20 µL of the control cells. Since the pViB plasmid carries resistance to both antibiotics, this test was performed to help determine if the cells were competent and if the antibiotics were viable.

The results of the transformation were inconclusive as to whether the cells were competent and whether the antibiotic was still active. To determine if the cells were, in fact, competent or if the antibiotic was no longer viable, another test was run. The transformed competent cells provided by Charlottesville Open Bio Labs were plated on two more plates inoculated with 3 µL of new ampicillin at a 1/100 concentration in water. In addition, transformed bacteria were grown on one Chloramphenicol plate, while control bacteria were grown on another. Competent DH5α *E. coli* provided by the University of Virginia were grown on one Luria-Bertani (LB) agar plate with ampicillin and one nutrient broth plate without antibiotic to determine if the antibiotic was viable. Another transformation using the DH5a *E. coli* was performed to determine whether the PViB gene was functioning and thus, not responsible for the experiment's failure.

Gel Electrophoresis. The next step was to practice transformation and electrophoresis before it was to be done to analyze the results from the helicase PCR (described below). The first series of transformations was carried out with four different genes: a promoter/ribosome binding site (RBS), a red fluorescent protein (RFP), a green fluorescent protein (GFP), and a Terminator from an iGEM DNA distribution plate, using competent cells provided by Open Bio Labs. First, the cells were added to nutrient broth to allow them to multiply for 24 h. After this time period, the cells were divided into 4 tubes and each tube was transformed with one of the desired genes. The transformed cells were then plated. The cells containing the promoter/RBS, RFP, and Terminator were all plated on chloramphenicol plates, since this was the antibiotic resistance carried on the plasmid. Similarly, the cells transformed with the GFP gene were plated on an ampicillin plate. One control plate of only nutrient broth was used for each gene. The cells were allowed to grow on the plates for 24 h before a miniprep was done to isolate the DNA from the cells. Using the DNA isolated from the cells during the miniprep, PCR was performed to amplify the isolated DNA. This was done with 12.5 µL of Master Mix at a 1x concentration, 1.3 µL of Forward Primer at a 0.5 µM concentration, 1.3 µL of Reverse Primer at a 0.5 µM concentration, ≈1 µL of Template DNA at < 1,000 ng, and enough nuclease-free water to bring the mixture to 25 µL. After amplification, the samples were run in an electrophoresis chamber. However, the first electrophoresis produced gels with no visible bands, so the entire procedure was repeated. For this test, DNA from the iGEM distribution trays were again re-hydrated. This procedure was performed with two sets of each gene—one set was leftover DNA from the first test, and the other from the second re-hydration. PCR amplification and electrophoresis was

practiced again, with hopes to estimate the DNA concentration of the samples. Two separate gels were used: one for the DNA from the first hydration, and one for the DNA from the second rehydration.

Competent Cell Procedure. After PCR amplification and electrophoresis did not yield gels with clearly visible bands, it was hypothesized that the possible problem was the cells' competence, or lack thereof. To test this theory, new competent cells were made. A 5 mL culture of cells was grown in LB broth overnight. This culture was then diluted with more LB broth to 25 mL. Twenty-five eppendorf tubes were placed on ice so they would be cold for a later step. The diluted culture was then divided into two test tubes each containing 12.5 mL and allowed to rest on ice for 10 min. The tubes were then centrifuged for 10 min. at 3,000 RPM. The liquid was pipetted out of the test tubes so that only the cell pellets remained. A vortex was used to resuspend the cells in 1.25 mL of Transformation & Storage Solution (TSS) Buffer, until the pellet was no longer stuck to the bottom of the test tube. One hundred µL of this liquid was then added to each of the cold eppendorf tubes, and the cells were placed in the freezer for later use.

Restriction Digest. The next step of the project was to practice a restriction digest, since this procedure would be needed to insert the desired gene into the plasmid for the project. The restriction digest was performed on the pViB plasmid using both EcoRI and PSTI restriction enzymes. The pViB plasmid provided by Carolina Biological served as a control for the restriction digest. First, this plasmid was amplified using PCR. It was then digested using three different combinations of enzymes, only EcoRI, only PSTI, and then both EcoRI and PSTI together. In each of the digests, 2.5 µL of Buffer and 10 µL of DNA were used. In the first control digest, 0 µL of both enzymes and 12.5 µL of water were used. In the second control digest, 0.5 µL of the EcoR1 enzyme was used, 0 µL of the PSTI enzyme was used, and 12 µL of water was added. In the final control digest, 0.5 µL of each enzyme was used, as well as 11.5 µL of water.

