5-15-2008

Cyclic Enzymatic Solid Phase Synthesis of DNA Oligonucleotides on an Epoxide-Activated Resin

Ahmed Mirza Khan
mahmedk@hotmail.com

CYCLIC ENZYMATIC SOLID PHASE SYNTHESIS OF DNA OLIGONUCLEOTIDES ON AN EPOXIDE-ACTIVATED RESIN

by

Ahmed M. Khan

Under the Direction of Dr. Markus W. Germann

ABSTRACT

Standard chemical DNA synthesis with isotope labels requires expensive reagents; moreover, a large excess of phosphoramadites (typically 50-100 fold) must be used. We developed a process where enzymatic cyclic solid phase synthesis of DNA allows for more economic reagent use. A DNA template was immobilized on an epoxy-activated solid support. This chemistry was chosen because the formed linkage is inert to high pH conditions. High efficiency of the covalent attachment was observed when the reaction was carried out in $MgCl_2$/CAPS buffer. It was found that Mg^{2+} enables the reaction to be completed over a period of 14 h, compared to 72 h under standard conditions. DNA synthesis was carried in a cyclic fashion on a support bound DNA using Klenow fragment.

INDEX WORDS: Cyclic enzymatic DNA synthesis, Epoxy-activated resin, Solid-phase synthesis, labeled DNA oligonucleotide

CYCLIC ENZYMATIC SOLID PHASE SYNTHESIS OF DNA OLIGONUCLEOTIDES ON AN EPOXIDE-ACTIVATED RESIN

by

Ahmed M. Khan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2008

CYCLIC ENZYMATIC SOLID PHASE SYNTHESIS OF DNA OLIGONUCLEOTIDES ON AN EPOXIDE-ACTIVATED RESIN

by

Ahmed M. Khan

Committee Chair: Markus W. Germann

Committee: Kathryn B. Grant
W. David Wilson
Markus W. Germann

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2008

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
dH_20	De-ionized water
DMT	Dimethoxy-trityl
dNTP	deoxynuceotide tri-phosphate
ESRA	Endonuclease sensitive repeat amplification
h	Hours
HPLC	High performance liquid chromatography
Kf	Klenow fragment
min	Minutes
NMR	Nuclear magnetic resonance spectroscopy
OD_{260}	Optical density at A_{260}
PAGE	Polyacrylamide gel electrophoresis
s	Seconds
TBE	Tris-borate ethylene diamine tetraacetic acid
u	Units
UV-Vis	Ultraviolet-Visible

Chapter 1: Background and Introduction

Nuclear magnetic spectroscopy is a powerful tool in solving solution structures for proteins and nucleic acids. However, spectral overlap in these macro molecules is a common problem. Specific-labeling with isotopes reduces the complexity and increases dimensionality of the spectra, enabling recording of nD (multidimensional) NMR experiments.

Various methods have been described to produce ^{13}C and ^{15}N labeled DNA oligonucleotides. Labeled oligonucleotides can directly be synthesized using a DNA synthesizer, but a large excess of expensive phosphoramidites must be employed. Endonuclease sensitive repeat amplification (ESRA) can be used to efficiently increase product yield [1]. While utilizing labeled dNTPs, this process amplifies labeled end-product amounts with the use of a method similar to PCR. Desired DNA, which is embedded in the growing strands, is acquired by cutting with restriction enzymes. Although efficient, ESRA products are double-stranded complementary DNA sequences [1]. Annealing of other complementary sequences containing mismatches of choice to the strands is not easily achieved. Also complications can occur during amplification if the desired product is intended to be a hairpin (i.e., self-complementary).

Another method to produce labeled products is described by Zimmers and Crothers where enzymatic synthesis with labeled dNTPs was done on a template [2]. The product is cleaved with a ribonucleotide at the 3' end under alkaline conditions. Even though considerable quantities of labeled single-stranded DNA can be produced, expensive triphosphates are not reused. Unincorporated labeled dNTPs were purified from template and products using a spin-column in buffer. The tri-phosphates are reused

but losses occur as they have to be desalted. This approach provides a novel way to make labeled DNA; however the method can be improved.

Synthesis of isotope-labeled DNA strands requires numerous steps. If these series of steps were done in repetitive cycles, more efficient yields could be achieved. This is accomplished by first attaching a DNA-template, used for new DNA fill-in synthesis, to an epoxide-activated resin. Previous studies indicate conjugation of the DNA to the resin requires prolonged amounts of time [3, 4]. In the work described in this thesis, a modified 26 base long DNA strand (Figure 1), bound to a support, was used as a template to synthesize and isolate a 10mer in a cyclic fashion.

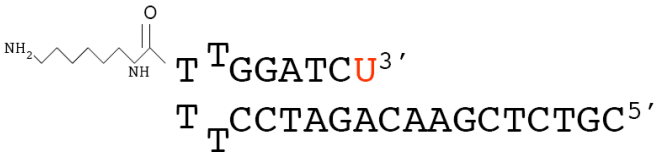


Fig. 1: Sequence of the structure of 26mer used template

This investigation introduces a cost-effective means to produce such strands. Additionally, it brings to light the proficiency of binding the DNA to epoxide-activated resin under conditions that makes this conjugation efficient. The cycle encompasses two major components that are investigated in detail. First, the binding of the 26mer DNA strand to the resin needs to be optimized before other reactions are performed. Secondly, the reactions which synthesize and isolate the 10mer are studied to maximize yield. To covalently attach the 26mer to an epoxide-activated support, a modification which provides a proper ligand-linker-arm is synthesized in the strand. The strand itself forms a hairpin due to its intra-strand complimentary sequence and contains a ribonucleic uracil at the 3' end. This unique feature allows it to function both as a site for 10mer

polymerization and subsequent cleavage. Isolation of 10mer after synthesis is achieved using the 3' terminus unique 2' hydroxyl of uridine nucleotide. Under alkaline conditions, this hydrolyzes the newly synthesized strand (Figure 2) [2].

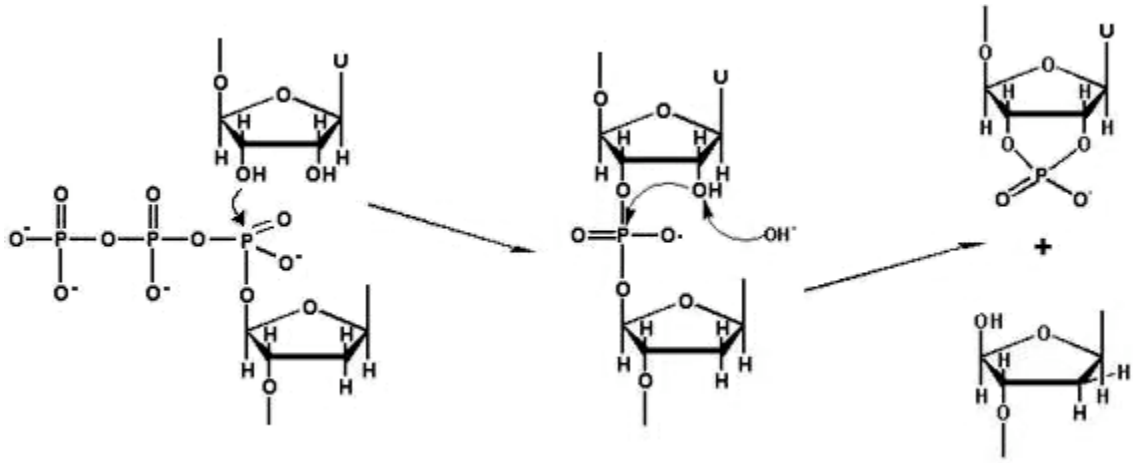


Fig. 2: Ribonucleic uracil at the 3' terminus is used both as primer and cleavage site

The modified thymidine nucleotide attached to the linker-arm, positioned in the hairpin loop, contains an alkyl primary-amine providing space between the DNA and resin to avoid steric constraints.

Amines have a pK_a of close to 9.0 and are therefore deprotonated (NH_2) at a higher pH (9-11). This primary-amine ligand facilitates a nucleophilic attack to the epoxide ring following the mechanism illustrated in Figure 3.

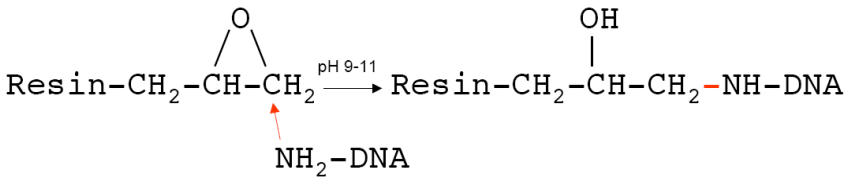


Fig. 3: Nucleophilic attack of primary amine at the end of linker covalently binds template to the resin

As previously described by Wheatley and Schmidt, binding of DNA possessing a linker-arm to an epoxide-activated support is not efficiently achieved [4]. Their proposed solution is that higher potassium phosphate (3 M K_2HPO_4) buffer concentration immobilizes 95% of DNA in 75 h [4]. However, other possible salt and buffer combinations which might be efficient were not explored. Monovalent cations such as Na^+ and K^+ were used in our study to provide DNA stabilization and screening. In spite of this, none or only slow conjugation was observed since the negatively charged DNA had to react with an also negatively charged resin.

The resin matrix (Figure 4), a branched polysaccharide made of many glucose molecules joined into chains of varying lengths, carries an overall negative charge under our binding conditions (pH 9.4) [5].

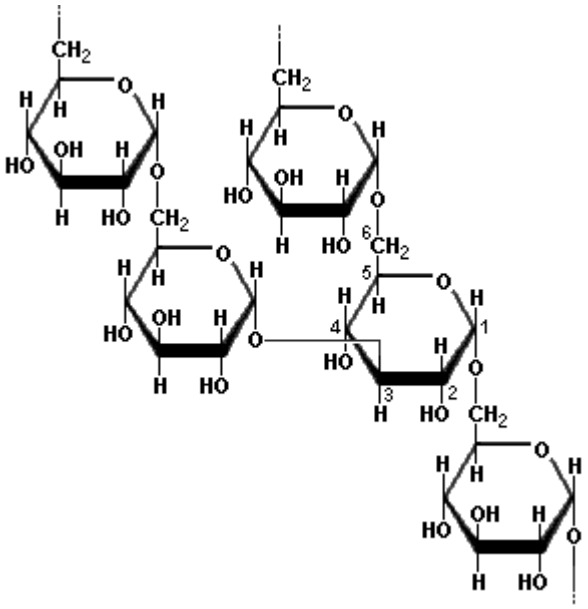


Fig. 4: Dextran matrix

The DNA itself is also negatively charged and is repelled by the matrix. This electrostatic repulsion negatively affects the rate of conjugation. The overall charge of the DNA can be reduced using multivalent cations, thereby facilitating the conjugation to the support. The divalent cation Mg^{2+} better screens the negatively charged DNA structure [6]. We find that Mg^{2+} enables the reaction to be carried out over a period of 14 h, compared to 72 h described previously [4].

