

METHYLATION OF DNA MAY BE USEFUL AS A COMPUTATIONAL TOOL: EXPERIMENTAL EVIDENCE

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Previously we have explained the abstract concept we call ‘aqueous computing’ and illustrated it with concrete wet lab results. Here, we explore the use of methylase enzymes to ‘write’ on double-stranded DNA molecules at sites where restriction enzymes will cut if, and only if, the sites have not previously been methylated. A site represents the bit zero (False, F) if the site has been methylated and the bit one (True, T) if it has not been methylated. ‘Reading’ is done by attempting a cut at each of the sites. We found 8 commercially available methylases and 8 corresponding restriction enzymes that would not cut after the action of one of the methylases. We were able to confirm that methylation by each of these 8 enzymes individually blocked cleavage only by the restriction enzyme associated with that site and not any other enzyme. We then used these enzymes to approach a 3-variable, 4-clause satisfiability (SAT) problem using either plasmid DNA (pBluescript) or PCR product made from the region containing the restriction enzyme sites on the plasmid. Pairs of methylases were defined to represent each of the states of the operators p , q and r , one methylase for p and another for p' , etc. We methylated the DNA in parallel at the two sites so either the p site was methylated (making p false) or the p' site was methylated (making p' false). We did that for the other two variables as well to create a set of logically consistent DNA fragments. Then we applied the 4 clauses using restriction enzymes to cut DNA fragments that did not satisfy them. At the end, we found evidence for intact DNA indicating an answer satisfying all of the clauses. To confirm the state of each of the Boolean operators, we used cleavage by the appropriate restriction enzyme. We found in the computation with both the plasmid and the PCR product, one site pair to show false in both sites; q and q' , for instance. This should not be possible. We suspected incomplete cutting during the clauses by one of these restriction enzymes, specifically BssHIII. In summary, we did successfully show the usefulness of DNA methylation in a scheme to do a mathematical computation. Thus, we have added to our arsenal of potential methods of performing DNA computing in the aqueous style.

1. Introduction

An increasing number of groups in the world are exploring the possibilities of using DNA as a computer register. This has at least one obvious advantage over traditional computers, namely there are 4 possible bits at each position – ‘A’, ‘C’, ‘G’, and ‘T’. Also, there are many naturally occurring enzymes that work with DNA that can be harnessed to facilitate DNA computing. Another advantage of using DNA in aqueous solution (i.e., in water) is that with a simple split-and-mix technique, problems that require an exponential number of steps to solve conventionally, involve only a linear number of steps with this approach. Previously we have explained this abstract concept which we call ‘aqueous computing’ and illustrated it with concrete wet lab results (for reviews see [3, 6]). Each aqueous computation begins with a vast number of molecules all of identical structure. Each of these molecules is used as a ‘nano-tablet’ having prescribed locations at which bits can be ‘written’ by specified means. This writing step requires an alteration such that bits where “writing” has occurred can be distinguished experimentally from “non-written” areas. We have generally done these steps in pairs to represent the two possible states of a variable (such as p and p' , etc.)

We have discussed various theoretical approaches to this, but up until now we have concentrated on the approach of using a set of three enzymes to do this writing. To write on DNA in this approach, it is cut first by a restriction enzyme that leaves overhanging ends of DNA at the cut site. Second, we use a DNA polymerase to fill in the overhang ends to make a blunt end. Then third we ligate those pieces back together using the third enzyme, DNA ligase. The DNA polymerase adds bases equal to the number of overhang nucleotides from the enzyme. This allows two things, first the site will no longer be cleaved by the original restriction enzyme and second it increases the size of the DNA by the number of added bases. Thus, this method writes on DNA and we can distinguish between written and un-written DNA by size and by its ability to be cleaved by a specific restriction enzyme. We implement the clauses for SAT problems on the DNA molecules by performing parallel digestions with specific restriction enzymes. In this problem, cleaved DNA does not satisfy the condition and is removed. If there is a molecule at the end that has remained uncut, it has satisfied all the conditions and is an answer to the problem. We read the answers for a problem by sequencing the DNA and/or by digesting it with the appropriate restriction enzymes. We have used this approach to address two classical problems

in DNA computing namely a 3-variable SAT problem and a 3×3 Knight problem [2–5].

