IN VITRO REPLICATION STUDIES OF OLIGONUCLEOTIDES CONTAINING

SITE-SPECIFIC DNA ADDUCTS IN FRAMESHIFT-PRONE SEQUENCES

By

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To the loves of my life, family, friends, and knowledge, may they never stop loving back…

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LIST OF ABBREVIATIONS

CCIQ   Carbocyclic IQ derivative

dA   Deoxyadenosine

dATP   2'-Deoxyadenosine triphosphate

dC   Deoxycytosine

dCTP   2'-Deoxycytosine triphosphate

dG   Deoxyguanosine

dGTP   2'-Deoxyguanosine triphosphate

DMSO   Dimethylsulfoxide

DMTr   4,4'-Dimethoxytrityl

DNA   Deoxyribonucleic acid

dNTP   2'-Deoxynucleotide triphosphate

Dpo4   *Sulfolobus solfataricus* P2 DNA polymerase IV

dT   Deoxythymidine

DTT   Dithiothreitol

EDTA   Ethylenediaminetetraacetic acid

ESI   Electrospray ionization

HCA   Heterocyclic aromatic amine

HPLC   High performance liquid chromatography

IQ   2-Amino-3-methylimidazo[4,5-*f*]quinoline

Kf^- Klenow fragment of *Escherichia coli* DNA polymerase I exo$^-$

LC-MS   Liquid chromatography mass spectrometry

MALDI-TOF	Matrix assisted laser desorption ionization – time of flight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to Charge
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
PAH	Polycyclic aromatic hydrocarbon
Pol II$^-$	*Escherichia coli* DNA polymerase II exo$^-$
PAGE	Polyacrylamide gel electrophoresis
Pol η	Human DNA polymerase eta
Pol ι	Human DNA polymerase iota
Pol κ	Human DNA polymerase kappa
TIC	Total Ion Current
TLS	Translesion synthesis
UDG	Uracil DNA glycosylase

CHAPTER I

INTRODUCTION

Part A: Cancer

According to the American Cancer Society, an American male has a one in two chance of being diagnosed with cancer in his life time and a female has a one in three chance. Fortunately, the five-year survival rate for Americans has risen approximately to 66% from 1996 to 2003, up from 53% for 1984 to 1986. For 2008, the American Cancer Society estimates that 1.4 million Americans will be diagnosed with a new case of cancer. Despite the increase in survival rate, 565,000 Americans will still die of cancer this year.[1] This number of yearly deaths related to cancer in the United States is nearly equivalent to the population of Nashville, TN.[2] Cancer deaths are the second leading cause of death in America, exceeded only by heart disease.[1] We must study and understand the facets of cancer in order to devise prevention and treatment strategies.

Cancer is the unregulated growth and spread of cells in the body and is believed to be initiated at the genetic level by the accumulation of mutations in the DNA on the cellular level. These mutations seem to be centered in two different, but related, sets of genes. These genes are the proto-oncogenes and the tumor suppressor genes.[3] In general, proto-oncogenes are involved in cell growth while tumor suppressor genes inhibit cell growth. Research indicates that cancer is dependent on these two classes of genes, which can be grouped into two different areas of concentration in the cell, in order to obtain its proliferation.[3,4] The first site is intracellular activity, which can be sub-categorized into

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agonist-induced signal transduction, DNA replication and repair, cell cycle control, and cell fate (survival, differentiation, senescence, and apoptosis). The second site is cell surface and extracellular functions, such as adhesion to molecules, proteases, and angiogenesis (formation of new blood vessels).[4] In order to understand some of these gene sites, we will briefly explore a few of them.

Cell cycle control is the ability of the cell to determine if the cell should pass through to the next stage of the cycle. The stages are divided into four distinct phases: gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M) (**Figure 1**). G_1 is characterized by growth of the cell size and preparations for DNA replications. S-phase replicates DNA, G_2 prepares the cell for mitosis, and M is the division of the parent cell into two daughter cells. The gate keepers between the various phases are cyclins and cyclin-dependent kinases (CDKs). If both are not working in conjunction with each other, the cell cannot proceed to the next phase, and cell division is arrested.[3]
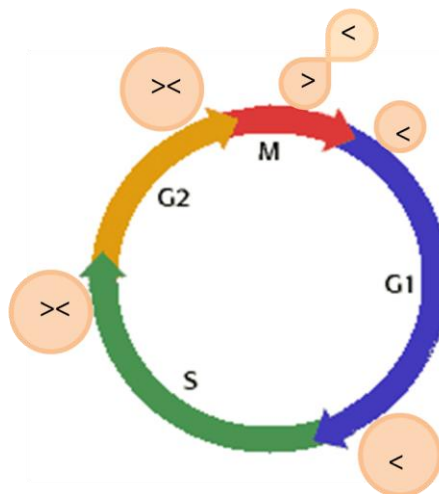
Figure 1: Cell cycle