Another primary-amine ligand with a different charge distribution was investigated as a control. Tryptophan, an amino-acid with a single negative charge under our reaction conditions (Figure 5), was treated with the resin in buffers used in DNA binding. Tryptophan can be quantified at 280 nm due to its aromatic side chain.

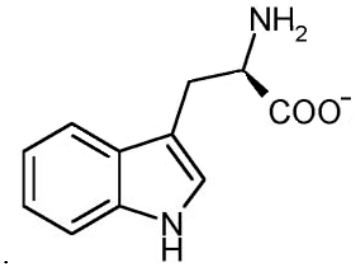


Fig. 5: A tryptophan molecule possesses a deprotonated primary amine at reaction pH

The simultaneous hydrolysis of the epoxide groups causes blockage of ligand binding sites over time (Figure 6) [7]. Our studies with tryptophan conjugation showed that Mg^{2+} also accelerates hydrolysis of the epoxide groups.

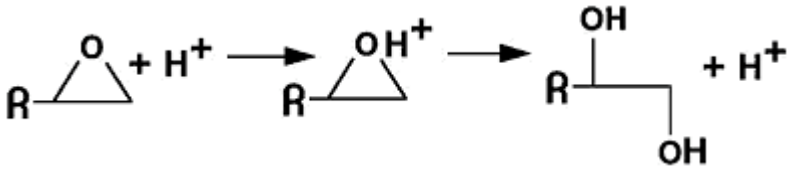


Fig. 6: Simultaneous hydrolysis of the epoxide groups hinders the site from further attack

Confirmation of binding larger amounts of DNA while using Mg^{2+} was assessed by keeping a dimethoxy-trityl (DMT) protective group at the 5' end of the template after synthesis. Once the oligonucleotide was found to be bound to the resin by quantifying supernatant wash-offs, trichloroacetic acid was introduced to the resin in order to cleave off the trityl group from the template bound to the resin (Figure 7).

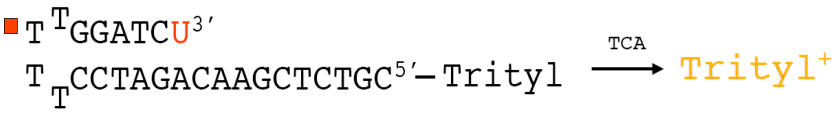


Fig. 7: Once conjugated to the resin, introduction of TCA cleaves the trityl group from DNA

Under these acidic conditions, the cleavage of the DMT group from the 5' end leaves it positively charged while producing a bright orange color (Figure 8) [8]. The supernatant was then quantified using UV-Vis at 498 nm; giving the amount of DNA bound to the resin.

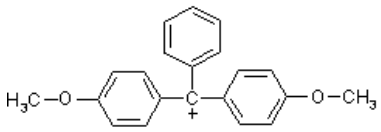


Fig. 8: Dimethoxy-trityl cation (λ_{max} = 498; ε = 70,000 M^{-1} cm^{-1})

After attachment of 26mer hairpin to resin, synthesis of 10mer on the template is completed using Klenow fragment, a 3'→ 5' exonuclease deficient mutant of *Escherichia coli* DNA polymerase I [2]. The enzyme utilizes the 3' hydroxyl of uracil at 3'end of the template to form the first phosphodiester bond to start polymerization (Figure 2). The

rate-determining step here is the enzyme locating the 26mer on the resin and attaching itself to it, after which the extension is done quickly using dNTP's [2]. Because of this, short terminated fill-in sequences are not expected. Figure 9 shows a schematic illustration of the cyclic process.

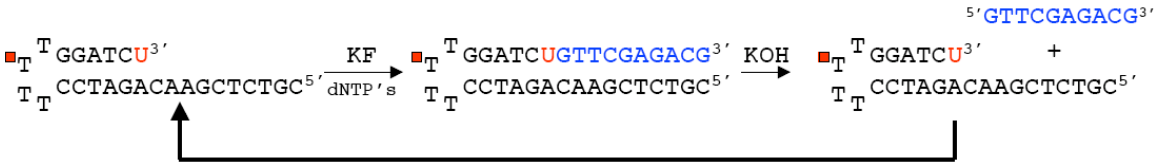


Fig. 9: Simple schematic diagram of the processes for cyclic 10mer isolation

The integrity of the released 10mer is assessed by polyacrylamide gel electrophoresis. Yields and efficiency were monitored throughout the process to ensure maximum usage of materials and solutions.

Numerous cleavage cycles expose the 26mer template to high pH over prolonged amounts of time. Exposure of DNA to high pH at high temperature causes modifications of some bases. Especially in the case of cytosine, alkaline hydrolysis is eminent causing the base to deaminate into uracil (Figure 10) [9].

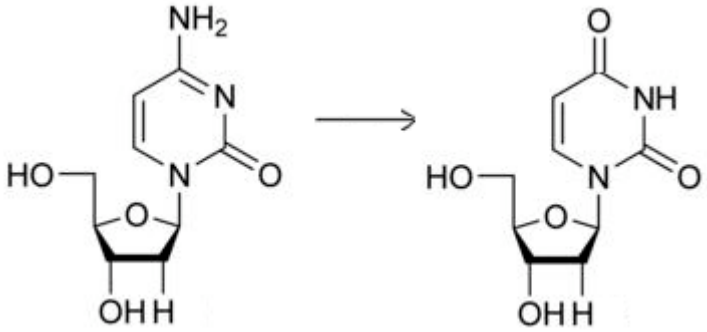


Fig. 10: High pH leads to the deamination of cytosine to uracil

The progression of deamination can be assessed with the use of nuclear magnetic resonance spectroscopy. These experiments were done in 0.2 M KOH at 55^{o} C. Protons H5 and H6 are unique to the cytosine base in ^{1}H-NMR experiments. The change in chemical shifts of the two protons can be monitored as the cytosine base is hydrolyzed to uracil. Proximity of the protons to a more electronegative nucleus causes a shift in position of the peaks. Relative integration of the peaks allows for quantification and rate determination.

Chapter 2: Experimental

2.1 Synthesis of control 26mer template

To better understand the polymerization and isolation of the 10mer from the template prior to resin attachment, the fill-in/cleavage reactions were first analyzed in solution. The 26mer template with 3' uracil was synthesized in an automatic DNA synthesizer (Applied Biosystems Model 391) using a 0.2 μM solid phase controlled-pore glass (CPG) support column and cyanoethyl phosphoramidite chemistry. Products were cleaved from the column with ammonium hydroxide (Fisher Scientific, Fairlawn, NJ) and then treated at 55^{o} C overnight for deprotection.

Prematurely terminated oligomers were purified from the using gel electrophoresis. This was done by running 15% polyacrylamide 8 M urea denaturing gel and by excising the strongest visible band under UV shadowing. The gel slice was then crushed and soaked in 1X TBE overnight. After a series of centrifugations at 5000 x g for 30 min at 4^{o} C, the supernatant was collected. The resulting supernatant of this series was then lyophilized. A sample was diluted in dH_2O and run on a G-25 sephadex column to desalt and further purify the DNA sample. The sample was injected into the *Amersham Pharmacia* AKTA purifier with dH_20 as mobile phase. Elutants after the void volume were collected and quantified using a Cary, Varian UV-Vis spectrometer.

After desalting and purification, 0.2 OD_{260} of 26mer was run on a 15% polyacrylamide 8 M urea denaturing gel for purity confirmation. Synthesis of 10mer in solution was done using approximately 1.0 μM 26mer DNA with 4.9 u of Klenow fragment (New England Biolabs) and 3 mM of each dNTP. The total volume was brought

to 50 µl and the reaction was run for 2 h at 37^{o} C in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The enzyme was then deactivated for 20 min at 70^{o} C. Starting 26mer and newly synthesized 36mer were analyzed on a 15% polyacrylamide 8 M urea denaturing gel to ensure complete 10mer polymerization on the template strand.

2.2 Cleavage of polymerized 10mer via alkaline hydrolysis

After fill-in reaction was achieved, cleavage of 36mer into 26mer and 10mer was carried out with varying concentrations of potassium hydroxide (KOH). Typically, 12 µl reaction aliquots were taken out after fill-in reactions and set as time zero. Enough concentrated KOH was then added to the reaction to bring to appropriate final concentrations. For example, to achieve a final KOH concentration of 0.2 M, approximately 11 µl of 1 M KOH were added to 38 µl of initial fill-in reaction volume. Cleavage reactions were carried out at 37^{o} C and 55^{o} C. As the cleavage reaction proceeded, 12 µl reaction aliquots were taken out at set time intervals. The progression of the cleavage reaction was analyzed after these samples were run on 15% polyacrylamide denaturing gels.

2.3 Purification of dNTPs upon reaction completion

Progression of the cleavage reaction was assessed by 15% polyacrylamide denaturing gels. Excess unused dNTPs caused a blur during UV shadowing, and purification of these dNTPs was necessary. A micro-spin column containing 500 µl of G-25 sephadex matrix was used to purify the reaction products from the triphosphates. After washing the matrix with 100 µl of H_20, 50 µl end-reaction volume was added to the

micro-spin column. The column was then centrifuged at 1000 rpm for 6 s and the flow through collected. Six more 50 µl aliquots of H_20 were added to the column one after the other with centrifugation after each addition. Each elutant was collected and quantified to determine the flow through of DNA products. DNA elutants after the 3rd and the 4th 50 µl H_20 wash were then run on 15% polyacrylamide 8 M denaturing gels.

2.4 Deamination studies

Prolonged exposure of DNA to alkaline conditions in potassium hydroxide can lead to modifications in DNA bases. Primary-amine containing bases such as adenine, guanine, and cytosine can be deaminated in high pH. Specially, cytosine is most sensitive to alkaline conditions [9].

Deamination was monitored using three nucleosides adenosine, guanosine, and cytidine which were subjected to 0.2 M KOH treatment in D_2O in 37^o C and 55^o C for 19 h. Proton degradation was monitored through nuclear magnetic resonance spectroscopy which enabled us to gauge conversion of H5 and H6 protons. Solutions containing 20 mM of each nucleoside in 500 µl 0.2 M KOH were heated over time while proton intensity was monitored on a Bruker AMX 600 NMR spectrometer**.** Also the cytidine nucleotide and a decamer with sequence $^{5'}$GCGAATTCGC$^{3'}$ were studied under similar conditions to quantify degradation of cytosine bases in a DNA strand.

2.5 Synthesis and purification of 26mer with linker-arm

The DNA template with the linker arm at the T7 position was synthesized similarly to the 26mer without the arm. The amino linker-arm used was acquired from Glen Research and is linked to C5 of the thymidine base. After synthesis, the support was washed with 1 ml of a 10% diethylamine/acetonitrile solution (Glen Research, Sterling, VA) to remove acrylonitrile, a reagent which activates transamidation of the amine [10]. Products were cleaved from the column with ammonium hydroxide (Fisher Scientific, Fairlawn, NJ) and then treated at 55^{o} C overnight for deprotection of the protection groups used during DNA synthesis.

