Blocking Oligomer Design Update (3/10-3/11)
 Modifications for the Inhibition of Φ29 (exo+) DNA Polymerase

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ABSTRACT

A primary objective of the UCSC Nanopore Lab is to develop a novel method to sequence DNA. The proposed method intends to detect each base of a DNA sequence as it is passed through a small sensor called a nanopore. Currently, a catalytic protein known as an enzyme is used to pass DNA through the nanopore. Various enzymes are used, however they are all DNA polymerases (DNAP), which are enzymes that replicate DNA. To control the DNAP-directed passing of DNA through the nanopore, our lab has designed modified DNA sequences, called Blocking Oligomers. Blocking Oligomers bind the DNA and act as a molecular switch that activates the DNA for replication only when captured on the nanopore sensor. DNA replication is used to pass the DNA base-by-base through the nanopore sensor.

This study presents the modifications made to a previous design of the Blocking Oligomer that inhibited the DNAP known as Klenow Fragment (KF DNAP). The Blocking Oligomer was modified for application with a novel DNAP, Φ29 DNAP. Our lab hopes that Φ29 DNAP can resolve numerous issues encountered with KF DNAP, thanks in part to a higher fidelity and processivity than KF DNAP. Numerous experiments explained here in detail were required to optimize the Blocking Oligomer for inhibition of Φ29 DNAP. Consequently, the new design of the Blocking Oligomer for Φ29 DNAP maintains its needed functions, but is also less costly than its predecessor.
1. Introduction

As the world strives to develop an affordable sequencing platform, the UCSC Nanopore Lab presents a novel method to whole genome sequencing has the potential of being low-cost.

DNA can theoretically be sequenced by recording the change in current across a nanopore sensor as a DNA template is replicated through the nanopore, base-by-base, by an enzyme known as a DNA polymerase (DNAP). [1,6,9,13] However, before sequencing can be considered, the UCSC Nanopore Lab must develop a reliable method to control the DNA replication reaction at the nanopore sensor. Our lab has already shown that DNA replication can be inhibited by annealing a modified oligonucleotide sequence, referred to as the Blocking Oligomer [1], to the DNA
template before the DNAP has an opportunity to bind. The Blocking Oligomer protects the DNA from replication until it is captured on the nanopore. Upon capture, the Blocking Oligomer is removed and DNA replication is initiated, which concomitantly passes the DNA through the nanopore. The goal of this project is to modify the current design of the Blocking Oligomer to inhibit a specific DNAP.

2 Background Information

2.1 DNA Replication

Contemporary sequencing approaches utilize DNAPs extracted from various organisms to simplify the work of the sequencers. The primary function of a DNA polymerase is to replicate DNA. To do so, it requires a template sequence of DNA to be replicated, a primer sequence of DNA, RNA, or protein to initiate replication by providing a binding site for the DNAP, deoxynucleotide triphosphates (dNTPs) to be added to the DNA polymer, and magnesium ions to activate the mechanism of the enzyme by coordinating the addition of dNTPs to the primer strand.

In addition to the polymerase activity, some DNAPs are also able to chew-up the primer strand with another enzymatic activity, known as an exonuclease activity. Exonucleases catalyze the hydrolysis of DNA into its monomers, deoxynucleotide mono-phosphates (dNMPs). The Klenow Fragment (KF) of *E.Coli* DNAP I lacks the typical 5’-3’ exonuclease activity that removes primers. This process, known as digestion, may seem counterintuitive to the polymerase activity that catalyzes DNA synthesis, but it serves an important role to proofread the DNA that was just replicated in the 5’-3’ direction. Doing so increases the accuracy of correctly replicated DNA, since in the case of KF, an erroneous base is added by accident every 1/10,000 base
pairings. [4] In such a case, the incorrect base would have been removed by the exonuclease activity of DNAP I, yielding another opportunity for addition of the correct base.

Our lab recently shifted towards the use of an enzyme that has a 5ʹ-3ʹ exonuclease activity. This enzyme is known as Φ29 DNA polymerase, Φ29 DNAP, and it possesses both the usual 5ʹ-3ʹ polymerase activity to replicate DNA and a 3ʹ-5ʹ exonuclease activity for proofreading an extended primer. [5,11] Φ29 DNAP is shown in cross-section below in Figure 2 to identify key DNA interactions within the enzyme.