But for a variety of reasons, we wished to explore other writing approaches for aqueous computing. The three enzymes involved each require different enzyme conditions (buffers and salts) and so must be used sequentially and not simultaneously. This approach also requires the DNA be purified between steps to remove the unwanted buffer and salt components. These steps take significant time and result in some losses of DNA material at each step. To alleviate some of these challenges, we explore in this paper the use of DNA methylation as a modification that can be used for DNA computation. DNA methylases are naturally occurring enzymes in both prokaryotic and eukaryotic organisms that add a methyl group either to an adenosine or a cytosine (reviewed in [1] and [7]). In the former, they are believed to protect the organism from infection by a virus. These prokaryotes carry restriction enzymes to cut specific DNA sequences and a corresponding methylase that modifies the same DNA sequence in the host DNA. This modification blocks the restriction enzyme from cutting host DNA while incoming foreign DNA (say from a virus) does not have the methylation modification at that specific sequence and so is cleaved. This cleavage generally renders the viral DNA non-infectious, thus protecting the prokaryotic organism. Eukaryotic cells tend to use DNA methylation to affect gene regulation by DNA binding proteins. In most cases, methylated DNA is not expressed by eukaryotic cells. Because of the commercial availability of methylases and restriction enzymes, we thought it would be worthwhile to test whether this process could be used for DNA computing. Thus, here DNA methylation is the writing step while discerning whether a restriction enzyme can cut the DNA is part of the reading phase. The fact that one enzyme was going to be used at this writing step (the methylase) instead of 3, we felt would potentially speed up the computation. Thus, in this report, we tested 8 sets of methylases and restriction enzymes and did two 3-variable SAT problems with these enzymes. We used both plasmid and double-stranded PCR products for these reactions. We found this system works fairly well with some limitations in obtaining the correct answers to these problems.

2. Materials and Methods

The starting plasmid DNA, pBluescript SKII (Stratagene) was initially used and also the PCR product derived from amplification of the multiple cloning site of this plasmid with the M13-20 and reverse primers. Methylases

were obtained either from New England Biolabs (BamHI methylase, dam methylase, EcoRI methylase, HaeIII methylase, HhaI methylase, and HpaII methylase) or Takara (HindIII methylase and ClaI methylase). Restriction enzymes and Taq polymerase were obtained from New England Biolabs. For methylation, generally 11–15 μg of DNA was incubated in 250 μl of the preferred enzyme buffer in the presence of 120 μM of S-adenosyl methionine and 24–48 units of the methylases. This was incubated for 12 hours at 37°C, heated at 65°C for 15 minutes and then the DNA purified using QIAquick PCR Purification Kit (Qiagen). Restriction enzyme digestions occurred with approximately 0.3 μg DNA in 10 μl of the enzyme’s preferred buffer with 4–20 units of the restriction enzyme. The samples were incubated at the optimal temperature for the enzyme (37°C for all enzymes except SmaI which was incubated at 25°C and BssHII which was incubated at 50°C) for 1 hour. To confirm cleavage or not of the DNA, it was separated on an acrylamide (10% acrylamide Ready gel from BioRad) or agarose gel (0.8% in TBE buffer 0.09M Tris, 0.09M borate, 2mM EDTA) and visualized with ethidium bromide. Images of the gels were taken using a Kodak Imaging system.

3. Results

3.1. *Explanation of the system set-up and approach*

DNA methylases modify DNA so that certain restriction enzymes can no longer cleave the site. We wanted to test DNA methylation as a possible means for DNA computations in the aqueous style we have previously used [2, 4–6]. The overall scheme is in two stages for a standard 3-variable SAT problem (shown in Figure 1).