Removal of protecting groups along with small failure sequences was performed on a 24 ml G-25 sephadex column. Samples were run with 10 mM NaH_2PO_4/100 mM NaCl (mobile phase; pH 7.4) buffer to ensure proper separation. Throughputs were collected then desalted again on a 15 ml G-25 sephadex column while using water as the mobile phase. Purity of targeted 26mer was assessed on a 15% polyacrylamide 8 M urea denaturing gel and a yield of approximately 30 OD_{260} was quantified using UV-Vis.

2.6 Conjugation of 26mer template in Phosphate buffer

Approximately 8 OD_{260} (30 nmol) of purified 26mer was dissolved in 3 M K_2HPO_4 (pH 4.9). After introduction of 5 mg Toyopearl AF-Epoxy 650 resin (Tosoh Bioscience LLC, Montgomeryville, PA), the pH of the solution was adjusted to 9.4, bringing the end reaction volume to 400 µl. A 20 µl aliquot was taken out and quantified by a UV-Vis spectrometer (Cary 100 Bio, Varian) as time zero (t_0). DNA and resin were

reacted at 45^{o} C for 72 h with slight agitation. Five 20 µl aliquots of the supernatant were collected at various time lengths to monitor progress of the reaction.

2.7 Conjugation of 26mer template in 800 mM $MgCl_2$ and 200 MM CAPS buffer

After observing slow binding efficiency, a similar reaction was carried out but using 800 mM $MgCl_2$ in 200 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (FisherBiotech, Fair lawn, NJ) for 14 h. Supernatant wash offs showed a greater decrease in a shorter time span compared to potassium phosphate.

2.8 Fluorescence imaging

Fluorescence imaging on a flouro-imager (Typhoon 9400, Amersham Bioscience) was carried out to visualize DNA bound to resin. A fluorescent dye SYBR green II which emits green light (λ_{em}= 522 nm) was mixed in with the resin on a 96-well micro-array plate. A green laser (λ_{exc} = 532 nm) was passed through the sample after which the signal was digitally converted, producing an image which shows the dye interactions with other materials.

2.9 Dimethoxy-trityl studies

To quantify the amount of DNA immobilized, another batch of 26mer with amino-linker was synthesized. The DMT protection group at the 5' end, which normally is taken off during synthesis, was left on to be later removed after conjugation to resin. After purification on a G-25 sephadex column, 4.9 OD_{260} of this 26mer were reacted with the resin in 800 mM $MgCl_2$/200 mM CAPS for 14 h. The resin was then washed with 200

ul 3% TCA/dichloromethane (Glen Research) four times to ensure all DMT groups were cleaved. Wash-offs were collected and compared to a control ran earlier in which trityl outputs from 2.0 OD_{260} of 26mer with 5' DMT were quantified at 498 nm.

2.10 Tryptophan conjugation

To investigate how another primary-amine ligand with low charge density binds to the resin under similar conditions, 400 µl of 1.5 mM tryptophan (ε = 5,500 M^{-1} cm^{-1}) were reacted with the resin in the same buffers used for DNA conjugation (Na_2HPO_4, K_2HPO_4, $MgCl_2$/CAPS). Reactions were carried out at 45^o C for times ranging from 24 to 52 h. Supernatant measurements were quantified at 280 nm.

2.11 10mer synthesis/cleavage on resin

After 2.49 OD_{260} were conjugated to 5 mg of resin, the resin was separated into five aliquots of 1 mg each. Each aliquot with 0.5 OD_{260} 26mer bound was washed in 1X Buffer 2 (New England Biolabs). Enzyme amounts ranging from 20 to 2 u and dNTP concentrations from 6 mM to 0.5 mM of each dNTP were added to the mixture bringing the volume up to 400 µl. The 1X Buffer 2 constitutes 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The sample was placed at 37^o C with constant shaking for 4 h after which the supernatant was washed off, and 50 µl of 200 mM KOH was introduced back into the vessel for 6 h at 37^o C to perform cleavage by hydrolysis. This cycle was repeated after a quick wash with antartic phosphatase (New England Biolabs) which cleaves the 3' phosphate left after cleavage. Isolated 10mers from each cycle were then quantified using a UV-Vis spectrometer. They were then assessed for

purity on a 15% polyacrylamide 8 M urea denaturing gel. To visualize DNA bands, the gels were stained with SYBR Green II in 1X TBE. Approximately 5 μl of stock SYBR Green were added in 50 ml of 1X TBE (1:10,000 dilution). The gel was then washed in this TBE bath for 30 min with constant shaking.

2.12 Incorporation of ^{13}C, ^{15}N labeled dGTPs into 10mer

After yields of the 10mer production were optimized, the synthesis and isolation of 10mer were carried out using ^{13}C, ^{15}N labeled dGTP (Cambridge Isotopes). Approximately 0.15 μmol of dGTP were added instead of the unlabeled tri-phosphate counterpart. The reaction progressed with Klenow fragment in the buffer at 37^{o} C. Successfully incorporated cleaved products were then run on a polyacrylamide gel.

Chapter 3: Results and Discussion

3.1 Polymerization and subsequent cleavage control studies

Quantification of 26mer showed yields of approximately 15 OD_{260} (57 nmol). A 50 µl fill-in reaction to polymerize 10mer on the 26mer template strand was done with approximately 1 µM DNA, 5 u of enzyme and dNTP concentrations of 3 mM each. Starting and end products were run on a 15% polyacrylamide 8 M urea denaturing gel (Figure 11).

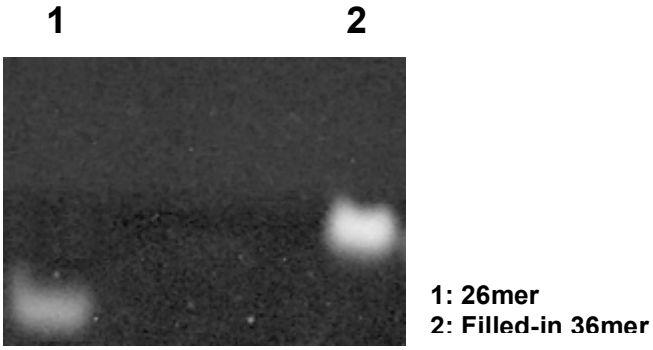


Fig. 11: 26mer template and filled-in 36mer after fill-in reaction

After fill-in was achieved, cleavage of 36mer into 26 and 10mers was performed in 0.2 M KOH for 4 h at 37^{o} C and run on a gel (Figure 12). Due to high salt concentrations in solution, bands in Lane 3 are distorted.

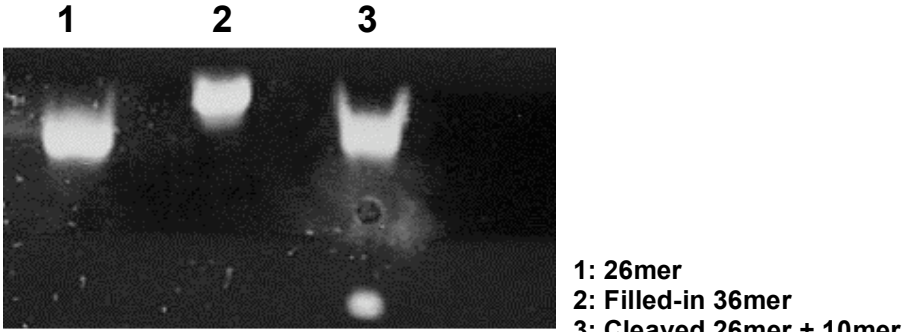


Fig. 12: 26mer template, filled-in 36mer and cleaved 26mer + 10mer

To optimize time limitations on the cleavage reaction, reactions were done at either 37° C or 55° C while KOH concentrations varied from 0.1 M to 0.3 M. The following gels represent cleavage reactions done at 37° C (Figure 13):

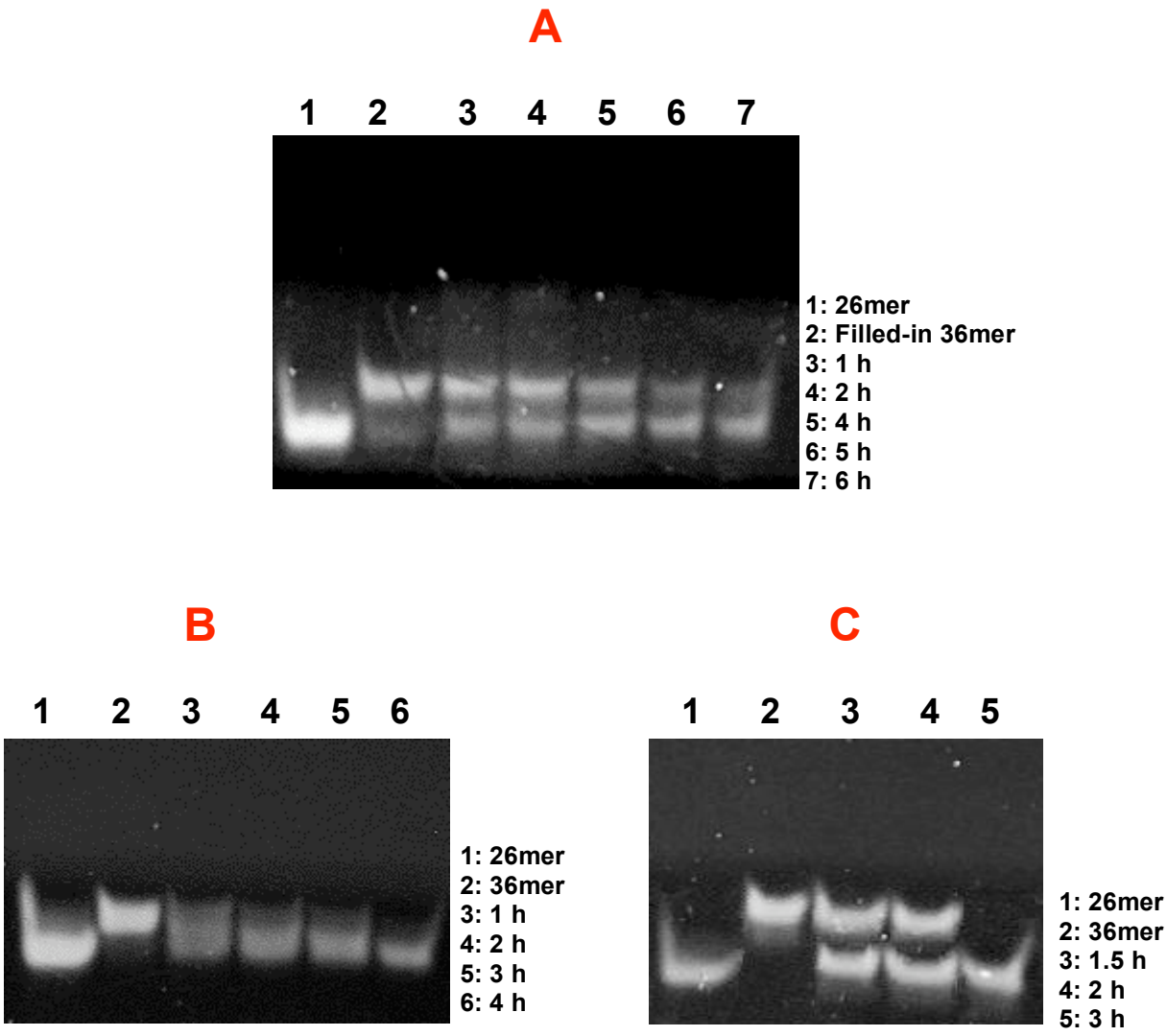


Fig. 13: 36mer filled-in products cleave at varying (a: 0.1 M., b: 0.2 M c: 0.3 M) KOH concentrations at 37° C