Apoptosis is the programmed death of a cell. If for some reason a cell has a problem that cannot be corrected, the cell can initiate a series of events that lead to apoptosis. Such problems may be the injury of chromosomal DNA or disruptions to tumor suppressor genes. While cell "suicide" may seem extreme, the potential threat posed by a cancer cells survival is far worse for the survival of the organism.[3]

Cells have been found to have a limited set of divisions that can occur, somewhere in the neighborhood of 55 divisions. After such time, the cells seem to stop dividing and are considered senescent. However, cells that have a slight mutation in the retinoblastoma (RB) or p53 gene could continue to replicate. After a period of time, the surviving cells would reach a second state, crisis, where they would die; although, a few will also survive this stage and become immortal, allowing for indefinite replication of the cell.[3] Given these multiple deterrents to cancer, it is understandable that years of accumulations of mutations could lead cancer to occur in normal, healthy humans. The question is, if mutations are the cause of cancer, then what causes the mutations?

Mutations to the DNA of a cell can occur from exogenous and endogenous sources. Endogenous sources have been found to be due to inherited mutations, hormones, immune conditions, and mutations that occur from metabolism. Exogenous sources have been found to be such things as tobacco, environmental or occupational exposure to chemicals, radiation, infectious organisms, and even diet.[5] However, Western industrialized countries can attribute over 80% of cancer to exogenous sources, with diet and tobacco accounting for nearly 65% of those cases.[6] With such a high degree of mutations being linked to exogenous sources, it has become imperative that the research community look beyond cancer treatment and focus on the processes which lead

to the formation of cancer. Given that exogenous sources may account for the majority of cancer cases, this area shall be explored furthered.

In order to understand the complex nature of exogenous exposure to carcinogens, one must understand that each person has a different susceptibility to carcinogens. Factors that affect ones susceptibility are gender, ethnicity, diet, age, and health condition. Given all these factors, it is no wonder that proving a specific compound as carcinogenic is such a daunting task. A list of selected carcinogens is provided with the cancer they are known to induce in **Table 1**. It is of note that metabolic activation (also referred to as xenobiotic metabolism) is required for many known and suspected chemical carcinogens. This was discovered when xenobiotic metabolism of carcinogens was shown to parallel the chemical reactivity toward cellular macromolecules.[7]

Table 1: Selected Human Chemical Carcinogens[8,9]

Compounds	Main Sources/ Uses	Target Organs/ Cancer Type
Anticancer Drugs		
Melphalan	Chemotherapy	Leukemia
Thiotepa	Chemotherapy (no longer in use)	Leukemia
Aromatic Amines/ Amides		
2-Napthylamine	Dyes; antioxidant (no longer in use)	Bladder
4-Aminobiphenyl	Dyes; antioxidant (no longer in use); Research tool	Bladder
Metals (and Inorganics)		
Arsenic	Natural ores; alloys; pharmaceutical agent	Skin, Lung, Liver
Cadmium	Natural ores; pigments; batteries; ceramics	Lung, Prostate, Kidney
Nickel	Natural ores; alloys; electrodes; catalyst	Lung, Nasal Cavity
Olefins		
Ethylene oxide	Glycol and polyester production; sterilization	Leukemia, Lymphoma
Vinyl Chloride	Plastics; co-polymers	Liver (angiosarcoma)
Trichloroethylene	Degreasing operations; adhesives; lubricants	Liver, Kidney
Paraffines/ Ethers		
Bis(chloromethyl)ether	Technical application (rarely used)	Lung
Mustard Gas (sulphur mustard)	Chemical warfare in WWI; research	Lung

For many chemical carcinogens, such as polycyclic aromatic hydrocarbons and aromatic amines, xenobiotic metabolism is required in order to form a short-lived electrophilic compound (often referred to as the "ultimate carcinogen"), which covalently modifies DNA and causes the subsequent carcinogenic effect.[10,11] A plethora of enzymes carry out this metabolic action, such as the cytochrome P450s, glutathione S-transferases, and sulfonyltransferases. As a result, oxidizing and conjugating enzymes can activate carcinogens that modify DNA.[10] However, the fact that a chemical compound, or its metabolite, can modify DNA does not prove that it is a carcinogen as other factors play a role. These other factors include the extent of DNA adduction, the stability of the adduct, and the repair rate of the adduct. The modification of DNA by a carcinogen can depend on the DNA sequence and the reactivity of the carcinogen to DNA.[12] But as noted above, DNA adducts can be repaired.