We first assign the six Boolean literals (p , p' , q , q' , r and r') specific sites on a given piece of double-stranded DNA (Figure 1A). These sites correspond to specific restriction enzymes sites much like we have done in other implementations [2, 4–6]. Initially, all of the sites can be cleaved by the restriction enzymes. We define a literal as being True if the site corresponding to this variable can be cleaved by the restriction enzyme. Thus, initially each literal is true which is not logically consistent (p and p' cannot both true). We created the collection of logically consistent DNA molecules by methylating the restriction enzyme sites to prevent them from being cleaved. We did this in pairs, each with half of the DNA (see Figure 1A where it is shown for variables p and p'). Thus, in half of the DNA, methylation is done on the p site while in the other half,

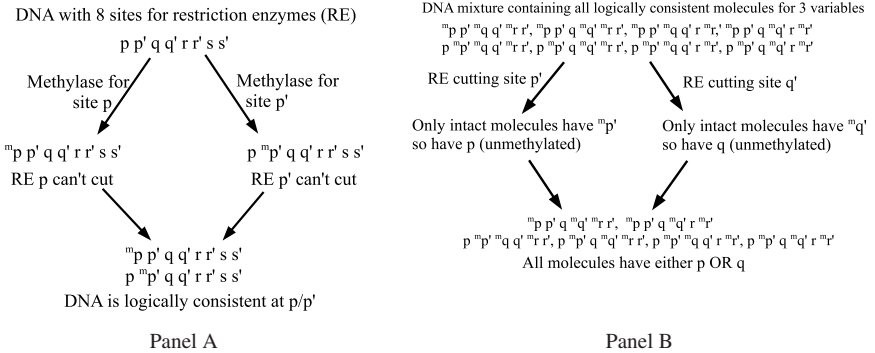


Figure 1. Representation of using methylation as a computation tool in a SAT problem. A. To create the logically consistent molecules at 4 variables we start with a plasmid DNA (or a PCR product) with 8 sites for distinct restriction enzymes (designated p , p' , etc.). Half of these molecules are treated with the methylase for the p site and half treated with the methylases for the p' site. The action of each methylase blocks the cutting by the corresponding restriction enzyme. When the product of those two reactions are recombined, the resulting DNAs are methylated either at p or at p' and so are considered logically consistent at this variable. This is continued for the other 3 variables to make a mixture of DNAs that are logically consistent at all 4 variables. B. To implement the clauses, we cut with the appropriate restriction enzymes to destroy molecules that do not satisfy the clause. In the example given, to implement p OR q , we separate the result from part A with logically consistent molecules at 3 variables into two pots. In one, we cut with the restriction enzyme for the p' site which should leave intact only those molecules where the p' site is methylated. In the other pot, we cut with the restriction enzyme for the q' site which should leave intact only those molecules where the q' site is methylated. When these two are combined the intact molecules have either the p site unmethylated (p' methylated) or the q site unmethylated (q' methylated) and so for the intact molecules p OR q has been satisfied. We continue with this approach for the other clauses.

methylation is put on at the p' site. When these two parts are recombined together, no DNA molecules contain both p and p' sites unmethylated and thus cleavable. By doing this reaction, we have enforced logical consistency for the pair of literals p and p' . In the problem, we then continue with the methylases corresponding to the sites for the literals q and q' and then the literals r and r' . The final resulting DNA is logically consistent with regard to each individual Boolean variable and should contain 8 distinct types of DNA molecules.

