A hypothetical protocol for DIYBIO DNA synthesis

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1 Introduction

DNA synthesis has traditionally been beyond the home enthusiast or small amateur groups. Most DNA synthesis these days involves the phosphoramidite method. [17] This involves a number of hard to acquire and often toxic chemicals. Some work has been done to reduce the burden of expense of these special chemicals and increase throughput by printing lots of different DNA sequences on one surface or by synthesis in small capillary chambers in microfluidic devices. [16, 11, 12] However for many DIYBIO enthusiasts the obstacle to DNA synthesis is not cost but the difficulty of finding a producer willing to supply these chemicals. Here we present a hypothetical method for synthesising medium to long DNA sequences with out resorting to rare and potentially toxic chemistry. It is our hope that those more expert than our selves will assist in refining and providing feedback on the feasibility of the scheme.

2 Key points of utility in the method

2.1 Terminal Deoxynucleotidyl Transferase (TDT)

TDT is structurally similar to DNA Polymerise. It's function is to add a random nucleotide to a single strand of DNA. [14] It has two isoforms a short and a long one the but only the short form is believed to add bases. TDT requires at least 3 nucleotides in a single strand of DNA to attach to perform it's function.

2.2 Thermolabile blocked dNTPs

Currently it's posable to purchase deoxynucleoside triphosphates who's 3 prime hydroxyl groups have been blocked with a thermolabile group. These groups prevent further addition to the DNA strand when incorporated by TDT. [5, 10, 1] However this group can be removed by simple heating restoring the 3 prime hydroxyl group. These dNTPs are typically used for hot start PCR to block the polymerase reaction until higher temperatures are achieved.

2.3 Piezoelectric Printheads

Unlike thermal inkjet technology piezoelectric printheads can deposit chemicals on surfaces in small well defined droplets with out breaking down the chemicals involved and has been previously used for printing DNA micro arrays as well as depositing functional enzymes in precise patterns. [11, 20] One posable issue is ensuring the viscosity of the printed fluid is high enough to form well defined droplets. A potential solution is to add glycerol, a common PCR additive, to increase the viscosity of a TDT or dNTP containing solution. [3] This has the additional advantage of inhibiting hairpin formation somewhat.

2.4 Type IIS restriction endonuclease

These restriction endonucleases typically cut outside of their recognition sequence. [13, 2] Consequently if one wishes to release a section of arbitrary DNA from a larger section of DNA bound to a solid support one can do so provided that large section of DNA has the appropriate recognition sequence immediately before the section to be released and a complimentary section of DNA for the recognition sequence is provided. In this case the the restriction enzyme will cut the arbitrary section of DNA away from the recognition sequence still bound to the solid support along with possibly a very short section of the complimentary DNA.

2.5 Ligase assembly

Ligase can join sections of DNA as little as 8 nucleotides long. [9] When relatively short sections of DNA are ligated there is a potential error checking effect as erroneously synthesised strands typically occur due to an omitted nucleotide in the sequence. In this case the erroneous strand will usual fail to anneal to the complimentary strand or be too short to properly ligate unless the complimentary strand features an omission in the same location. In this case one can achieve some degree of error correction by mixing the correct and with them erroneous oligos for ligation then purifying the resultant fully ligated product by length. This will hopefully exclude most errors and give the desired sequence.

3 Protocol

1. Preprepared short DNA strands are attached at the 5 prime end to a glass slide. Preferably using a template with holes in it laid on the slide to ensure than only certain areas are treated.

2. A piezoelectric print head layers TDT and selected Thermolabile blocked dNTPs on the slide in a precise pattern.

3. The glass slide is washed clean.

4. A solvent is deposited on the slide which is then heated to remove the Thermolabile blocking groups.

5. The slide is washed clean.

6. Repeat steps 2 to 5 as needed.

7. The slide is removed and a template laid on top of it forming wells that contain several different printed DNA spots.

8. These wells are filled with a solution containing a type IIS restriction endonuclease enzyme and optionally the complimentary DNA that binds at the recognition site. Alternatively the DNA synthesised can be chosen to form a hairpin, where the desired DNA forms the loop and adjacent DNA, and the double stranded section the recognition site.

9. The DNA containing solutions are transferred out of the wells into separate containers and the endonuclease purified out or heat inactivated.

10. The separate DNA samples are subjected to a ligation reaction.

11. The desired ligated DNA strands are separated out and retained using standard methods (for example PAGE electrophoresis).