Figure 1: Structural comparison of KF DNAP (exo-) and Φ29 DNAP (exo+) bound to DNA duplexes. Shown on the left, DNA duplex of primer and template strands bound by KF(exo-) [1,2,4]. Shown on the right for comparison, DNA duplex bound by Φ29 DNAP (exo+). [12]

Figure 2: DNA replication within Φ29 DNAP (exo+). (A) 3-dimensional plot of DNA duplex entering through an upstream duplex tunnel, where the primer and template are split apart to reveal the 3’ hydroxyl terminus of the primer to initiate DNA replication, and thus primer extension. [11]
For this project, it is important to denote exonuclease proficiency of the different DNAPs used in my research by referring to enzymes as being either exonuclease proficient (exo+), that is to say they do possess an exonuclease activity, or being exonuclease deficient (exo-). Therefore, this paper shall henceforth refer to Φ29 DNA polymerase as Φ29 DNAP (exo+) and the KF used as being either KF DNAP (exo-) or KF (exo+) for clarity.

2.2 Sequencing on the UCSC α-Hemolysin Nanopore

The nanopore sensor is constructed from alpha-hemolysin (α-HL), a lipid-membrane protein isolated from *Staphylococcus aureus*. α-HL can readily insert into a lipid membrane, forming a 1.5nm passive channel that allows for the transport of a buffered salt solution through the channel and across the lipid membrane. A voltage is applied across the bilayer that drives ionic current through the nanopore. This aqueous solution simulates the *in vivo* conditions for DNA replication within a cell by maintaining a neutral, biologically-relevant pH for enzyme activity to be observed. Figure 3 below shows the annealing, or binding, of a DNA Template to a DNA Primer, and the primer-template junction. The enzyme binds at the Primer/Template junction (circled below), in order to extend the primer into a complementary strand of DNA bound to the template.

![Figure 3: Attachment of primer to template DNA. [1]](image)

The general construction of the nanopore begins with the formation of a lipid bilayer over an aperture approximately 25 micrometers wide. Diluted α-HL toxin is then added over the lipid bilayer and a single α-HL molecule is needed to insert into the bilayer to form the nanopore channel. The channel formed is 1.5nm wide, which is just wide enough to accommodate a single
strand of DNA. The negatively charged DNA template’s single-stranded 5’ terminus is driven through the pore when the voltage is applied across the nanopore channel, following the direction of the resulting current (Figure 4).

![Figure 4: Duplex DNA captured on the α-HL nanopore. [1,6]](image)

As the DNAP extends the primer on the pore, the template is pulled back up the pore, allowing the enzyme to read the next position on the template. Template DNA base’s physically block the pore and impede the circuit relative to their individual sizes. As shown below in Figures 5 and 6, the template movement can be monitored in real time by measuring the change in current through the pore as the DNA polymerase raises the template.

![Figure 5: Idealized structure of the nanopore platform, showing the nanopore sitting in a lipid bilayer with the directions of charge flow indicated by direction of positively charged Potassium ions and negatively charged Chloride ions. [6]](image)

![Figure 6: Proposed sequence of detection of nanopore DNA capture events. Nanopore recordings upon binding of Φ29 DNAP (exo+) to DNA substrate, and subsequent distance of a certain sequence of DNA (red) passing through the α-HL nanopore. [13]](image)
2.3 The Blocking Oligomer

To prevent replication of the DNA primer in the presence of enzyme before the DNA is captured on the nanopore, it is necessary to regulate when the enzyme replicates DNA. Doing so allows us to activate DNA replication only on the nanopore. By controlling the time when the DNAP replicates the DNA template, the nanopore experiments can have a definitive start and thus theoretically sequence the DNA completely, without missing the initial bases added to the primer. This is accomplished by adding the Blocking Oligomer to the DNA duplex before it is added to the nanopore with DNAP. This protects the exposed 3’ OH of the primer strand, and therefore inhibits the enzyme from attaching to and polymerizing the primer (Figure 7).