Figure 14 (a, b, and c) are gels showing cleavage reactions done at 55° C:

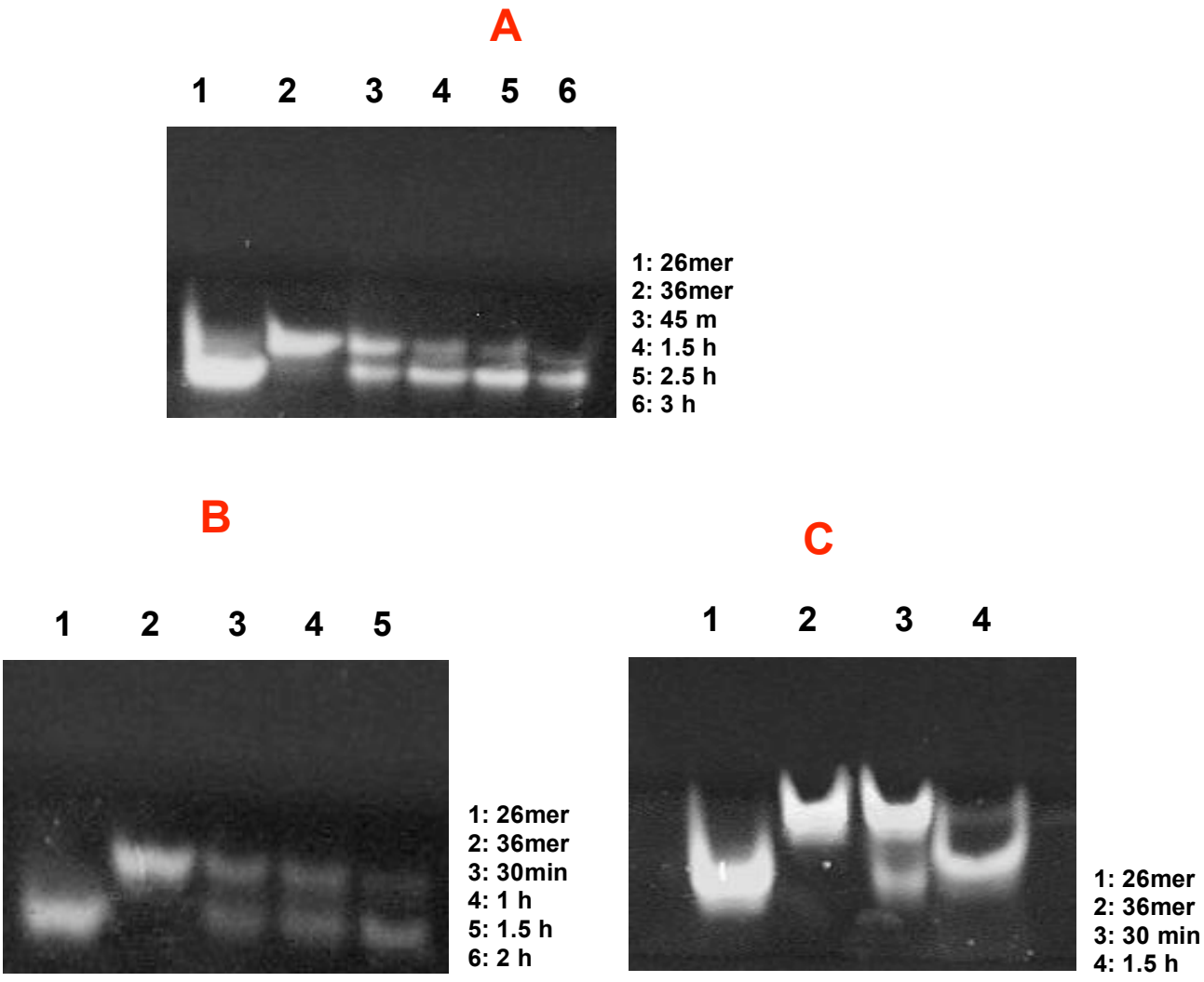


Fig. 14: 36mer filled-in products cleaved at varying (a: 0.1 M., b: 0.2 M c: 0.3 M) KOH concentrations at 55° C

Concentration and temperature seem to be directly proportional to the amount of time it takes to cleave the 36mer. Figure 15 summarizes the concentration and temperature course studies.

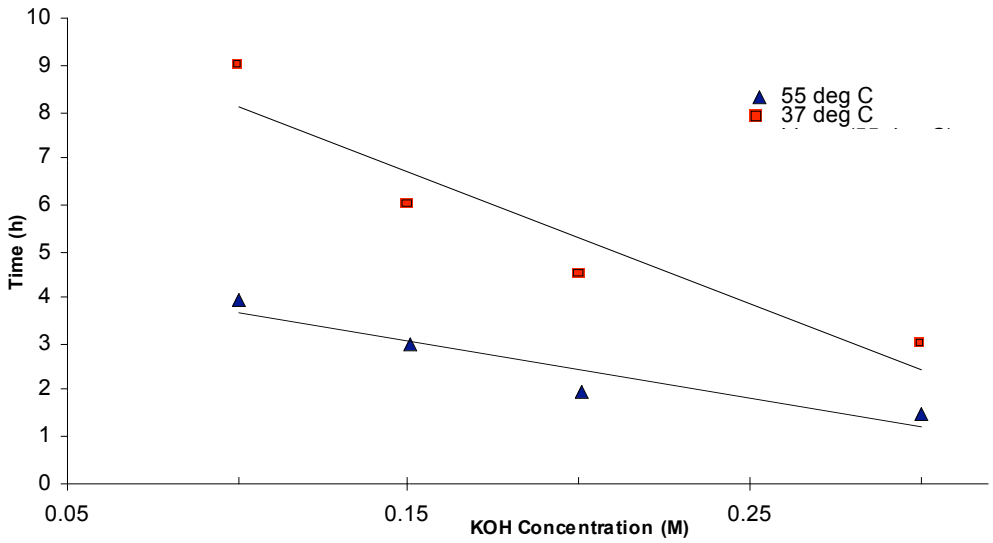


Fig. 15: Cleavage-time dependence on temperature and KOH concentration

From Figures 13, 14 and 15 it can be noted that increase in temperature and KOH concentration leads to the increase in rate of cleavage. But the extent of the increase needs to be studied since high temperature and pH can modify DNA bases.

3.2 Deamination of cytosine by alkaline hydrolyses

Exposure of DNA to high pH under high temperatures causes modifications of some bases. Especially in the case of cytosine, alkaline conditions causes deamination to uracil.

Since the 36mer filled-in DNA was to be cleaved at high pH, modifications of adenosine, guanosine and cytidine nucleosides were studied under alkaline conditions through the use of ^{1}H-NMR. Proton shifts for H5 and H6 for cytosine occur at as doublets at approx δ 6.4 and δ 8.2 ppm respectively. Upon hydrolysis, these doublets shift upfield to about δ 6.2 and δ 8.0 ppm respectively. The amount of conversion is directly related to

the integral of the corresponding peaks. These peaks are integrated in relation to each other. By taking the ratios of the shifts relative to each other, the percent change (% of cytosine deaminated) was quantified:

$$\% \text{ change} = \frac{I_U}{I_C + I_U} \times 100 \qquad \text{Eq. 1}$$

The percent deamination (% change) was calculated by taking the ratio of the intensity of the uracil proton peak (I_u) and the sum of the intensities of the cytosine (I_c) and the uracil (I_u) peaks (Equation 1).

Table 1: Peak integration reveals deamination of dC, dpC and C8 in 0.2 M KOH at 55° C

	dC		dpC		C8 in Decamer	
Time (h)	H6 (% change)	H5 (% change)	H6 (% change)	H5 (% change)	H6 (% change)	H5 (% change)
0	< 1	< 1	< 1	< 1	< 1	< 1
1	3.5	3.4				
3	7.4	6.7	3.7	2.9	1	< 1
6	14	13				
9	20	19	7.2	5.5	4.0	3.0
15	30	30				
19			12	8.3		

There was no significant change observed over 40 h for adenosine and guanosine nucleotides at 55° C. But as expected, proton peaks for cytosine (dC), the nucleotide cytosine triphosphate (dpC) and the internal C8 had shifted.

Table 1 shows that about 30% of dC is converted to dU in 15 h at 55° C. The nucleotide dpC on the other hand, is a more accurate depiction of the cytosine base in the DNA since it is phosphorylated. The nucleotide shows more resiliency towards high pH, with about 10% modification in 19 h. The cytosine in the decamer was studied at 55° C as

well and showed even more resiliency in high pH, with deaminition occurring only to 4 percent of the sample in 9 h. Half-lives of dC, dpC and C8 can now be predicted to be 26, 84 and 120 h respectively. The cytosine in the decamer is most stable and depicts the most real-life scenario since it is part of a DNA strand.

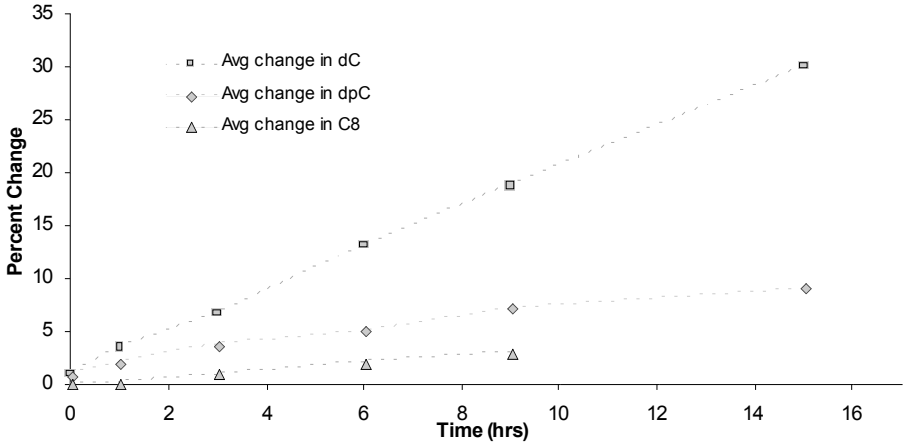


Fig. 16: Percentage of cytosine nucleoside, nucleotide and terminal C8 deaminiated in 0.2 M KOH at 55^{o} C (integrated values for the two protons H5 and H6 are averaged from Table 1)

Exposure of DNA to high pH can lead to base modifications such as cytosine deamination to uracil. Stabilities of individual nucleosides, nucleotides and an oligonucleotide chain were studied under alkaline conditions (0.2 M KOH). Peak integration of the protons affected by hydrolysis show that DNA is quite stable in high pH for more than 40 h at 55^{o} C. Moreover, cytosine bases in the oligonucleotide chain show greatest stability compared to the nucleotides and nucleosides.