After transforming the newly created competent cells with the pViB plasmid provided by Carolina Biological, a miniprep was performed to isolate the DNA. The DNA was then amplified via PCR and was digested using the same combinations of enzymes as the control plasmid. The DNA was concentrated at 0.05 µg per 10 µL of water. In each digest, all materials were mixed together by pipetting up and down and then incubating the reaction for 1 h at 37°C. To test whether the restriction digests were successful, electrophoresis was performed. The gels were made with 0.21 g of agarose, 3.5 µL of loading dye, and 35 mL of tris base, acetic acid, and ethylenediaminetetraacetic acid (TAE) Buffer. In each individual sample, 1 µL of dye was added to 10 µL of DNA.

Designing the DNA Strand. The goal of the research was to test if DNMT1 and helicase-dependent amplification combined can replicate a methylated DNA strand while preserving the methylation pattern. In order to test this theory, a methylated DNA strand was designed. According to the specifications required for helicase-dependent amplification, the desired length of the designed strand was between 70 and 120 bp, consisting of a forward primer, methylated DNA region, and reverse primer. Heidelberg 2013 iGEM team's methylated DNA region was used as the methylated DNA portion of the strand. Next, the primer was designed following the criteria specified by the IsoAmp® II Universal tHDA kit (New England Biolabs, Ipswich MA). First, a length of 24 to 33 bp was required, with an optimal length of 27 bp. In addition, the primers needed a GC% content of 35%–60%, with an optimum at 44% in order to ensure the appropriate melting temperature of the primer. The final designed DNA strand for this experiment was 119 bp long, with a 30 bp forward primer and 30 bp reverse primer. The primers had 50% GC content and a melting temperature of 61.4°C.

HDA. Next, Helicase PCR was practiced using a control DNA strand provided in the IsoAmp® II Universal tHDA kit. Conventional PCR was also run with the same DNA strand for comparison of amplification results. For Helicase PCR, the first step was to set up the reaction by mixing 27.5 µL of water, 2 µL of RNA template, 5 µL of Annealing Buffer at a 10x concentration, 0.75 µL of Forward Primer, 0.75 µL of Reverse Primer. 1.75 µL of $MgSO_4$, 4 µL NaCl, 3.5 µL of IsoAmp dNTP Solution, and 3.5 µL of IsoAmp Enzyme Mix. The reaction was mixed by pipetting up and down. Then 50 µL of mineral oil was overlaid on top of the reaction. The reaction was placed on ice. Then the reaction was incubated at 65°C for 90 min. using a water bath. In the conventional PCR reaction, 5 µL of EZ PCR mix, 0.75 µL of Forward Primer, 0.75 µL of Reverse Primer, 2 µL of DNA template, and 18.5 µL of water were mixed for a total of 27 µL. Both the Helicase and the PCR reactions were run in an electrophoresis gel with 0.35 g of agarose, 3.5 µL of stain, and 35 mL of TAE Buffer at 100 V for 25 min. to determine the concentration of DNA and compare the degree of amplification for the two procedures.

Results and Discussions

Many of the results were inconclusive. Some of the plates in the competence test did not display growth, and some of the controls did have growth. In the many gel electrophoresis experiments that were conducted, a ladder was visible on most of the gels, but while some of them had visible bands showing the different plasmid parts we were testing for, the majority of the time, the ladder was the only thing visible. The GFP gene did not show up on any of the gels. The results of the restriction digest were similarly inconclusive as the ladder migrated, but there were no other bands visible on the gel.

The HDA results were also inconclusive. We ran two gels, and both times, neither the helicase product nor the PCR product were visible. On one of the gels, not even a ladder was visible. The other gel displayed a ladder, but the expected bands from the HDA product and the PCR product were not present.

Testing Competence. Before we began, we performed a transformation to test our antibiotics and competent cells. We used a PViB plasmid for our transformation, which, if successful, would cause the cells to glow, making the success of the procedure easy to determine. The plasmid also contained a resistance to the antibiotic ampicillin, with which we inoculated our plates, thereby preventing growth of all bacteria without the plasmid. However, in transformation, bacteria grew on both the control and experiment plates, even though the control bacteria did not have the PViB plasmid. The cause of this was unclear, so we did an additional 9-plate transformation to see what went wrong. The first two plates were a repeat of the original transformation experiment. The next three plates had ampicillin added on top of the plates. We hypothesized that one thing that might have gone wrong in the original transformation was that the ampicillin might not have been distributed throughout the entire plate evenly or might have not been viable any longer. To confirm this theory, we also used two plates with Chloramphenicol, another antibiotic, and, as expected, bacteria grew on the plate with PViB and not on the control plate, meaning that the Chloramphenicol is viable. However, the bacteria grew on both ampicillin plates, meaning that our ampicillin was not working. Finally, we plated two plates coated with ampicillin with DH5α *E. coli*, a type of competent cell containing no PViB gene, and bacteria grew on both plates, further confirming that our ampicillin was not viable.