Chemical carcinogens have been found not to react with DNA randomly; rather, carcinogens react with certain areas of DNA and nucleotides with higher frequencies. Sequence dependence has been observed to be of importance, with areas where DNA is prone to adduction known as hot spots. Two well known hotspots for mutation are a run of G's (GGGGG) and a dinucleotide repeat of alternating G's and C's (GCGCGC). These hotspots were found with reactive forms of acridines and induced frameshift mutations.[13,14] The reaction of DNA with a chemical carcinogen has been proposed to be one of the earliest steps in the initiation of cancer.[15] Fortunately, nature has developed mechanisms that recognize and repair damaged DNA, albeit with vastly different efficiencies. Repair of modified DNA can be accomplished by such repair mechanisms as mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair

(NER).[16] However, if these adducts are not repaired, a buildup of these modifications can lead to mutations, of which the outcome may lead to numerous alterations, such as the switching on or off of genes, irregular protein expression, or alterations in cell cycle control.[15]

In order for cancer to appear, a set of events must happen; these events include adduction of DNA and mutation of specific genes. The way the adduct works is by reacting with DNA and avoiding repair enzymes. Here, the adduct is incorrectly replicated by a DNA polymerase leading to miscoding errors, i.e. mispairing, deletion, translocation.[17] It is also possible for spontaneous mutations from base substitution or frameshift mutation to occur. These miscoding events must avoid repair, but they must also not cause apoptosis. If there are too many mutations or if the mutations are in critical genes, apoptosis can be activated, and the cell does not proceed to a cancerous state. This series of events are summed up in Figure 2. As stated above, the mutations must occur in two sets of genes in order to cause cancer, proto-oncogenes and the tumor suppressor genes. In the end, multiple mutations are needed in these two areas to cause cancer.[18]
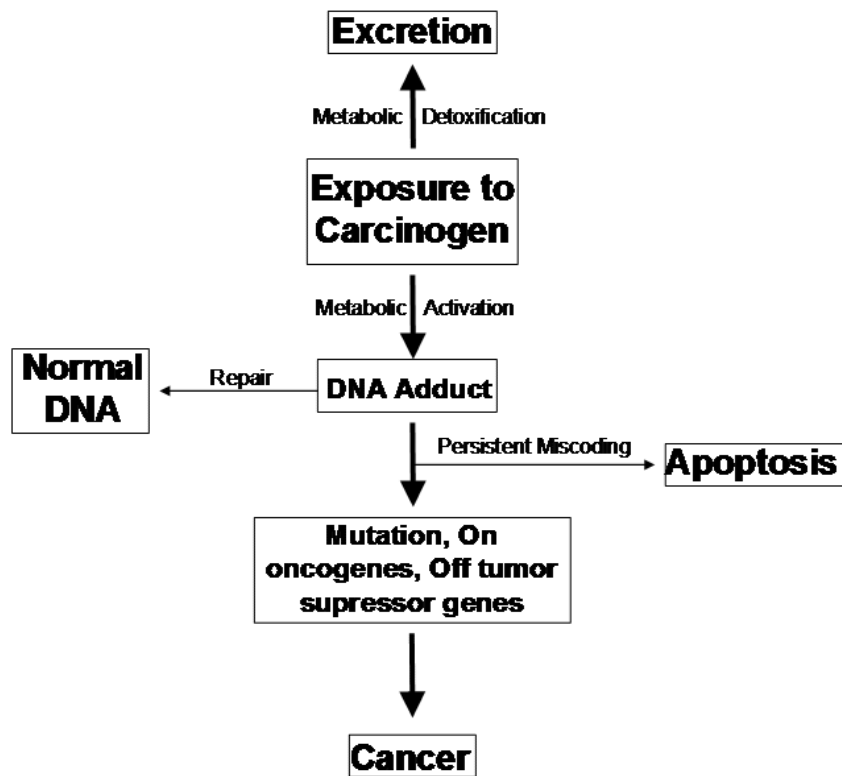
Figure 2: Chemical Carcinogen Route to Cancer