![Figure 7: Blocking Oligomer binding to DNA duplex. Yellow indicates the diacridine residues of Blocking Oligomer, and red indicates the DNA residues. Binding of the Blocking Oligomer blocks the enzyme-binding site, and thus DNA replication is inhibited until the Blocking Oligomer is removed. [1]](image)

The current Blocking Oligomer design (Figure 8) relies on covalently binding two acridine molecules (diacridine, ZZ) to the 5’ terminus of the Blocking Oligomer, followed by 6 Locked Nucleic Acid (6LNA) [1,3] modified nucleotides that inhibit KF DNAP (exo-) binding, which is an expensive modification.

![Figure 8: Previous design of Blocking Oligomer to inhibit KF DNAP (exo-), including the diacridine modification and the 6LNA’s. [1]](image)
The two acridine molecules intercalate between the bases of the template DNA at the Primer/Template junction. The LNA’s are modified nucleotides induce the loss of the template DNA’s B-form structure, literally incorporating a kink that relaxes DNA into an A-form conformation. [3] Since DNA polymerases bind to specific substrates with specific structures (ie: B-form DNA), DNA polymerases such as KF or Φ29 DNAP (exo+) that likely inhibits these enzymes from extending DNA primers.

The gel image in Figure 9 shows the results of a primer extension assay (see Methods 3.3) to compare different Blocking Oligomer designs against KF DNAP (exo-). The assay results were qualitatively compared by primer band intensities to estimate the amount of primer that was protected. It can be seen that the Blocking Oligomer used in lane 6 unequivocally inhibits polymerase activity, compared to other Blocking Oligomer designs, such as the diacridine alone design in lane 4, which failed to inhibit KF DNAP (exo-) activity. For this reason, the current Blocking Oligomer (lanes 5/6) is chosen over other designs to inhibit KF DNAP (exo-) polymerase activity.

![Figure 9: Inhibition of primer extension by KF DNAP (exo-) using various Blocking Oligomers. As seen in lane 6, the Blocking Oligomer with both modifications, the diacridine (ZZ) and the 6LNA, was capable of inhibiting primer extension far beyond that of the diacridine alone design in lane 4, for example. Complete image available in Supplement, Figure S1.](image-url)
Currently, this Blocking Oligomer has been designed only to inhibit KF DNAP (exo-)’s polymerase activity, however with the recent shift towards Φ29 DNAP (exo+) the Blocking Oligomer must be redesigned to accommodate the exonuclease proficiency of the enzyme, which is where my thesis research begins.

3 Research Methods

3.1 Enzymes
The D355A, E357A KF DNAP (exo-) was ordered from and produced by New England Biolabs at a concentration of 100,000 U ml\(^{-1}\) with a specific activity of 20,000 U mg\(^{-1}\). Also, wild-type Φ29 DNAP (exo+) was ordered from and produced by Enzymatics at a concentration of 833,000 U ml\(^{-1}\) with a specific activity of 83,000 U mg\(^{-1}\).

3.2 DNA Oligonucleotides
DNA oligonucleotides, namely the primer, template, and Blocking Oligomers, were synthesized at Stanford University Protein and Nucleic Acid (PAN) Facility. They were purified upon delivery following a standard protocol for our lab, as listed in Appendix A. After purification, quantification of purified DNA oligonucleotides was done using a Nanodrop ND1000 UV spectrophotometer to measure absorbance at various concentrations and then following a linear regression plot to calculate actual concentrations.

3.3 Primer Extension and Excision Assays
A 23mer DNA primer was used to examine Blocking Oligomer efficacy for enzyme inhibition by analyzing whether or not the primer was protected. The primer was labeled with 6-FAM on the 5’ terminus for post-PAGE-visualization purposes. Reactions were conducted with 1 µM annealed DNA and 0.75 µM Φ29 DNAP (exo+) in 10 mM K-HEPES, pH 8.0, 0.3 M KCl, 1 mM EDTA, 1 mM DTT, and 10mM MgCl\(_2\) added when indicated (absent in enzyme activity
controls). Exonuclease activity was examined by removing dNTPs from the aforementioned mix, and polymerase activity was observed by adding dNTPs to a final concentration of 100µM per nucleotide. At such a high concentration, polymerase activity dominates exonuclease activity. Reactions were incubated at room temperature for the indicated times and were terminated by the addition of buffer-saturated phenol. Following extraction and ethanol precipitation, reaction products were dissolved in 7M urea, 0.1X TBE and resolved by denaturing electrophoresis on gels containing 17% acrylamide:bisacrylamide (19:1), 7 M urea, 1X TBE. Extension products were visualized on a UVP Gel Documentation system using a SybrGold filter.