Transformation/Electrophoresis Practice/Trouble Shooting. The next step was to practice transformation and electrophoresis before we obtained the IsoAmp® II Universal tHDA kit. We wanted to be very skilled at basic lab procedures before beginning our main lab work. We decided to use four different genes from iGEM DNA distribution plates in order to practice these skills. The genes used were a promoter gene, a GFP, a RFP, and a terminator gene. Open Bio Labs supplied competent *E. coli*, which was transformed and allowed to grow in nutrient broth. All but the GFP tube had visible growth. The cells were then plated. The three tubes of cells containing the RFP, promoter, and terminator genes were all plated on ampicillin plates (we obtained new viable ampicillin), while the GFP was plated on a chloramphenicol plate. The type of antibiotic used matched the resistance carried on the gene's plasmid. There was no growth on any of the plates. The growth in the nutrient broth may have been due to contamination from the environment. At this point, we began to suspect that the competent cells we were using had lost their competency. The next steps were to do a Mini Prep in order to isolate the DNA from our transformations, then to do PCR and gel electrophoresis. The results from the gel electrophoresis were not very clear. The bands in the first gel were fuzzy and not well defined. Also, the GFP gene did not migrate at all; there was no band in its lane. One reason for the inconclusive results may have been that we did not let the DNA rehydrate for long enough. Because of this, we rehydrated the same DNA wells a second time. Then, the entire test was done again on the DNA from the second hydration. The results from the second electrophoresis were clearer than the first, but again there was no GFP band on the gel. The GFP gene only had a resistance to Chloramphenicol and no Ampicillin resistance, so it was grown on Chloramphenicol plates. This may have played a part in why there was no GFP growth or electrophoresis band. Another hypothesis was that maybe our competent cells were not actually competent.

Restriction Digest and Electrophoresis. We performed a restriction digest of the pVib plasmid. The results were dissatisfying. The ladder migrated, but the control DNA did not, or if it did, the bands were quite faint and hard to see. The "transformed" had two bands but they didn't show either. Our hypotheses for why this happened are as follows: we believe that one of two things happened. Either we did not let the gel run enough, or we did not add enough loading dye. At this point, we are not sure if the restriction digest worked.

Designing the DNA. Since HDA works best with DNA that has certain characteristics, we decided to design the DNA that we need for HDA. There are several criteria for the reaction to work effectively. First, since HDA requires a temperature of at least 60°C, the strand had to be stable at this temperature. The New England Biological website (from which we ordered the HDA kit) gives the ideal strand length, which is between 80 and 120 bp, and the necessary GC%, which is between 35% and 60% for this HDA kit. For our strand, we attached primers to Heidelberg's strand, because we needed our strand to have methylation sites. Using a primer analyzer from Integrated DNA Technologies, we designed two primers of 30 bp each. It took a lot of trial and error to find primers that would work with HDA, since we had a lot of factors to consider: they had to fit the requirements for HDA, and they also had to be free of hairpins. Hairpins are places where one side of the DNA can bend backwards and anneal to itself; this is undesirable in a DNA strand that is meant to be unzipped for amplification. Finally, we found two primers that each fit the criteria for HDA. We then ordered the completed DNA strand, which turned out to be 97 bp, from Integrated DNA Technologies (IDT).

We also requested for the DNA to be methylated on one of the methylation sites; Heidelberg's DNA has two restriction enzyme cut sites, each with two methylation sites. The reason only one is methylated is that when a restriction digest is performed, we will be able to see if the pattern was maintained or if all of the sites are methylated. A digest that results in two fragments indicates the preservation of the pattern, whereas a digest that results in one fragment indicates new methylation, since the methyl group would block the cut site. Finally, a digest that results in three fragments shows that none of the cut sites have been blocked by a methyl group. In the future, we plan to use our designed DNA to perform HDA, so we will be able to see whether HDA combined with Heidelberg's PCR 2.0 will preserve methylation.

HDA and Electrophoresis. Our HDA gels were inconclusive. We ran the DNA through electrophoresis twice, and both times, no bands of DNA were visible. On one of the gels we did get a DNA ladder band, but not on the other. One possible reason for this is that in the HDA procedure, mineral oil is overlaid on top of the DNA, so when we put the DNA into the gel, it is possible that we only got mineral oil. Another possible reason for the inconclusive results is that the gels might not have been made correctly. This is something we have had problems with in the past. There also might not have been enough loading dye in the DNA samples to make them appear on the gel. In order to determine the cause of our inconclusive results, we plan to conduct more tests.

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