Part B: Translesion Synthesis (TLS)

As mentioned above, mutations may be caused by DNA polymerases. Polymerases are enzymes that replicate duplex DNA into two new duplexes; each duplex consists of one of the original strands and one newly replicated strand, a process better known as semiconservative replication. In order for a cell to be able to pass on its genes, the ability to replicate DNA becomes important. These replicative enzymes have extremely high fidelity, which is the accuracy of the replication, and high processivity, which allows for multiple rounds of catalysis with the enzyme before the enzyme disassociates from the DNA.[19] Mutations can arise when a DNA polymerase misincorporates across from an adducted base or when slippage occurs. DNA

polymerases can also spontaneously incorporate the wrong base, thus causing replication errors.[20]

As mentioned previously, a type of replication error of interest is frameshift mutation. Frameshift mutations are caused by the insertion or deletion of bases in a given sequence. By inserting, or deleting one or two bases, the reading frame is shifted and thus can lead to the change in translation of the genetic code. These mutations can be classified by the direction and number of bases that affect the sequence. The insertion of a single base is known as a +1 frameshift; while, the deletion of two bases is known as a -2 frameshift. A model for frameshift mutation that is well accepted is the Streisinger slippage model. The model proposes that the misalignment of the primer and template leads to a local bulging of the DNA during replication. Bulging is where DNA bases are extruded outside of the normal double helix. It is through this bulging that misalignment can occur and potentially lead to mutations.[21,22] It is hypothesized that DNA adducts can stabilize the slipped mutagenic (bulged) intermediate and can thereby promote frameshift mutations. An example of this is the aromatic amide, N-acetylaminoflourene (AFF), which can be seen in **Figure 3**.[22]

Figure 3: -2 Frameshift Mutation with AAF

Most replicative DNA polymerases share three common features: fingers, thumb and palm domains, which can be seen in **Figure 4**. The fingers interact with the incoming nucleoside triphosphate and the template base. As for the thumb, it positions the DNA and it also plays a role in the process and translocation associated with the enzyme. The palm catalyzes the phosphoryl transfer reaction.[23] Even though they have these similar regions, the kinetics, mechanism, fidelity, and processivity differ greatly between the different polymerases.[24]
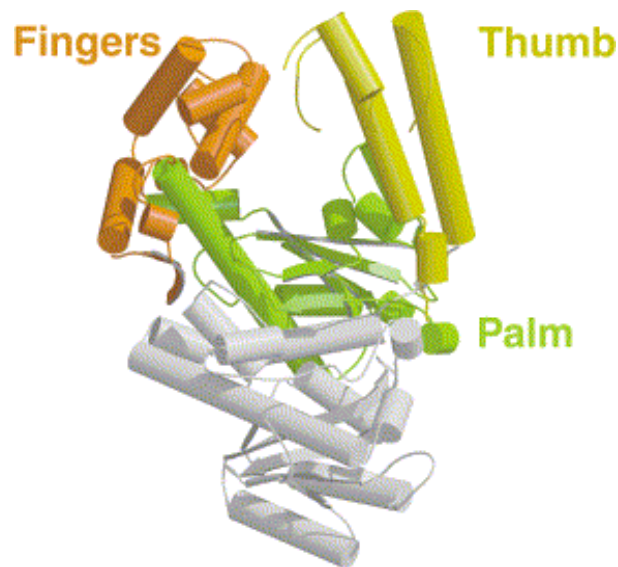
Figure 4: Regions of DNA Polymerases[25]

Depending on the DNA polymerase, mis-insertion and local environment of the sequence, error rates for single base nucleotide substitution vary from 10^{-3} to $>10^{-6}$.[26] DNA adducts can inhibit DNA replication and cause the replication fork to be halted. Recent data suggests that a gap is left where the replicative polymerases cannot replicate and then replication is carried on past the adduct.[27] A class of DNA polymerases has

recently been discovered that are able to conduct TLS and replicate through damaged DNA. These enzymes are largely members of the Y-family DNA polymerases.