In the second stage, we proceeded with various cleavage steps to invoke the chosen clauses of the SAT problem being tested. The problem we used here was to determine if there is a truth setting for the Boolean variables p , q , r for which each of the four clauses p OR q , p' OR q OR r' , q' OR r' ,

$p' \text{ OR } r$ is True. Simple written work will show that the answer to this is Yes and the truth setting is p is False, q is True and r is False. To weed out DNAs that do not satisfy specific clauses we used the restriction enzymes associated with each of the sites. To represent the clause $p \text{ OR } q$, we poured the logically consistent DNA (above) into two tubes (Figure 1B). In one tube, we cut at the p' site (only those DNAs where p' is True will be cut), thus only DNA molecules where p' is False (p is therefore True) will be uncut or intact. In the other tube, we cut at the q' site (only those DNAs where q' is True will be cleaved), thus only DNA molecules where q is True will remain uncut or intact (Figure 1B). Then, we unite the content of these tubes and note that, the intact, uncut molecules have the property of $p \text{ OR } q = T$. We continued in a similar manner with each other clause. At the end of all the clauses, we determined if there is any intact, uncut DNA left and if so, what is the methylation status (and thus the designation) of each literal site. In this way, we hoped to be able to use methylation instead of the three-enzyme protocol we had used previously [2, 4, 5].

3.2. *Testing available methylases and restriction enzymes*

We found 8 commercially available methylases and the corresponding restriction enzymes that we used to confirm whether this system could be used as a binary modification on DNA and therefore a potential technique for DNA computations. These enzymes are listed in Table 1.

We first needed to confirm the specificity of each of the methylases for its target site and only that site on the DNA. For this experiment, we methylated the DNA first with a specific methylase, then we confirmed that the DNA was completely methylated using the target restriction enzyme. This was done by showing this restriction enzyme could not cut the DNA. Then, we cut the methylated DNA with all of the other restriction enzymes to confirm that these others could cleave this methylated DNA. All 64 combinations were tested (8 differently methylated DNA with the 8 different restriction enzymes), most both on plasmid and on PCR product. Checking of the *dam* methylase was only done on PCR product as the plasmid DNA was obtained from a *dam* positive host thus the DNA was already methylated at that site. In all cases, once completely methylated DNA was obtained, only the appropriate enzyme was blocked from cleaving the DNA. Some representative gels are shown in Figure 2.

We had at times difficulty in obtaining completely methylated DNA due to apparent low activity of some of the DNA methylases. We found that in

Table 1. Listing of methylase and restriction enzymes used for computation

Methylase	Seq. modified ¹	Restriction enzyme blocked & sequence recognized
BamHI methylase	G-G-A-T- ^m C-C	BamHI GGATCC ²
Dam methylase	G- ^m A-T-C	DpnII GATC ²
ClaI methylase	A-T-C-G- ^m A-T	ClaI ATCGAT
EcoRI methylase	G-A- ^m A-T-T-C	EcoRI GAATTC
HaeIII methylase	G-G- ^m C-C	NotI GCGGCCG
HhaI methylase	G- ^m C-G-C	BssHII GCGCGC
HindIII methylase	^m A-A-G-C-T-T	HindIII AAGCTT
HpaII methylases	C- ^m C-G-G	SmaI CCGGG

¹Sequence after methylase modification with the ^mA or ^mC indicating a methylated adenine or cytosine residue, respectively.

²Although the BamHI methylase and dam methylase overlap in their sequence specificity, the BamHI restriction enzyme is not inhibited by dam methylase modification while the DpnII restriction enzyme is not inhibited by the modification of the BamHI methylase. NdeI, Sau3A and MboI also cut at this site. MboI is not sensitive to methylation.