4 Redesigning the Blocking Oligomer for Φ29 DNAP (exo+)

The current design of the Blocking Oligomer (Figure 10) is comprised of three key features that inhibit KF DNAP (exo-) polymerase activity: 5’ diacridine, 6-LNA’s, and the non-complementary 7-C tail. These three components of the molecule effectively inhibit DNA replication, while allowing for in situ removal during nanopore experiments with KF DNAP (exo-) thanks to the non-complementary tail. Evidence of this was shown in previous literature from the lab [1].

However, as discussed earlier, the switch to Φ29 DNAP (exo+) requires additional consideration to accommodate for a number of differences between itself and KF DNAP (exo-). At the beginning of my research, there were two key differences that needed to be considered. First and foremost was the Blocking Oligomer’s efficacy in the inhibition of a DNA polymerase with an exonuclease activity. The other factor to consider was whether or not low-cost alternatives to the expensive 6-LNA modifications in the current Blocking Oligomer could be used. These two questions led to the first few experiments that I ran to better understand the interaction between the Blocking Oligomer and Φ29 DNAP (exo+).
4.1 Can the ZZ-6LNA Blocking Oligomer Inhibit KF DNAP (exo+)?

The first question was to whether or not the ZZ-6LNA Blocking Oligomer could inhibit Φ29 DNAP (exo+). However, before jumping to an entirely new enzyme, KF DNAP (exo+) was used to yield a preliminary assessment of possible gel results with an exonuclease-proficient enzyme. As is shown in Figure 10 below, the ZZ-6LNA Blocking Oligomer inhibits both the polymerase and exonuclease activities of KF DNAP (exo+). This is seen in regards to polymerase activity inhibition in lanes 3 and 5, where the presence of the ZZ-6LNA Blocking Oligomer inhibits primer extension. Also in lane 5, both the strength of the primer band and the lack of hydrolysis bands identify that the primer is being protected from exonuclease activity, and so the ZZ-6LNA Blocking Oligomer design inhibits KF DNAP (exo+).

![Figure 10: Comparison of primer extension between KF DNAP (exo-) and KF DNAP (exo+) to identify the efficacy of the ZZ-6LNA Blocking Oligomer against exonuclease proficient enzymes. Primer extension was inhibited in lanes 3 and 5, relative to the control in lane 2 that shows where the band for complete primer extension appears, absent Blocking Oligomer. Complete image available in Supplement, Figure S2.](image)

This assay also identified the difference between an exonuclease deficient enzyme and an exonuclease proficient enzyme, as discussed theoretically earlier in the paper. This comparison is left out of the image above, however it can be found in Supplementary Figure S2, and is shown by the presence of a series of bands leading towards the bottom of the gel, indicating the
presence of smaller and smaller DNA chains in the reaction product that was ran in the denaturing PAGE-gel. The next step is to test the ZZ-6LNA Blocking Oligomer against Φ29 DNAP (exo+) with KF DNAP (exo+) controls.

### 4.2 Can the ZZ-6LNA Blocking Oligomer Inhibit Φ29 DNAP (exo+)?

The ZZ-6LNA Blocking Oligomer was tested against Φ29 DNAP (exo+) in primer extension and excision assays with a KF DNAP (exo+) control to standardize the results. Since both enzymes are exonuclease-proficient, both exonuclease (Figure 11a) and polymerase (Figure 11b) activities were assessed to ascertain if primer was protected from both activities.

As Figure 11 above shows, the 6-LNA Blocking Oligomer does inhibit Φ29 DNAP (exo+) polymerase and exonuclease activities, however it is still considered to be too expensive to be used practically. And so, less-costly Blocking Oligomer designs were tested for efficacy.