In order to understand the complexity of TLS (of which much is still unknown) a simplified figure has been provided to walk through the steps of TLS (**Figure 5**). When an adduct is detected, two proteins are recruited, Rad18 and Rad6, and they signal for the cleavage of USP1, which is involved in the removal of ubiquitin from the proliferating cell nuclear antigen (PCNA), and allows for ubiquitination of PCNA. After PCNA has been ubiquitinated, the binding constants of the TLS polymerases are increased enough to allow for replacement of the higher binding, high fidelity polymerases (i.e., pol δ) with a Y-family polymerase (i.e., pol η). Translesion synthesis is carried out past the adduct, after which the higher fidelity polymerases are allowed to carry out the rest of the synthesis.[27]
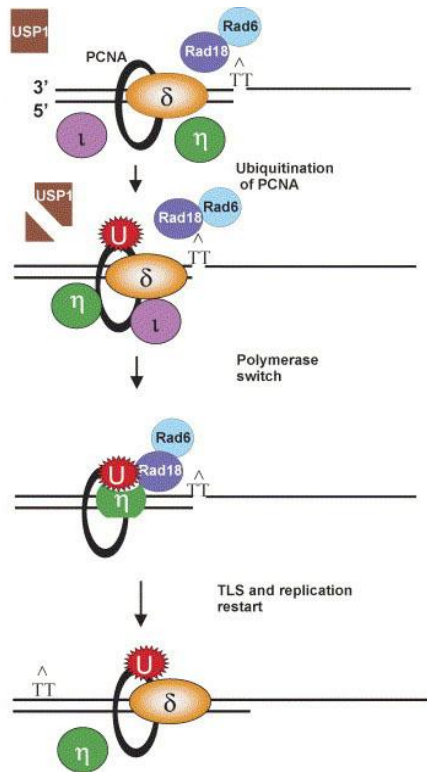
Figure 5: Model for TLS[27]

In contrast to replicative DNA polymerases, which can synthesize DNA with higher efficiency and accuracy, the Y-family DNA polymerases have a significantly higher error rate. The lower accuracy can be attributed to the fact that many of these TLS polymerases do not have exonuclease activity, which acts as a proofreader for the polymerase. In order to make up for this lack of an exonuclease, these polymerases have a little finger domain that helps facilitate the association of DNA, catalytic efficiency, and interactions with auxiliary factors.[28]

Given the complexity of TLS shown above, which is based on the synthesis in eukaryotes, simpler models have been devised in order to study the replication of these polymerases. Archaea have been studied for their mechanism of replication of DNA, and are believed to be similar to human replication, but at a simpler level.[29,30] The

advantages of using the archaea system is that they are structurally simpler, and there are proteins that are used in the analogous synthesis, which are structurally close to eukaryotes.[31] *Sulfolobus solfataricus* P2 has a polymerase known as DNA polymerase IV (Dpo4), which has been used as a model of the eukaryote polymerase η, although it is more closely reflected to pol κ.[32] Given the low fidelity and processivity, Dpo4 shows all the classical signs of Y-family DNA polymerases, including the lack of an exonuclease domain. Because of these characteristics, Dpo4 has helped in the study of replication with regards to mutation.

Like eukaryotes, bacteria are susceptible to modification of their DNA. One highly conserved protein is the Klenow fragment of *Escherichia coli* polymerase I. Klenow fragment has two enzymatic activities, the polymerase and the exonuclease. The polymerase activity works in the 5'→3' direction, while the exonuclease works in the 3'→5' direction, where the mis-incorporated nucleotides are edited.[33] A widely used form of the Klenow fragment lacks a functional exonuclease domain (Kf⁻). Although removing the function of the exonuclease can alter the overall base substitution activity of the enzyme, it is an attractive model for studies of DNA replication with an adducted oligonucleotide.[34]

E. coli has another highly conserved protein that is used in TLS, polymerase II (pol II). This prototype of the B-family of polymerases is one of the enzymes in the SOS system of bacteria. The SOS system also includes DNA polymerases II, IV, and V, which participates in TLS. It has been proposed that pol II is the enzyme that catalyzes the restart of the synthesis of damaged DNA, along with replication through a myriad of different adducts.[35]

The human Y-family polymerase η is a well studied polymerase. Pol η is known to be of extreme importance for the replication past TT dimers (a product of UV radiation), by inserting two A's opposite the dimer.[36] In the genetic disease known as a variant form of xeroderma pigmentosum, in which the risk of sunlight induced skin cancer is greatly enhanced, the subjects typically are deficient of pol η.[37,38] It is believed that when pol η is not available, that pol ι can serve as a backup polymerase for T-T dimmer bypass. Pol ι is far less efficient at correctly replicating DNA past TT dimers.[39]