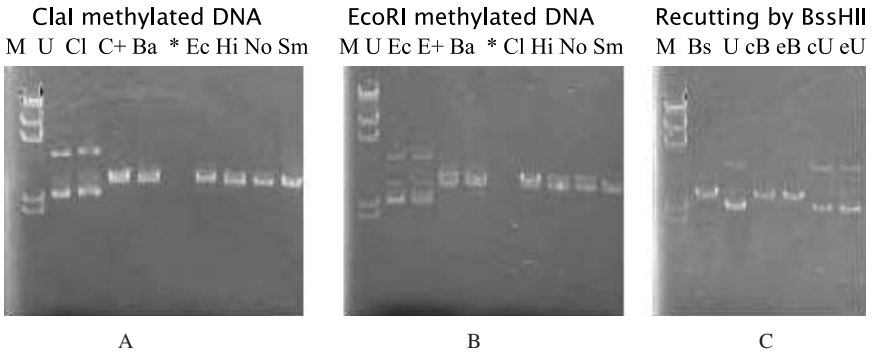


Figure 2. Confirmation that methylation does not affect the other restriction enzymes. Plasmid DNA was methylated with either ClaI methylase (panel A and C- labeled cB and cU) or with EcoRI methylase (panel B and C- labeled eB and eU), then left uncut (U, cU, eU) or cut with either ClaI (Cl), BamHI (Ba), EcoRI (Ec), HindIII (Hi), NotI (No), SmaI (Sm) or BssHII (cB, eB), then separated on a 0.8% agarose gel with a λ HindIII cut DNA marker (M). As a positive control, unmodified plasmid DNA was cut with Cla (C+), EcoRI (E+) or BssHII (Bs). Uncut DNA shows up as multiple bands on these gels representing open circular and supercoiled DNA while cut DNA shows up as one band at 3Kb.

some cases the DNA needed to be incubated with the methylase 2 or 3 times before the site was completely methylated (measured by complete lack of cleavage by the corresponding restriction enzyme). This was especially true of BamHI methylase and HindIII methylase. We tried longer incubations

and higher concentrations of S-adenosyl methionine and methylases but it did not always make any difference in the outcome. Thus, we showed that each of the individual methylases modified only their corresponding site and did not disrupt the cleavage by restriction enzymes recognizing different sites, so we could begin with the computations.

3.3. 3-variable SAT computation with plasmid DNA and PCR product

We tried two different schemes for doing the same satisfiability (SAT) problem with 3 variables and 4 clauses. In one scheme, we used circular plasmid DNA pBluescript that contains a multiple cloning site containing all the restriction enzyme/methylase recognition sites and in the other case, a PCR amplification product of this same region. The assignments of methylases for the individual Boolean variables for both the plasmid and PCR products are given in Table 2.

In both cases, we first created the self-consistent set of DNAs (ones where either p or p' is true, etc.) by incubating two portions of the DNA in parallel with the two appropriate methylases, for instance the p and p' methylase (scheme in Figure 1A). At each stage, we confirmed that each individual methylase had completely modified the DNA by showing the lack of cutting by the corresponding restriction enzyme before recombining the two halves. We then separated into two equal portions to continue with the other two methylases to represent the q and q' literals, followed with the methylation by the enzymes corresponding to the r and r' literals. In the final tube, we expect to have only logically consistent molecules (p or p' , etc) and all 8 possibilities of the different independent combinations. Following the preparation of this pool of different DNAs, we then performed restriction enzyme cleavage steps to remove those DNAs that did not satisfy the clauses using the scheme described above and in Figure 1B. Following

Table 2. Boolean variable assignments used

	For plasmid		For PCR product
p	HindIII site	p	BssHII site
p'	SmaI site	p'	NotI site
q	NotI site	q	BamHI site
q'	BssHII site	q'	DpnII site
r	ClaI site	r	ClaI site
r'	EcoRI site	r'	EcoRI site

the reduction in complexity of the DNA molecules using the clauses, we determined whether there was an answer (intact, uncut DNA) and if so, what the truth settings for the answer was for the 3 Boolean variables.