3.3 26mer immobilization on epoxide-activated resin in phosphate buffer

Following a recommendation from the resin manufacturer, the 26mer with the linker-arm was reacted with the resin in 3 M K_2HPO_4. After several attempts using sodium salts (NaOH, NaCl, and Na_2HPO_4) no conjugation was observed. However, the potassium phosphate buffer seemed to provide ample cation strength for stabilizing the DNA strand and making the reaction possible. As observed from UV-Vis readings of supernatants collected, it took close to 75 h to immobilize 38% of DNA. Figure 17 shows the absorbance readings at different times as the reaction proceeded.

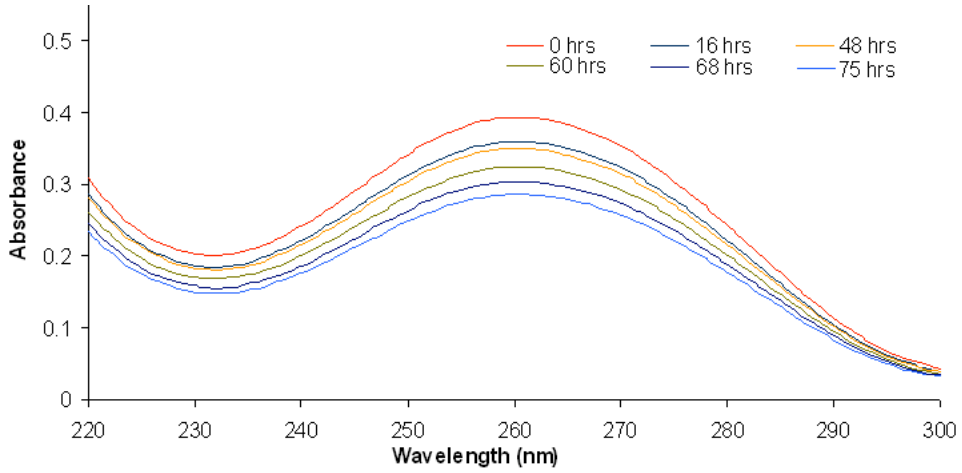


Fig 17a: UV-Vis spectrum obtained from supernatant wash-offs

Table 2: Amount of DNA bound to resin as reaction proceeds

Time (h)	Abs	Conc (OD/ml)	Supernatant OD_{260}	Amt. of DNA bound	% of Starting amt. bound
0	0.39	20	7.8	0	0.0
16	0.36	18	7.2	0.7	8.7
48	0.35	17	6.6	1.4	18
60	0.32	16	5.7	2.1	27
68	0.30	15	5.6	2.3	29
75	0.29	14	4.9	3.0	38

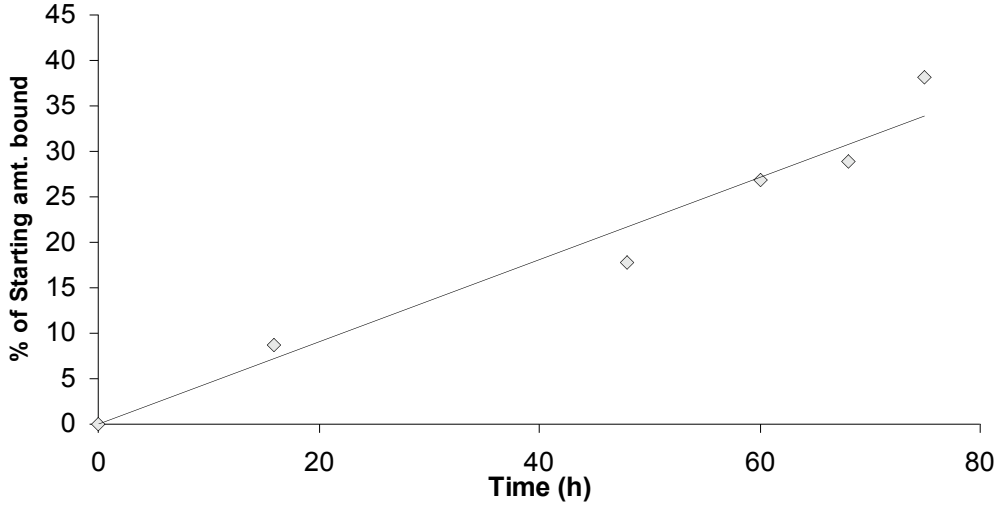


Fig. 17b: Increase in the amount of DNA bound to resin as reaction progresses in K_2HPO_4

As outlined in Table 2 and Figure 17b, 38% of the starting DNA binds to the resin after 75 h. Numerous trials with varying buffer concentrations, reaction volumes and resin amounts proved not to make a significant difference. However, all buffers used contained monovalent cations (K^+, Na^+). This low efficiency may be caused by repulsion between DNA and the negatively-charged matrix. The matrix, a branched polysaccharide of varying chain lengths, carries an overall negative charge under our binding conditions (pH 9).

3.4 26mer immobilization in magnesium chloride and *N*-cyclohexyl-3-aminopropanesulfonic acid

A divalent cation such as Mg^{2+} could solve the problem by reducing electrostatic repulsion as it neutralizes more charges on the DNA [3]. A salt/buffer combination of 800 mM $MgCl_2$ and 200 mM CAPS was used in this investigation.

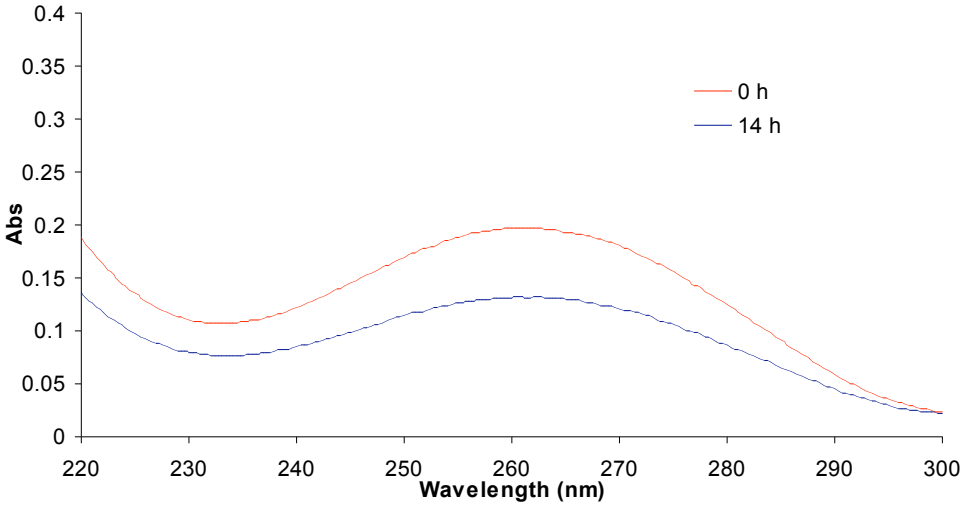


Fig. 18a: UV-Vis spectrum obtained while quantifying supernatant wash offs in 800 mM $MgCl_2$

Table 3: Amount of DNA in supernatant as reaction progresses in 800 mM $MgCl_2$/ 200 mM CAPS at pH 9.4

Time (hrs)	Abs	Amt. in supernatant (OD_{260})	Amt. of DNA bound	% of Starting amt. bound
0	0.19	4.9	0	0.0
14	0.13	2.5	2.4	49.0

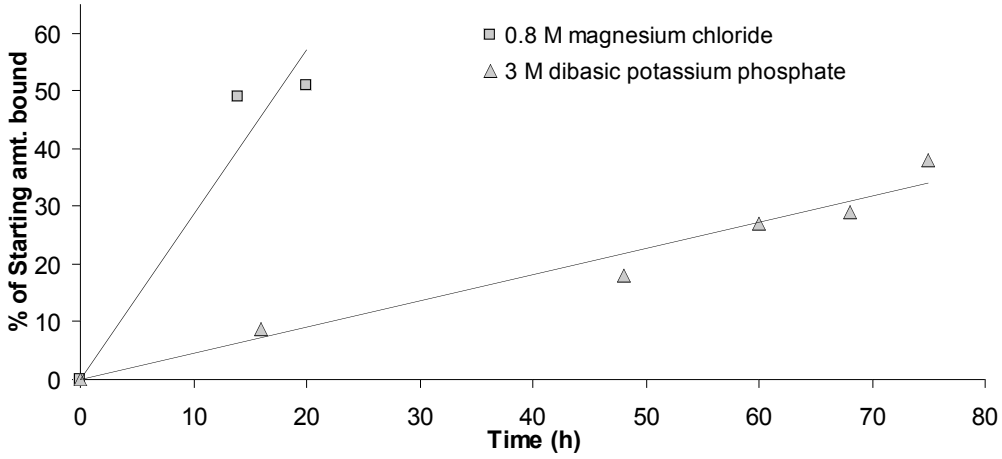


Fig. 18b: Comparative of DNA conjugation in 800 mM $MgCl_2$/200 mM CAPS buffer and potassium phosphate

It is evident from Figures 18 a and b that 49% decrease in absorbance is significant in 14 h compared to 38% in 75 h obtained previously. In the $MgCl_2$/CAPS buffer about 2.5 OD_{260} bind in 14 h whereas 3 OD_{260} bind in 75 h in the potassium phosphate buffer. The rate of conjugation is about 4 times faster in 800 mM $MgCl_2$/ 200 mM CAPS compared to the standard conditions of 3 M K_2HPO_4.

Table 4: Summary of all buffers used; corresponding supernatant quantification after 14 h of conjugation

Buffer	Starting Amt. DNA OD_{260}	Ending Amt. DNA OD_{260}	DNA amt. bound	% of Starting amt. bound
0.1M NaOH*	6.7	6.7	0	0.0
3M NaCl*	5.8	5.7	0.10	1.7
3M Na_2HPO_4*	6.1	6.0	0.10	1.6
3M K_2HPO_4	7.8	7.3	0.50	6.4
800 mM $MgCl_2$/ 200 mMCAPS	4.9	2.5	2.4	49

* Detailed data not shown

To gauge the affect of CAPS concentration on DNA conjugation, the reaction was carried out in 800 mM $MgCl_2$ and 20 mM CAPS buffer as well. Table 6 shows that no significant difference was observed compared to the 200 mM CAPS buffer.

Table 6: DNA conjugation to resin in 800 mM $MgCl_2$ and different concentrations of CAPS

Mg^{2+}	CAPS	Starting Amt. DNA OD_{260}	Ending Amt. DNA OD_{260}	DNA amt. bound	% of Starting Amt. Bound
800 mM $MgCl_2$	200 mM	4.9	2.52	2.38	48.6
800 mM $MgCl_2$	20 mM	4.1	2	2.10	51.2

3.5 Conjugation and hydrolysis studied with a tryptophan control

Tryptophan was reacted with the resin in solvents Na_2HPO_4, K_2HPO_4, and $MgCl_2$ to investigate how a primary-amine ligand possessing a single negative charge behaves under DNA binding conditions.