12. The extracted DNA is combined in an assembly PCR reaction producing the final DNA product. [19]

4 Details and Points of Interest

4.1 The hairpin versus complimentary additive method

The advantage of forming a hairpin to release the DNA is that both the sides of the recognition site can be synthesised de novo which means the initial DNA bound to the solid support can be arbitrary DNA of varied sequences provided it's of a suitable length. There is no need to order oligos from a company to start the process. If the released section of DNA is short enough erroneous deletions may prevent the hairpin forming. If the delegations are in the recognition site they will likely prevent the erroneous DNA from being released. In this case restriction endonuclease that leave a long 5 prime overhang are ideal as they allow the released section to form part of the recognition site. Potential enzymes we've identified for this purpose are BbvI, BcoDI, BsmAI, BsmFI, FokI, HgaI, SfaNI, BbsI, BfuAI, BsaI, BsmBI, BspMI, BtgZI, EarI, SgeI, BslFI, BsoMAI, Bst71I, FaqI, AceIII, BbvII and BveI. [2] Of these the shorter ones with a larger number of unspecified nucleotides are largely to be favoured to allow greater flexibility in the design process and higher yields of correct released DNA. The issue with hairpin release is the possibility the

strand ends hybridising with the base of a neighbouring strand releasing a section of DNA with part of the recognition sequence attached. Consequently it's important to design this sequence to prevent unintended ligation. On the other hand achieving DNA release through use of a complimentary strand added with the restriction digest mix is simpler. Also if preprepared oligos are used all or most of the recognition site can be part of the initial oligo bound to the solid support. Therefor only released DNA is synthesised by TDT which should improve the yield of the correct DNA sequence considerably. Also if these oligos are ordered from a professional supplier they can be preprepared for attachment to the solid support simplifying this process. in this case a restriction endonuclease which creates blunt ends or a short 3 prime overhang is preferable to ensure the cut section of complimentary DNA will be too short to take part in ligation. The potential enzymes we have identified as posable candidates are MnlI, HphI, HpyAV, MboII, MlyI, BciVI, BmrI and BmuI. [2] As with the hairpin method restriction endonucleases with a large number of arbitrary nucleotides in their recognition sequences are to be favoured but length is less important as the recognition site is not de novo synthesised. It should be noted that because it may be necessary to have at least one nucleotide of the complimentary DNA overlap with the released DNA product it may be necessary to use 4 variants of the complimentary strand. Ostensibly the DNA for preprepared oligos must be ordered from a conventional oligo supplier. However it is at least theoretically posable to purify the sequence from a plasmid derived from cell culture if the DNA with the sequence was adjacent to DNA that formed an aptamer. [15] However this DNA would be methylated and so would require a further PCR reaction involving another hairpin to act as a primer to acquire unmethylated DNA. Alternatively using a yeast cell to replicate the oligo might avoid this. [4] All In all a long winded means of a acquiring an oligo involving multiple restriction digests and affinity chromatography. However possibly worth it to acquire large amounts of oligo with out primers by leveraging cell replication to make lots of DNA. Indeed this would be one way of acquiring large amounts of primer with out ordering oligos.

4.2 Potential issues

One of our main concerns is that after PAGE purification of the ligation products there is a risk that not enough DNA will be recovered for assembly PCR. The amount of DNA produced from a non porous glass surface may be quite small and not all of the synthesised DNA will be released and not all of the released product will be able to ligate. Also the choice of how the DNA is divided up between print spots and different wells will be critical as well as design of the recognition sites. The ligation between each strand in a given well must be absolutely specific with no possibility of cross talk with the wrong strand particularly not any unannealed recognition site complimentary DNA. Likewise any areas of similarity in the over all sequence must lie towards the centre of the ligation product from each well to ensure that annealing of strand ends in assembly PCR adheres to a unique sequence. [19] Its very likely the final product will need additional PCR to get a

usable amount of DNA.

4.3 Availability of dNTPs with Thermolabile blocks

Currently individual dNTPs with with Thermolabile blocks can be purchased under the brand name CleanAmp from TriLink biotechnologies. [1] In the future this product may be withdrawn or possibly only sold with the bases premixed. Therefor it is highly desirable that a DIY method for adding removable 3 prime blocking groups to dNTPs be developed. Blocks that can be removed with out resorting to aggressive chemicals that would require the dNTPs to have other protecting groups added. It's often been suggested that finding a way to synthesise DNA synthesis reagents would be a good idea but the chemistry involved is complex and the number of reagents large. However since this method does not involve any special non enzyme reagents finding a way to synthesise reversibly blocked dNTPs from standard dNTPs would seem a more manageable challenge compared to synthesising reagents for the phosphoramidite method. It has also not escaped our attention that blocked dNTPs facilitate sanger sequencing. [18]

4.4 Availability of enzymes

Since this method is dependant on enzymes and potentially consumes a large amount of them even if the enzymes involved remain readily available the cost may be prohibitive. However these enzymes are easy enough to synthesise using recombinant techniques. [6] One could easily envision a kit of preprepared cell cultures each capable of producing the enzymes needed for this process. These enzymes could be purified from the lysed cells possibly with a method as simple as nickel affinity chromatography. [6, 8] In this case sharing the technology would be as simple as offering some one samples of your cell cultures to establish their own. Of course it would still be necessary to construct an enzyme printer but piezoelectric print heads are readily available in epson printers and Epson's own patents provide sufficient information to drive them. [7] It may even be posable to purify oligos and primers from cell culture although as mentioned the endeavour would seem some what vexed.