For plasmid DNA, we had 3 different ways to look for intact DNA. We could look for uncut circular DNA as we had in the previous work (see [5]). Alternatively, we could look for DNA able to transform bacteria as the pBluescript plasmid contains an origin of replication for *E. coli* bacteria and an ampicillin resistance gene for selecting bacteria containing the plasmid and only circular, intact plasmid DNA will efficiently transform bacteria (see [5]). Finally, we could use PCR to amplify the full multiple cloning site using primers annealing to the two different sides of this region (M13 reverse primer and M13 -20 primer). Practically, we found too little of the plasmid DNA to detect on a gel directly but we did find enough circular DNA to transform bacteria (data not shown) and to produce a PCR product (Figure 3, lane 2).

Thus, there is an answer that satisfies all the clauses. To determine the status of each literal, we cleaved the plasmid DNA first with each of the six restriction enzymes, then performed PCR using the primers that were located on either side of the multiple cloning site. If a site is methylated, the enzyme should not cut (site is defined as False) and a PCR product should

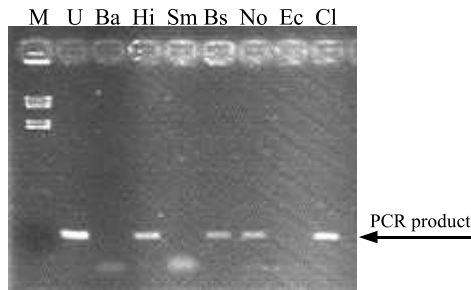


Figure 3. PCR product from treated plasmid DNA following SAT computation using methylation. Circular plasmid resulting from the SAT computation was either untreated (U) or cut with various restriction enzymes (BamHI=Ba, HindIII=Hi, SmaI=Sm, BssHIII=Bs, NotI=No, EcoRI=Ec, ClaI=Cl) then amplified using PCR with M13 -20 and reverse primers and the product separated on a 1.5% agarose gel with the λ DNA cut with HindIII as molecular weight marker (M). If a PCR product is present, that indicates the plasmid is intact and the site was not cut (presumably due to the site being methylated) and therefore the site would be considered False. If there is no PCR product produced, that indicates the restriction site was cut and therefore the site would be designated True. Uncut DNA and BamHI cut DNA act as positive and negative controls, respectively.

be formed. If a site can be cleaved (non-methylated and is True), the sites for the two opposing PCR primers are separated from each other and no product is formed. We used this strategy to detect the status of the 6 sites involved (Figure 3). The final product of the clauses can act as a template for successful PCR to produce a 300 bp fragment. Cleavage by an enzyme found in the multiple cloning site but not used in the computation shows that cleavage blocks the ability to form a PCR product (Figure 3, lane 3). Then the figure shows the pairs of sites next to each other on the gel. It is clear that cleavage by HindIII and ClaI still allow the production of a PCR product suggesting those sites are methylated. On the other hand, cleavage by enzymes SmaI and EcoRI block the ability of the PCR to produce a product (Figure 3, lanes 5 and 8). These sites are thus defined as true. This makes both p and r to be False as SmaI and EcoRI represent p' and r' , respectively. Unfortunately as can be seen in the figure, both NotI and BssHII cleavage result in some PCR product although in both cases the amount of product is decidedly less than that detected after HindIII or ClaI cleavage. The NotI and BssHII enzymes represent the literals q and q' , respectively and thus we do not expect both to be false as represented by the data presented (Figure 3). The expected answer for the problem is p is False, q is True, and r is False. We found the correct settings for both p and r , but not for q where the results call both q and q' as False.

We also performed the satisfiability problem with PCR product from an amplification of the multiple cloning region containing all of the sites used (see above). At the end of the clauses, we separated the resulting DNA on an acryl-amide gel to determine if any full length PCR product still existed (Figure 4B, lane 6). The full-length PCR fragment was detected on the gel along with many smaller cleavage products that had not been removed from the mixture following the clauses. Next, to determine the status of each of the literals, we incubated the remaining DNA with different restriction enzymes followed by acrylamide gel electrophoresis (Figure 4). Cleavage by EcoRI results in significant reduction in the amount of full-length PCR product detected relative to cleavage by the alternative enzyme site, ClaI (Figure 4A). Likewise, cleavage by DpnII results in more full-length DNA than cleavage by the enzyme BamHI (Figure 4B, lanes 2 and 3). When the DNA is incubated with either NotI or BssHII, more of the full-length PCR product was detected after cleavage by the latter enzyme than the former although there is still significant full-length PCR product in the case of the sample treated with NotI. Also, still some full-length DNA is visible in the EcoRI and BamHI digested samples suggesting incomplete cleavage