Table 5: Absorbance readings of supernatant wash-offs from tryptophan conjugation to resin

Buffer		Buffer Conc.	Time	ODs of W bound	% of starting amt.bound
	Na_2HPO_4	3 M	24 h	**3.0**	100.0
	K_2HPO_4	3 M	24 h	**3.0**	100.0
200 mM CAPS	$MgCl_2$	800 mM	24 h	0.74	24.6

***Red indicates completion**

At pH 9.4, a tryptophan molecule possesses a primary-amine and is negatively charged. Table 5 shows that tryptophan binds more efficiently in the presence of phosphate buffer. Only 24% of the tryptophan binds in $MgCl_2$/CAPS buffer compared to 100% in phosphate buffers (Table 5). This shows that in the presence of the phosphate buffers, hydrolysis of the epoxide groups occurs slower than hydrolysis in 800 mM $MgCl_2$/ 200 mM CAPS. This was confirmed by pre-incubating the resin in 3 M K_2HPO_4 and in 800 mM $MgCl_2$/ 200 mM CAPS buffers for 24 and 52 h. After pre-incubation, 3.0 OD_{280} of tryptophan were introduced into the resin for conjugation in 3 M K_2HPO_4.

Table 6: Tryptophan conjugation to resin after pre-incubation in reaction buffers

Pre-incubated with		Amt. bound (OD_{280})	% of starting amt.bound
3 M K_2HPO_4	for 52 h	**3.0**	100.0
$MgCl_2$ 200 mM CAPS	for 24 h	1.5	50.0
$MgCl_2$ 200 mM CAPS	for 52 h	0.4	14.0

As seen in the Table 6, all of the 3.0 OD_{280} bind when the resin was pre-incubated with 3 M K_2HPO_4 for 52 h. Lesser amounts of tryptophan bound (1.5 OD_{280} and 0.4 OD_{280}) when the resin was incubated in 800 mM $MgCl_2$/ 200 mM CAPS. This is due to the epoxide groups being hydrolyzed faster in 800 mM $MgCl_2$/ 200 mM CAPS than in the 3 M K_2HPO_4.

3.6 Visualization of resin-bound DNA with Fluorescence imaging

To visualize DNA covalently bound to resin, fluorescence imaging of the resin was performed using a fluoro-imager. Figure 19 is a picture taken of the DNA bound resin in the presence of 100 mM NaCl, which limits non-specific interaction of the dye with the resin (wells 4 and 6).

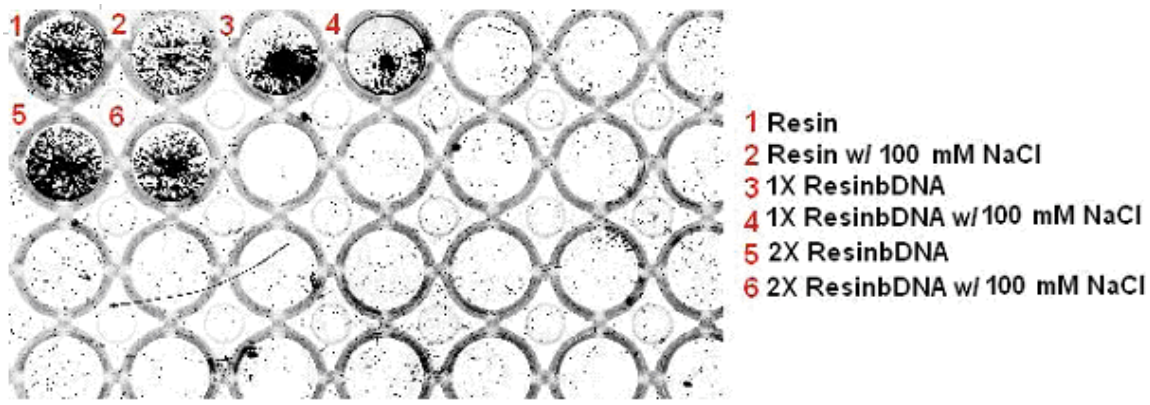


Fig. 19 Fluorescence image of DNA bound to resin (1X: 0.01 mg resinbDNA, 2X: 0.02 mg resinbDNA)

DNA bound to resin can be visualized in wells 3 and 5 which contain resin samples that are thought to be bound with the template. This is possible in the presence of SYBR Green II, a fluorescent dye, which interacts with DNA to provide such images.

Unfortunately, the dye also interacts with the resin itself (well 1), and salt (100 mM NaCl) minimizes these interactions (well 2). Though low in intensity, the control well 2, in which the bare resin is present with salt, shows a proper conclusion cannot be made even if DNA was bound since salt does not seem to completely inhibit the resin-dye interactions. Wells 4 and 6 which contain resin bound DNA with 100 mM NaCl do show intensity, but a proper conclusion as to whether it is from the DNA-dye interaction cannot be made. Consequently this approach was abandoned.

3.7 Quantification of resin-bound DNA through dimethoxy-trityl

As an alternate method to quantify the amount of DNA bound to resin, the DMT protective group was left attached to the 5' end of the strand. Before the reaction was carried out, a control of 26mer with 5' trityl group was dissolved in 1 ml of 3% trichloro acetic acid to cleave off the trityl group. DNA was thoroughly washed and supernatant was quantified with a UV-Vis spectrophotometer:

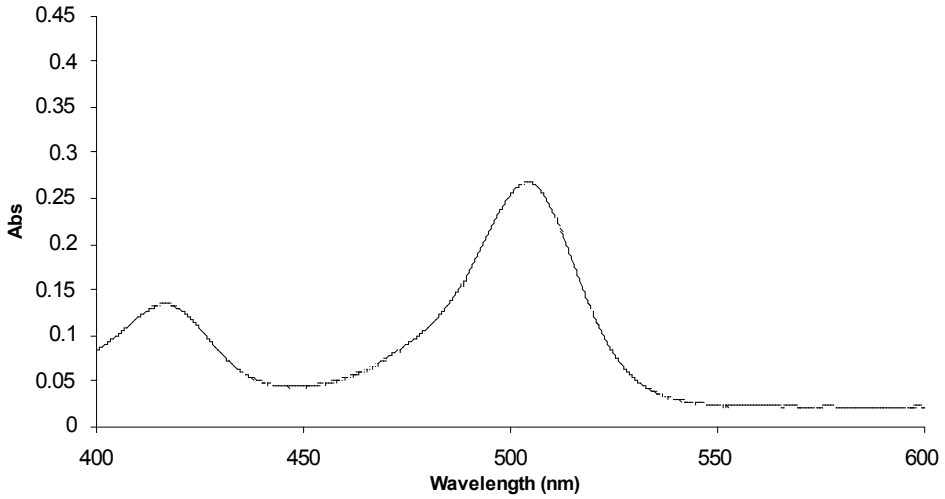


Fig. 20: UV-Vis spectrum of dimethoxy-trityl cation cleaved from 2 OD_{260} 26mer in solution (control)

Figure 20 is the UV-Vis reading taken of the trityl wash off from 2.0 OD_{260} (7.5 nmol) of DNA. With an absorbance reading of 0.24 at 498 nm, a control is set and this can now be used as a conversion factor to quantify the amount of DNA present. A fresh batch of 3.0 OD_{260} 26mer with 5' DMT in 800 mM $MgCl_2$/ 200 mM CAPS solution was mixed with resin and agitated for 14 h at 45° C/pH 9.4. A significant drop in DNA concentration in the supernatant was observed.

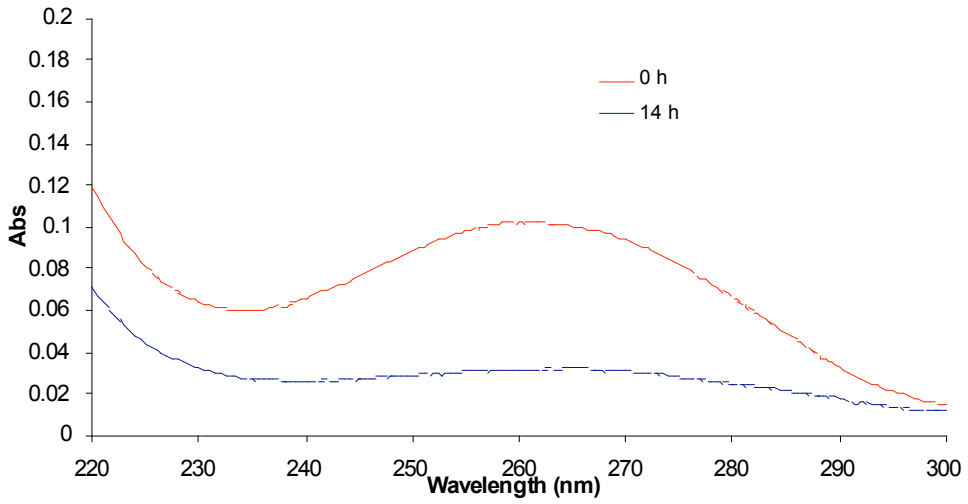


Fig. 21: UV-Vis spectrum of 26mer with 5'DMT binding to resin over 14 h

Table 7: Decrease in DNA amounts shown from supernatant wash-offs

Time (hrs)	Abs	Amt. in supernatant (OD_{260})	Amt. of DNA bound (OD_{260})	% of Starting amt. bound
0	0.10	4.56	0	0.0
14	0.06	2.3	2.3	49.6

Figure 21 and Table 7 show that 2.26 OD_{260} (8.7 nmol) of DNA have bound to the resin in 800 mM $MgCl_2$. If the trityl group was released, an absorbance reading of about

0.27 theoretically should be observed as per the control run prior to the reaction. The DMT groups were washed off and quantified at 498 nm (Figure 22).

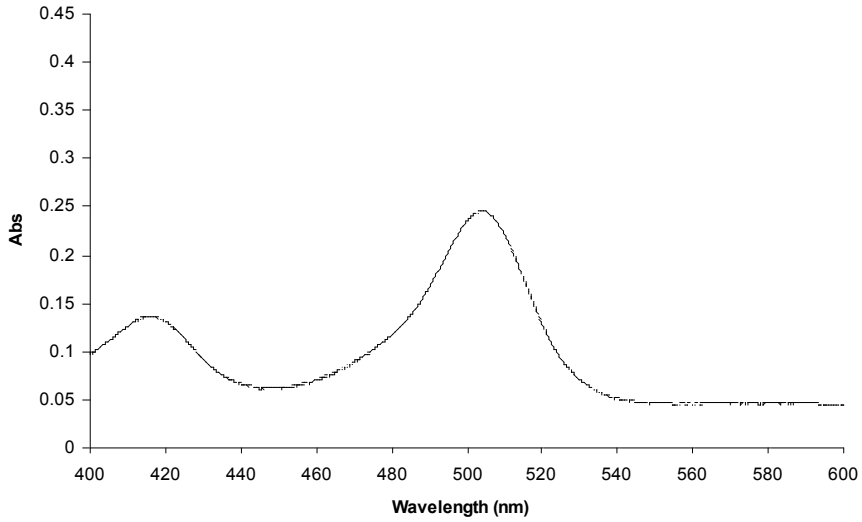


Fig. 22: UV-Vis absorbance showing trityl cleaved attached to 2.26 OD_{260} 26mer bound to resin

Quantification of this supernatant at 498 nm showed that 8.31 nmol of DNA were covalently linked to the support, corresponding to 2.16 OD_{260} bound DNA. This confirms the amount bound in 14 h in 800 mM $MgCl_2$/ 200 mM CAPS buffer is significantly higher than the amount bound (0.6 OD_{260}) in the same time using 3 M K_2HPO_4.