**Figure 11:** (a) Exonuclease Inhibition. Comparison of primer excision with Φ29 DNAP (exo+) to KF DNAP (exo+) to identify that the ZZ-6LNA Blocking Oligomer inhibited primer hydrolysis (lanes 3 and 5). This is known because the control hydrolysis bands in lane 2 disappear in lanes 3 and 5. (b) Polymerase Inhibition. Lanes 2 and 3 show complete extension products produced by the polymerase activity. Compared to lanes 3 and 5, we see that this activity was inhibited, and that exonuclease activity is not observed as seen in (a). Complete image available in Supplement, Figure S3.
4.3 Can a less costly Blocking Oligomer be used to inhibit Φ29 DNAP (exo+)?

In the previous assays, the Blocking Oligomer inhibited primer extension by Φ29 DNAP (exo+) to a greater extent than either form of KF. It is possible that the 6LNAs are not needed, and that perhaps the diacridine modification alone can effectively inhibit Φ29 DNAP (exo+). Without the LNAs, a significantly lower-cost Blocking Oligomer could be used. This can be tested by adapting the initial primer extension assay ran against KF DNAP (exo-) (Figure 9) to test the necessity of the 6LNA modifications for effective Φ29 DNAP (exo+) inhibition.

![Image of primer extension assay](image)

**Figure 15:** Primer extension with Φ29 DNAP (exo+) to compare Blocking Oligomer designs. As lanes 3 and 4 show, primer extension inhibition only required the diacridine-modified (ZZ) 5’ terminus, making the expensive 6LNA modification unnecessary. Complete image available in Supplement, Figure S4.

As is shown in the results of the assay below (Figure 15), the 6LNAs are indeed not needed to inhibit Φ29 DNAP (exo+). Adding only a 5’ modification of diacridine (lane 4), without incorporating the 6LNAs, the Blocking Oligomer was able to efficiently inhibit Φ29 DNAP (exo+) from extending the primer.

4.4 Can the exonuclease activity of Φ29 DNAP (exo+) be inhibited?

As discussed earlier, a primary concern with the utility of Φ29 DNAP (exo+) is the exonuclease activity. In previous assays, we only examined the fate of the Fluorescently labeled primers in each reaction, however it is also important to know the fate of our Blocking Oligomer
and Templates. Clearly, the Blocking Oligomer did protect the primer from excision. Although, the Blocking Oligomer itself may have had its non-complementary tail digested, and subsequently been extended to complement the template. If so, this would make it impossible to remove on the nanopore, since the tail is required to act as the lever to facilitate the removal of the Blocking Oligomer on the nanopore.

If the Blocking Oligomer can be protected from digestion, then so to would the primer. For the next assay, I tested a certain modification commonly used by our lab for other purposes block the exonuclease activity of Φ29 DNAP (exo+). This exonuclease-inhibiting modification will be known as “Mod X” in this paper to avoid patenting issues with a coming publication from our lab (details follow below in the Discussion).

![Figure 16 Excision Assay results for the utility of Mod X to inhibit the exonuclease activity of Φ29 DNAP (exo+). Complete image available in Supplement, Figure S5.](image)

The above gel image in Figure 16 shows the results of an assay to test the efficacy of Mod X against Φ29 DNAP (exo+) under exonuclease-favoring conditions, as discussed earlier in Methods (Section 4.3). The results clearly show that the Blocking Oligomer lacking Mod X was digested (lane 2), whereas the Blocking Oligomer with Mod X remained intact (lane 4 compared
to the DNA alone control in lane 3). Therefore, it is reasonable to conclude that Mod X successfully inhibited Φ29 DNAP (exo+).

4.5 Is the diacridine modification needed to inhibit Φ29 DNAP (exo+)?

When the diacridine modification was first incorporated in the design of the Blocking Oligomer to inhibit KF DNAP (exo-), it functioned to inhibit enzyme binding at the primer-template junction by tightening the gap between the DNA primer and Blocking Oligomer. It has been shown by the results of my previous assays that inhibition of primer extension by Φ29 DNAP (exo+) binding does not require the 6LNAs (Figure 15). Upon further analysis, the results suggest enzyme the Blocking Oligomer may also not need the diacridine modification (ZZ) on the 5’ terminus, which functioned to inhibit primer extension by inhibiting enzyme binding and subsequent DNA replication. To test this hypothesis, a primer extension assay can be run against Blocking Oligomers with and without diacridine modifications, the designs for which will also include the “Mod X” that inhibits exonuclease activity (Figure 16).