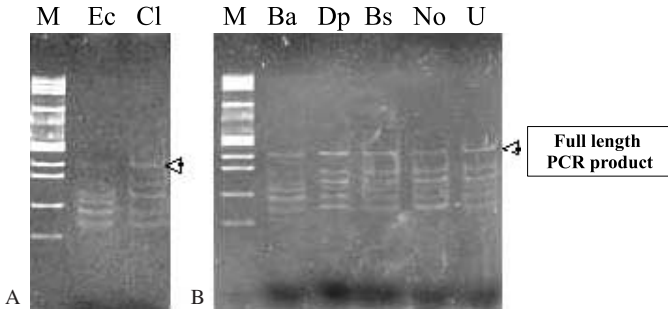


Figure 4. Analysis of DNA following SAT computation using methylation of PCR product. DNA fragments resulting from the SAT computation using PCR product as the initial template was either untreated (U) or cut with various restriction enzymes (EcoRI=Ec, ClaI=Cl, BamHI=Ba, DpnII=Dp, BssHII=Bs, NotI=No,) then separated on a 10% acrylamide gel with the θ X174 DNA cut with HaeIII as molecular weight marker (M). The arrowheads mark the full length PCR product. If the full-length PCR product is present, that indicates the DNA is intact and the site was not cut (presumably due to the site being methylated) and therefore the site would be considered False. If there is less full-length PCR product than in the untreated sample, that indicates the restriction site was cut and therefore the site would be designated True. Uncut DNA acts as a positive control.

of the final product and potentially incompletely methylated DNA. Thus, through the literal assignments in Table 2, we come up with the answer p is False, q is True and r is False with the caveat that there is still a significant amount of uncut DNA in all samples.

4. Discussion

To analyze the possibility of using the bacterial DNA methylation system as a means for DNA computations, we first had to explore whether the methylase enzymes were functioning and specific, meaning they would alter only the recognition of their linked restriction enzyme and would not alter the recognition by any of the other restriction enzymes we used. This appeared to be the case. We found evidence that the DNA methylase enzymes were sequence specific and no evidence of cross-methylation to affect the other restriction enzyme sites used in this study. We then used these 8 methylases and 8 restriction enzymes in two DNA computations in the aqueous style of our previous work. With this approach, we were able to perform two 3-variable SAT problems using 4 clauses. In both cases, we found evidence for an answer, however, the answer provided contained two possible states for a single given variable. Simple analysis of the chosen clauses did

not indicate two possible answers. Thus, we found some problems with the implementation of our approach.

There are at least three explanations for the lack of complete success of these SAT problems using the methylation approach, incomplete methylation during preparation of the logically consistent DNA set, incomplete digestion during implementation of the clauses or incomplete digestion during the reading phase of the computation. If the original DNA was incompletely methylated during the preparation, some DNAs in our mixture might contain both paired sites available for cleavage. As stated above, we often had problems getting completely methylated DNA during the preparation of the logically consistent DNAs and had to repeat the methylation step 2 and 3 times for two enzymes. We did not proceed with the computation unless we observed complete lack of cutting by the restriction enzyme associated with the methylation site. But, potentially if a small amount of incompletely methylated DNA were still present, we may not have seen it on our gels yet it may have carried through the clause steps. However, in this case, we would have expected no answer as more DNA would have been destroyed during the implementation of the clauses than should have been due to the lack of a methylated (protected) site. What we found were answers that had both True and False as states of the variable which is not as easy to explain through lack of complete initial methylation. One alternative explanation for the partial lack of success is less than complete digestion by the restriction enzymes during the implementation of the clauses. This would allow some DNA to persist that should have been removed during these steps. Related to this explanation is the one where incomplete cutting may have occurred during the reading phase of the computation after all the clause steps had been completed. Either of these explanations would likely be consistent with the appearance of a DNA answer that should not have been maintained in the population.