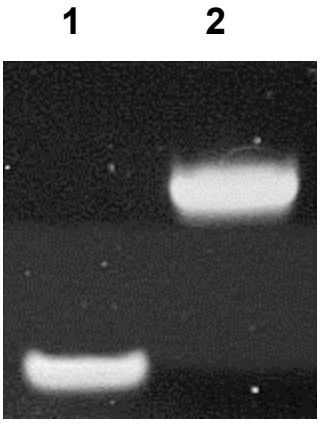
Table 8: Trityl readings corresponding to DNA washes

Sample run	DNA amount (OD_{260})	Trityl Abs @498
Control (in solution)	2.0	0.24
Experimental (off resin)	2.3	0.26

3.8 Synthesis and isolation of 10mer from resin-bound template

After washing the resin with buffer, the fill-in reaction to polymerize 10mer was carried out with 5 u of Klenow fragment and 6 mM each dNTP. This was followed by

introduction of 200 mM KOH with resin to cleave off the 10mer under alkaline conditions. Neutralization and then desalting with a G-25 sephadex purified the 10mer from excess salts. Prior to this, the crude yield was determined to be 0.12 OD_{260}. A small aliquot was run on a 15% polyacrylamide native gel:



1: Cleaved 10mer
2: 26mer (marker)

Fig. 23: Isolated 10mer (0.01 OD_{260}) run on 15% polyacrylamide native gel run with 26mer marker

Though Figure 23 shows the successful isolation of the desired 10mer product, low yields were obtained even though proportional scaled-up amounts of enzyme were used. Using 5 u of enzyme and 6 mM each dNTP for 2.49 OD_{260} (10 nmol) bound DNA to 5 mg of resin, only 0.1 OD_{260} (1 nmole) of 10mer were synthesized and cleaved off, indicating suboptimal yield.

3.9 Optimization of 10mer yields

The 5 mg resin bound with the DNA template was separated into 5 equal amounts (1 mg each). The fill-in reaction with 10 u of Klenow fragment and 6 mM each dNTP was then run at 37^{o} C each at variable times. The final concentrations of 6 mM dNTP provided 500 times (500X) the amount of each dNTPs required to fill-in the 26mer.

Better yields were observed (Table 9). Increasing the amount of enzyme to 20 u of enzyme further increased the 10mer yield.

Table 9: Amounts of 10mer isolated using 10 and 20 u of Klenow fragment

	10 u Klenow frag.		20 u Klenow frag.	
Time (h)	OD_{260}	mol	OD_{260}	mol
0	0	0.0E+00	0	0.0E+00
0.5	0.02	2.1E-10	0.03	2.5E-10
1	0.06	6.2E-10	0.07	6.9E-10
2	0.11	1.1E-09	0.13	1.3E-09
4	0.14	1.4E-09	0.15	1.5E-09
6			0.16	1.6E-09
24			0.07	7.0E-10

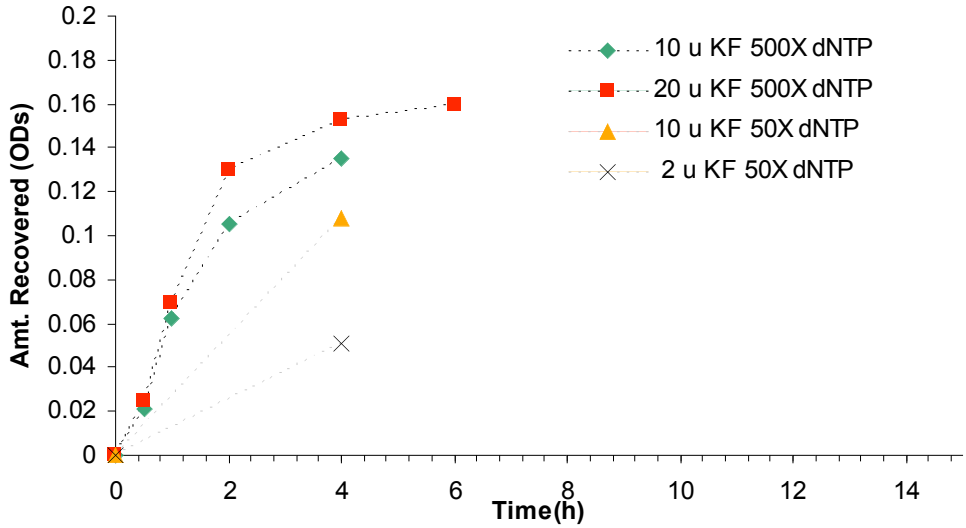


Fig. 24: Higher amounts of enzyme used during synthesis showed increased amounts of 10mer (50X dNTP: 50 times the required amount)

Figure 24 shows higher yields of the10mer are obtained when larger amounts of enzyme are used. Maximum synthesis occurred within 4-6 h of reaction time, but when the reaction was prolonged (24 h), yields went down (Figure 24). This could be due to the enzyme retaining its 3' to 5' exonuclease activity. Also, the released pyrophosphates after

the integration of the nucleotides into the growing chain have been shown to inhibit enzyme activity [11]. This observation suggests that extended reaction times should be avoided and pyrophosphates should be removed. All 10mer wash-offs were run on polyacrylamide denaturing gels to visualize purity of samples.

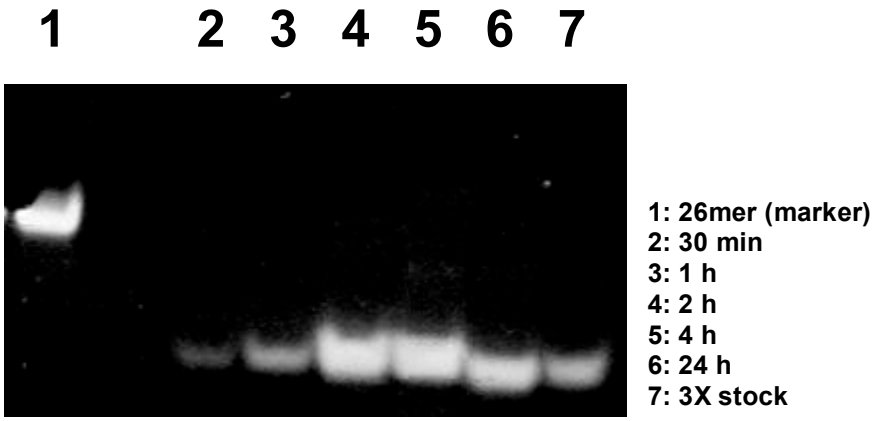


Fig. 25: Isolated 10mer from each aliquot using 10 u Klenow fragment (3X stock: ~ 0.05 OD_{260} stock 10mer)

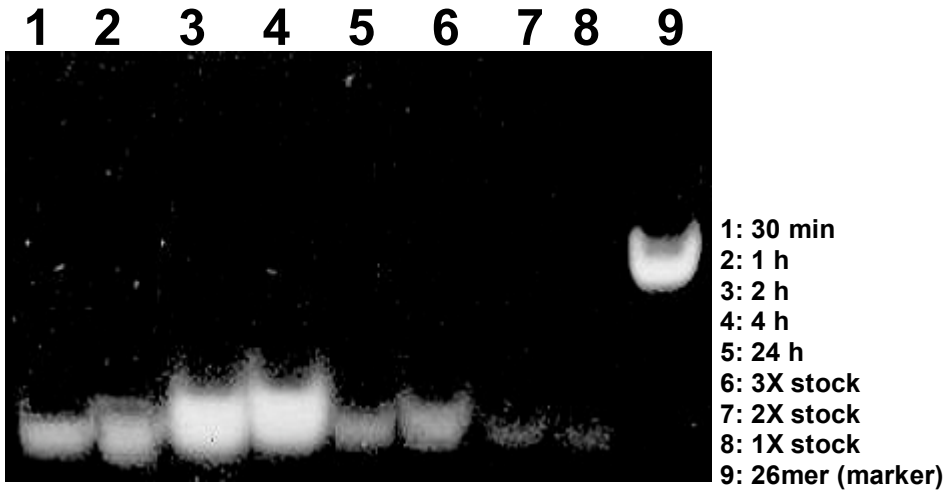


Fig. 26: Isolated 10mer from each aliquot using 20 u Klenow fragment (1X stock: 0.01 OD_{260} stock 10mer)

The synthesis and isolation of 10mer was carried out on the DNA bound epoxide-activated resin. Increase in enzyme concentration during polymerization increases yield.

The corresponding gel pictures (Figure 25 and 26) show the purity of the isolated products with 26mer marker. Stocks of 10mer isolated from a previous trial confirm concentrations.

To properly asses the number of times the enzyme and dNTPs can be reused, a set of experiments (A through D) were carried out.

Experiment A: A fill-in reaction was carried out on 1 mg of resin bound 0.5 OD_{260} template with 20 u of enzyme and 6 mM (500X) each dNTP (reaction 1). The supernatant from this reaction was used to synthesize on a new aliquot of 1 mg resin with bound DNA (reaction 2). After the 2^{nd} reaction, the supernatant was reused again for the 3^{rd} and 4^{th} times on fresh resins with bound template. As expected, after every reaction, the amounts of 10mer synthesized decreased due to the inactivity of the enzyme after its initial run.

Experiment B: A second experiment (Exp. B) was run but fresh 20 u of enzyme were added before the 2^{nd} fill-in reaction. It was observed that instead of getting similar amounts of 10mer compared to the 1^{st} reaction, much lower yields were recovered. Addition of fresh enzyme should have at least given the same yields as for the 1^{st} reaction if not more. Larger amounts of enzyme (20 u) increase 10mer yield (Table 9, Figure 24), but it seems from this experiment excess enzyme (40 u) decreases the yield.

Experiment C: Reactions using 10 u of enzyme and 0.5 mM each dNTP (50X the required amount) were done. After the addition of 10 u enzyme after the 1^{st} reaction, yields did not lower by a considerable amount. Lowering dNTP amounts by 10 fold did not have significant impact on 10mer yield compared to excess dNTP (Figure 27).

Experiment D: Lower yields are observed when enzyme concentrations are lowered (2 u), but 10mer amounts remained constant after addition of fresh 2 u before the 2nd reaction. This showed that enzyme concentration had a direct relation to the amount of 10mer produced.