Figure 17 Results of the primer extension assay to test necessity of the diacridine modification (ZZ) in the Blocking Oligomer against Φ29 DNAP (exo+). As lanes 3 and 4 show, the Blocking Oligomers with and without the modification were both equally capable of inhibiting primer extension. Complete image available in Supplement, Figure S6.
As the results of the assay show in Figure 17 above, the Blocking Oligomer design no longer requires the diacridine modification to inhibit primer extension by Φ29 DNAP (exo+). This is shown by successful inhibition of polymerase activity with and without the diacridine modification (lanes 3 and 4), since relative to the complete extension product band (lane 2), no significant extended product is detected. Moreover, this assay corroborates the ability of the X-modification (Mod X) to inhibit primer excision because no significant hydrolysis products are detected in lanes with Mod X. On the other hand, absent the Blocking Oligomer (lane 2), some hydrolysis did occur, despite being under conditions favoring polymerase activity over exonuclease activity, as described earlier in Methods (see section 4.3).

5 Discussion

As my study has revealed, a Blocking Oligomer can be used to inhibit Φ29 DNAP (exo+) DNA polymerase, the design for which is shown below, in Figure 18. Moreover, this new Blocking Oligomer cost less to synthesize because it no longer requires the modifications of the diacridine or the 6LNAs. This final design is more practical and cost-effective for use on the α-HL Nanopore.

![Figure 18: Final design of Blocking Oligomer to inhibit Φ29 DNAP (exo+), Mod X represented as a box for patenting issues. [Adapted from 1]]

One key issue that has not been addressed in this paper was the precise structural nature of the “Mod X”-modification that protected the Blocking Oligomer from the exonuclease activity
of Φ29 DNAP (exo+). Mod X will soon be revealed in a coming publication from our lab (publication in preparation), under the primary authorship of Gerald Maxwell Cherf. Until then, future work is still needed to optimize the Blocking Oligomer further for increasing throughput on the Blocking Oligomer.

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References
A. DNA Purification Protocol

All DNA was ordered from and synthesized at the Stanford PAN-oligonucleotide synthesis facility using phosphoramidite chemistry. Due to the inefficiency of the synthesis reactions, the DNA needed to be purified upon delivery by poly-acrylamide gel electrophoresis. The protocol is listed below.

- Re-suspend Blocking Oligomers in appropriate volume of PAGE extension buffer solution of 7M urea and 0.1x TE.
- Denature Blocking Oligomers by heating to 95°C for 3 minutes. (upon cooling, the Blocking Oligomers should remain denatured due to the urea).
- Mix, pour, and polymerize gel solution: 7M Urea, 1xTBE, and 13% acrylamide/bis-acrylamide. Gel plates = 12”x7”, spacers between plates = 2mm.
- After gel is polymerized, load wells with denatured Blocking Oligomer samples.
- Gel run: 28W for ~ 1.5hours
- DNA recovery: cut out and elute bands in ~700µL of buffer (0.3M NaOAc, 1mM EDTA, pH 5.2-5.5) in eppendorf tubes.
- Leave tubes rotating overnight in buffer to elute DNA.
- Centrifuge all tubes.
- Remove liquid from each tube and place into separate, new eppendorf tubes.
- Add one volume of phenol to each tube.
  - If DNA contains acridine, add one volume of a mixture of (2/3) chloroform and (1/3) phenol. Acridine with dissolve in pure phenol and you will not be able to precipitate the DNA in future steps.
- Vortex and centrifuge tubes
- Remove organic phase (at the bottom of each tube).
- Repeat previous phenol-chloroform extraction once.
- Add one volume of pure chloroform.
- Vortex and centrifuge tubes
- Remove organic phase (at the bottom of each tube).
- Repeat previous chloroform extraction once.
- Add 2.5-3x volumes of 100% ethanol to each tube.
- Vortex and centrifuge tubes
- Store tubes at -80°C for 10 minutes to facilitate precipitation.
- Centrifuge tubes at 15,000 rpm at 4°C for 30 minutes.
- Remove ethanol from each tube with micropipette.
- Centrifuge tubes.
• Remove remaining ethanol from each tube with micropipette.
• Place all tubes in an eppendorf tube rack, open all tubes and cover with a fresh sheet of aluminum foil.
• Let tubes sit until all the ethanol has evaporated out of each tube.
• Re-suspend pellets in 30µL of 1x TE final volume.
• Store in -20°C freezer.