In both attempts at the SAT problem, the enzyme pair NotI and BssHII was associated with the variable having the two possible answers. The latter of these two enzymes requires cutting at 50°C while the other works at 37°C. During the implementation of the clauses, we digested the DNA with one or the other of these enzymes to destroy the appropriate DNA molecules. In reading the final answers, we digested with these two enzymes as well before either performing PCR (with the plasmid) or before running on an acrylamide gel (for the PCR product). In neither case, did we confirm complete digestion of the material during clause implementation potentially allowing a small bit of incompletely digested DNA to persist in the mixture.

In the end, we only assess the presence of complete, uncut DNA from the original mixture and if incompletely cut material has carried over from previous steps, we cannot easily distinguish this from the appropriate answer without imposing our own biases. In both cases where the answers obtained showed two possible states of the variable, we were using the methylases HhaI and HaeIII and the restriction enzymes BssHII and NotI to test these sites, respectively. We expected the final answer molecule in both cases to be methylated at the BssHII site and have the NotI site unmodified. However, the molecule with the reverse arrangement seemed also to be present in both cases. Both answers maintained an intact molecule with a BssHII site unmethylated when this should have been destroyed during the clauses or the reading. If at either point, the BssHII enzyme did not cut the material completely, it would appear to be methylated at this site. Again, due to the challenges with this restriction enzyme, we feel that either incomplete digestion by BssHII during the clause or reading phase may explain our lack of complete success. Other methylases or an isoschizomer for BssHII that cuts more reproducibly would help make this approach more practical.

We undertook this work to find an alternative to our 3-step, 3-enzyme reaction to modify the state of each variable to make logically consistent DNA molecules [2, 4, 5]. We hoped that the use of a single enzyme, namely the methylases, would significantly speed up our processing. This has happened. However, our new approach has a number of limitations. In some cases, the methylase enzymes were not as effective in modifying the DNA and we found we needed to repeat certain methylation steps 1 or 2 times before completely methylated DNA at a site was obtained. We were also limited by the amount of material left at the end of the computation in our procedure making detection sometimes difficult. With these reactions, it is not possible to amplify the DNA material before the end of the computation as the action of the Taq polymerase to copy the strands during PCR alleviates the methylation state at these sites. It would be necessary therefore, to start with enough material to obtain a clear answer at the end. With the necessity of re-methylating some of the samples 1 and 2 times, the loss of material became more acute. There may be a way to remethylate the required strands after PCR as some DNA modification enzymes have been isolated that will modify hemi-methylated DNA to make both strands methylated [8]. This would have to be done after each round of PCR to reinstate the methyl groups at the positions of the parent strands. This may make it possible to expand this approach to make it easier to detect the answer with a small amount of material. We did use PCR but only in the last

reading step where we were ascertaining the existence of an intact plasmid molecule after cleavage. The methylation approach is also limited by the number of commercially available methylases and corresponding restriction enzymes. Our previous approach only required a restriction enzyme that left an overhang after cleavage that could be filled in through the action of a DNA polymerase [2, 4, 5]. There are many hundreds of these types of restriction enzymes that are available. In our new method, we used 8 methylase enzymes and their corresponding 6-base cutting restriction enzymes. Many more methylases are known but are not yet commercially available. Thus we have a difficult limitation to our technique. It is possible other DNA modifications or the use of DNA binding proteins to protect certain sites from methylation could be used in a further expansion of this technique. Thus, this advance may make it possible to expand this approach to a much larger number of Boolean operators in the future.

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