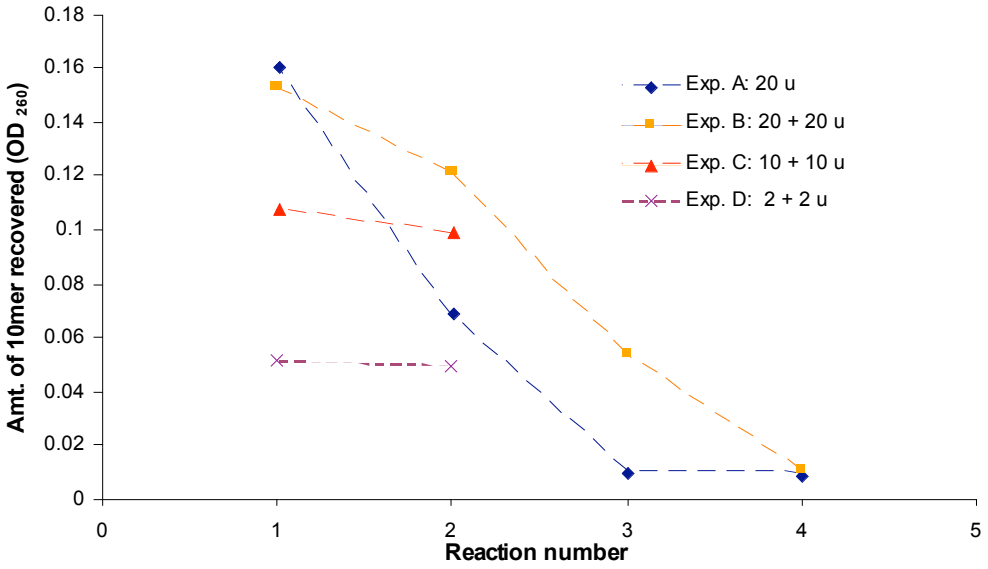


Fig. 27: 10mer yield after the four cycles where run (reactions 3 and 4 were unnecessary for Exp. C and D)

Figure 24 (Pg. 32) shows that using large amounts of enzyme will yield higher amounts of 10mer but enzyme amounts may be increased to a certain limit. When 40 u of enzyme were used to fill-in 0.5 OD_{260} bound template in a 400 µl reaction, lower than expected 10mer yields were recovered (Exp. B; Figure 27). Overcrowding of reaction with enzyme and dNTPs leads to a decrease in 10mer yield.

3.10 Further optimization while making the process cyclic

A plateau was reached after 30 u of enzyme were added to synthesize 10mer from 2.4 OD_{260} template (Figure 28). These reactions were being carried out in 400 µl reaction

volumes. To resolve this limitation, the resins were washed in 10 u antartic phosphatase and fill-in reactions repeated, but with fresh dNTPs and an increase to proportional volumes (Table 10).

Table 10: Increase in volume makes for better yields due to lower overcrowding by enzyme

Bound template (OD_{260})	KF (units)	10mer recovered (OD_{260}) 400 µl	larger volumes	
0.4	10	0.14	0.12	400 µl
0.8	15	0.18	0.24	800 µl
1.2	20	0.25	0.39	1.4 ml
2.4	30	0.33	0.72	2.0 ml

← Fill-in reaction **D**

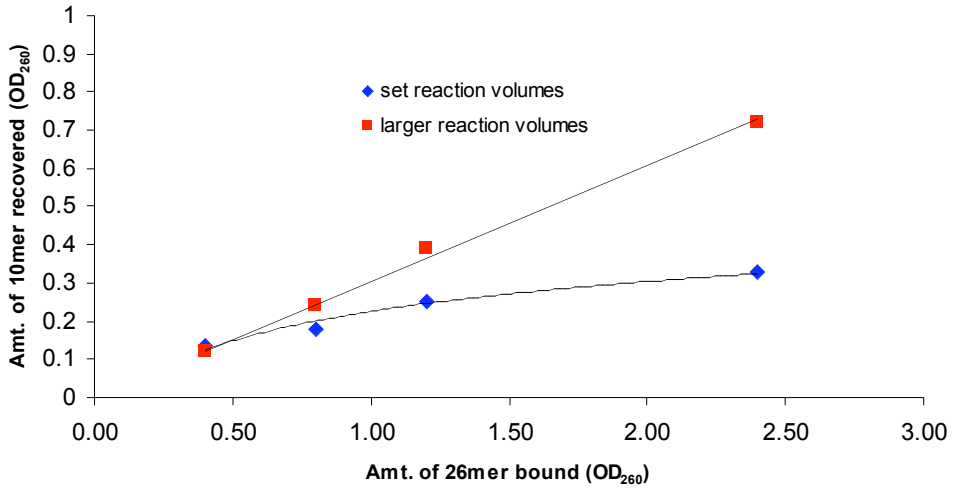


Fig. 28: Increase in enzyme amount should accompany increase in reaction volume

High concentrations of enzyme during fill-in reaction caused overcrowding and hence decreased 10mer amounts recovered. Figure 28 shows that this can be overcome by simply increasing the reaction volumes.

3.11 Reuse of template and dNTPs

The process was made cyclic by reusing the supernatant from fill-in reaction D (Table 10). After 0.72 OD_{260} of 10mer were recovered, the resin with 2.4 OD_{260} bound

DNA was washed with 20 u antartic phosphatase for 1 h at 37^{o} C to remove the 3' phosphate. The supernatant containing 30 u of Klenow fragment and unused dNTPs from reaction D along with a fresh batch of 30 u of enzyme were reintroduced back into the resin to perform fill-in reaction in 2.5 ml. A wash with 0.2 M KOH at 37^{o} C for 2 h cleaved the 10mer, which was washed off and quantified using a UV-Vis spectrophotometer. About 0.62 OD_{260} of 10mer were recovered.

These experiments set a standard for the amounts of enzyme and dNTP required to obtain proper 10mer yields from this type of solid phase synthesis. Depending on the desired amount of 10mer, they allow for optimum conversion factors to be available to calculate proper reagents necessary to perform synthesis.

3.12 10mer syntheses using labeled dGTP

A 10mer containing ^{13}C, ^{15}N labeled dGTPs was synthesized in solution on 26mer template. The other three nucleotides (dATP, dCTP, dTTP) were non-labeled.

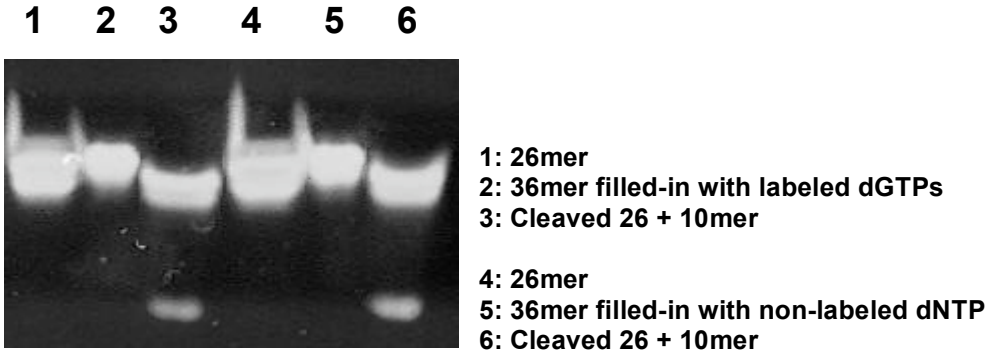


Fig. 29: Successful incorporation of labeled dGTP into growing strand (lane 3)

Labeled dGTPs were successfully incorporated into the growing strand (Figure 29, lane 3). Simultaneous fill-in reaction with non-labeled dNTPs (lanes 4, 5, 6) was

carried out and set as positive control. Since guanosine is the first nucleotide added, synthesis of 10mer can only carry-on after its successful incorporation. The cleaved 10mer present in lane 3 contains labeled guanosine bases.

Proper polymerization on template can not be monitored through loss of dNTP's in solution since only small amounts are used during the reaction, making quantification a challenge. For example, with 0.5 mM of each dNTP in the 400 µl reaction mixture, every cycle would use 12 µM of each dNTP. After about 5 cycles of fill-in reactions 0.44 mM of dNTPs would be left in solution (Table 11).

Table 11: Theoretical concentrations of each dNTP left in supernatant after every fill-in cycle

Cycle	dNTP concentration in solution
0	0.5 mM
1	0.49 mM
2	0.47 mM
3	0.46 mM
4	0.45 mM
5	0.44 mM

Chapter 4: Review

Synthesis and isolation of 10mer was carried out successfully using a template bound to solid support. Along the process, it was found that conjugation of a DNA ligand with linker-arm to epoxide-activated resin can be more efficiently achieved by using a Mg^{2+} divalent cation. In doing so, the hydrolysis of the epoxide groups speeds up as well therefore DNA conjugation slows down after 14 h. This was confirmed when tryptophan was conjugated to the resin in buffers used to bind DNA.

The 10mer yields were optimizing through a series of experiments where enzyme and dNTP amounts were varied to get maximum yield. It was found that high enzyme concentrations lead to higher 10mer yields. After conjugation of 2.4 OD_{260} (10 nmol) template, fill-in reaction was done with 30 u of Klenow fragment, 1 μmole of each dNTP in a 2 ml reaction volume (Figure 28). A total of 0.72 OD_{260} (7 nmol) of 10mer were recovered. A 2^{nd} cycle using the same supernatant with fresh enzyme was run in 2.5 ml, and 0.62 OD_{260} (6 nmol) of 10mer were recovered (Pg. 37). Multiple cycles using unincorporated dNTPs can now be performed to amplify product yield.

References

1. Gronenborn, A J. Louis, R. Martin, G. Clore. *Preparation of Uniformaly Isotope-Labeled Oligonuceotides for NMR Spectroscopy.* The Journal of Biological Chemistry, 273 (1997) 2374-2378.

2. Zimmer, D. and Crothers, D. *NMR of Enzymatically Synthesized Uniformly $^{13}C^{15}N$-Labeled DNA Oligonucleotides.* Biochemistry, Vol. 92, pp. (1995) 3091-3095

3. Bauer-Arnaz K, E. Napolitano, D. Roberts, J. Montali, B. Hughes, D. Schmid Jr. *Salt-InducedIimmobilization of Affinity Ligands onto Epoxide-Activated Supports.* The Journal of Chromatography, 803 (1998) 73-82

4. Wheatley, J. and Schmidt, D. *Salt-induced Immobilization of Affinity Ligands Onto Epoxide-Activated Supports.* The Journal of Chromatography, 849 (1999) 1-12

5. Hagi, N, Nishimura, S and Oyama, K. *Immobilization of Proteins on Toyopearl Gels 25 Vol.* 2 1981 13

6. Bloomfield, V. *DNA Condensation by Multivalent Cations.* Biophysical Journal, 74 (1998) 269-281

7. Long, F and Pritchard, J. *Hydrolysis of Substituted Ethelene Oxides in H_20 Solutions.* The Journal of American Chemical Society, 78 1956 2663-2667

8. Applied Biosystems *Manual for the use of Model 391 DNA synthesizer. 3-17*

9. Lindahl, T and Nyberg, B. *Heat-Induced Deamination of Cytosine Residues in DNA.* Biochemistry, 13 (1974) 3405

10. Glen research. *Methods to Avoid Inactivation of Primary Amines.* Glen Research Technical support bulletin, (2001).

11. Deutsher, M, and Kornberg, A. *Enzymatic Synthesis of Deoxynucleic Acid.* The Journal of Biological Chemistry, 244 (1968) 3019