B. Protocol for Primer Extension Assays/Blocking Oligomer Efficacy Assays
After purifying the Blocking Oligomers, they were individually tested against the enzymes to determine if they effectively inhibited their DNA polymerase and exonuclease activities. Each test contained a 22.5µL mixture containing the following concentrations of each reagent:
• 1.3µM primer DNA
• 1.3µM template DNA
• 1.6µM Blocking Oligomer
• 100µM d(A,T,C,G)TP
• 1µM Enzyme
• 0.3M KCl
• 0.01M K·Hepes (pH 8)
• 10mM MgCl₂
• 0.3x TE
In the control primer extension assays, the Blocking Oligomer was left out of the above mixture. Each experiment was run in the presence of enzyme for 60 minutes at room temperature (23°C) and then terminated with 200µL phenol. The resulting DNA was extracted from each experiment for further analysis by following the DNA extraction protocol listed in the appendix (C).

C. DNA Extraction Protocol
After testing the efficacy of Blocking Oligomer designs against enzymes, DNA was extracted for further analysis with the following protocol:
• Terminate each reaction by adding 200µL of phenol to each tube containing the reaction.
  o If Blocking Oligomers contain acridine, add 600µL Chloroform to each tube as well. Without the chloroform, the acridine will dissolve in the phenol phase and you will not be able to precipitate the DNA in the later steps.
• Add 180µL of a premixed solution of NaOAc[0.3M] and EDTA[1mM] to each tube.
• Vortex and centrifuge all tubes.
• Remove organic phase (at the bottom of each tube) using a micropipette.
• Centrifuge all tubes again.
• Remove remaining organic phase (at the bottom of each tube).
• Add 200µL chloroform to each tube.
• Vortex and centrifuge each tube.
• Remove organic phase (at the bottom of each tube) using a micropipette.
• Centrifuge all tubes again.
• Remove remaining organic phase (at the bottom of each tube).
• Add 1.5µL of glycogen to each tube to increase precipitation.
• Add 2x volumes of 100% ethanol to each tube.
• Store tubes at -80°C for 10 minutes to facilitate precipitation.
• Centrifuge tubes at 15,000 rpm at 4°C for 30 minutes.
• Remove ethanol from each tube with micropipette.
• Centrifuge tubes.
• Remove remaining ethanol from each tube with micropipette.
• Place all tubes in an eppendorf tube rack, open all tubes and cover with a fresh sheet of aluminum foil.
• Let tubes sit until all the ethanol has evaporated out of each tube.
• Re-suspend DNA pellets at the bottom of each tube in 10µL of a premixed denaturing PAGE-extension buffer solution containing 7M urea and 0.1x TE.
• Refrigerate tubes at 4°C until needed for PAGE analytical gel.

D. PAGE DNA Analysis Protocol
After extracting the DNA from each assay reaction (see Appendix B), to analyzed the state of the DNA the following protocol was implemented:
• Denature DNA samples in each tube by heating tubes to 95°C for 3 minutes. (upon cooling, the Blocking Oligomers should remain denatured due to the urea).
• Mix, pour, and polymerize gel solution: 7M Urea, 1xTBE, and 17% acrylamide/bis-acrylamide. Gel plates = 12”x7”, spacers between plates = .75mm.
• After gel is polymerized, load wells with denatured Blocking Oligomer samples.
• Run gel for approximately 1.5 hours at 18W, until Bromophenol Blue loading dye is an inch from the bottom of the gel.
• Visualize gel via UV-irradiation to see DNA with Fluorescent modifications.
Some gels were also stained with SybrGold to visualize all DNA bands, so as to assess the fates of all DNA from the reactions. This was necessary to determine which components of the Template-Primer-Blocking Oligomer complexes the (exo+) enzymes digested.