5 Closing Comments

It is our hope that this laying out of ideas on the topic will prove beneficial for those with an interest in synthesising their own DNA. We are seeking feedback on the feasibility of the method from those more expert than our selves. Indeed we would be interested in building a synthesis machine based on the proposed protocol which we propose to refer to as a ?tyndale?. However we lack the resources to do this at present but would be more than willing to collaborate or assist any labs, bio hacking collectives or hackspaces wishing to pursue this methodology.

References

[1] Cleanamp hot start pcr. http://www.trilinkbiotech.com/cleanamp/, 2016.

[2] Enzyme finder. https://www.neb.com/tools-and-resources/interactive-tools/enzyme-finder?searchType=6, 2016.

[3] Pcr problems try an additive. http://bitesizebio.com/24/pcr-problems-try-an-additive/, 2016.

[4] Floriana Capuano, Michael Mülleder, Robert Kok, Henk J Blom, and Markus Ralser. Cytosine dna methylation is found in drosophila melanogaster but absent in saccharomyces cerevisiae, schizosaccharomyces pombe, and other yeast species. *Analytical Chemistry*, 86(8):3697–3702, 04 2014.

[5] Castillo, Fung, Lin, Park, Jang, Najjar, Orrico, Poch, Sigal, Wong, Wong, Yeh, Lutz, and Medvedik. De novo enzyme mediated oligonucleotide synthesizer. http://2014.igem.org/Team:Cooper_Union/TdT_project, 2014.

[6] Aysha Divan and Janice *Tools and techniques in biomolecular science*. Oxford University Press, 2013.

[7] T. Fukano. Ink jet printer. http://www.google.com/patents/US6758544, 2004.

[8] Paul N Hengen. Purification of his-tag fusion proteins from escherichia coli. *Trends in Biochemical Sciences*, 20(7):285–286, 7 1995.

[9] Daniel R. Horspool, Robin JN Coope, and Robert A. Holt. Efficient assembly of very short oligonucleotides using t4 dna ligase. *BMC Research Notes*, 3(1

[10] Inna Koukhareva and Alexandre Lebedev. 3-protected 2-deoxynucleoside 5-triphosphates as a tool for heat-triggered activation of polymerase chain reaction. *Analytical Chemistry*, 81(12):4955–4962, 06 2009.

[11] Christopher Lausted, Timothy Dahl, Charles Warren, Kimberly King, Kimberly Smith, Michael Johnson, Ramsey Saleem, John Aitchison, Lee Hood, and Stephen R. Lasky. Posam: a fast, flexible, open-source, inkjet oligonucleotide synthesizer and microarrayer. *Genome Biology*, 5(8

[12] Cheng-Chung Lee, Thomas M Snyder, and Stephen R Quake. A microfluidic oligonucleotide synthesizer. *Nucleic Acids Research*, 38(8):2514–2521, 05 2010.

[13] Shaun M Lippow, Patti M Aha, Matthew H Parker, William J Blake, Brian M Baynes, and Daša Lipovšek. Creation of a type iis restriction endonuclease with a long recognition sequence. *Nucleic Acids Research*, 37(9):3061–3073, 05 2009.

[14] Edward A. Motea and Anthony J. Berdis. Terminal deoxynucleotidyl transferase: The story of a misguided dna polymerase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1804(5):1151–1166, 5 2010.

[15] Branislav Nastasijevic, Nicole A Becker, Susan E Wurster, and L James 3rd Maher. Sequence-specific binding of dna and rna to immobilized nickel ions. *Biochem Biophys Res Commun*, 366(2):420–425, Feb 2008.

[16] A C Pease, D Solas, E J Sullivan, M T Cronin, C P Holmes, and S P Fodor. Light-generated oligonucleotide arrays for rapid dna sequence analysis. *Proceedings of the National Academy of Sciences*, 91(11):5022–5026, 1994.

[17] Colin B. Reese. Oligo- and poly-nucleotides: 50 years of chemical synthesis. *Organic & Biomolecular Chemistry*, 3(21):3851–3868, 2005.

[18] F. Sanger, S. Nicklen, and A. R. Coulson. Dna sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74(12):5463–5467, 1977.

[19] Willem P. C. Stemmer, Andreas Crameri, Kim D. Ha, Thomas M. Brennan, and Herbert L. Heyneker. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene*, 164(1):49–53, 10 1995.

[20] Tianming Wang, Chris Cook, and Brian Derby. Fabrication of a glucose biosensor by piezoelectric inkjet printing. In *Sensor Technologies and Applications, 2009. SENSORCOMM'09. Third International Conference on*, pages 82–85