Polymerase ι is a very unique polymerase, in that it is the only polymerase believed to use Hoogsteen base pairing for replication.[40] This gives a good explanation for why pol ι replicates through N^2 adduct, these compounds affect the N^2 position of dG. As will be discussed later, pol ι has been studied with the N^2 adduct γ-hydroxy-1,N^2-propano-2'-deoxyguanosine (γ-HOPdG). Another reason for the interest of pol ι is that it incorrectly incorporates dGTP opposite A at higher rates than the correct base dTTP. [41] However, after pol ι has inserted a base opposite the adduct, it is very inefficient at further extension past the adduct. Pol ι has been proposed to insert a base opposite the lesion and then allows another polymerase, such as pol κ, to carry out the extension.[42] With regard to human health, no known human or mammalian conditions have been found that relate to individuals lacking pol ι.[43]

One enzyme that has been implicated in working in conjugation with pol ι is pol κ. A hypothesis for this states, "that an important biological function of pol ι is to incorporate nucleotides opposite minor-groove DNA adducts of purines and that pol κ is to carry out the subsequent extension reaction."[42] Another important correlation made with pol κ is that when knock-out cells of pol κ were made, UV light sensitivity was

observed.[44] Since pol κ is believed not to be the major polymerase involved in the bypass of adducts related to UV exposure, it is believed to be involved in NER. This is due to NER deficient cells being susceptible to UV light mutations.

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C8-DEOXYGUANOSINE 2-AMINO-METHYLNAPHTHOL[1,2-*d*]IMIDAZOLE ADDUCT

Part A: Introduction

The heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is one of the most potent compounds ever tested in the Ames assay of *Salmonella typhimurium*, having been shown to be 20 fold more mutagenic than aflatoxin B_1, and is found to be present in ppb to ppm levels in a variety of cooked meats and fish.[1,2,3] Heterocyclic amines (HCA's) have been known to cause mutations in different organisms. For example, HCA's have been found to cause frameshift mutations in CG repeat sequences in bacteria, while point mutations have largely been found in mammalian cells.[4] Also studied in relationship to IQ was a carbocyclic analogue of IQ, 2-amino-3-methylnaphtho[1,2-*d*]imidazole (CCIQ, **Figure 6**). CCIQ was found to be more than 5 orders of magnitude (10^5) less mutagenic for frameshift than IQ with Ames assays.[4] The difference in mutagenic effects can be deduced by the removal of the nitrogen in blue, thus showing the importance of it.
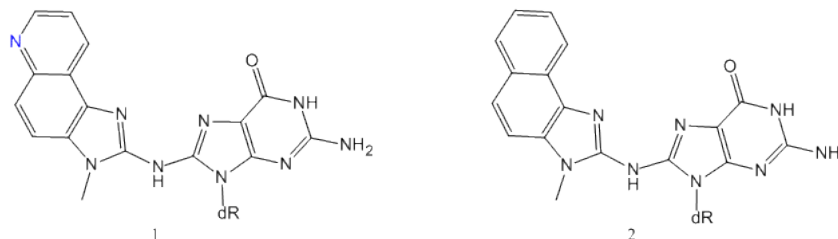
Figure 6: Structures of IQ and CCIQ

Due to the presence of IQ in the diet of most Americans and its mutagenic activity, it is likely that **1** plays a role in the etiology of some cancers. IQ has been studied in rodents and was found to induce tumors of the liver, skin, forestomach, and colon, among others.[5] As mentioned, some mutagenic compounds require activation to their final mutagenic form; IQ is one of these compounds. Activation of IQ occurs mostly in the liver, where cytochrome P450 1A2 oxidizes IQ to a hydroxylamine. N-Acetyltransferase is involved in the subsequent esterification that gives the N-acetoxy ester. Solvolysis of the N-acetoxy ester generates an aryl nitrenium ion, which is the DNA modifying agent (ultimate carcinogen). The major adduct that is formed from IQ is at the C8 position of 2'-deoxyguanosine (dG), while the minor adduct is the N^2-dG adduct.[6]

As mentioned earlier, HCA's are known to cause frameshift mutations at CG repeats. For this reason, the frameshift prone *Nar*I recognition ($G_1G_2CG_3CC$) will be used. This sequence is known to be a hotspot for -2 frameshift mutation induced by arylamines.[7]

In previous research from the Rizzo group, **1** was incorporated in at the G_1 and G_3 position of the *Nar*I sequence and the *in vitro* replication was examined with three different polymerases: Dpo4, Kf⁻, and pol II⁻. Replication by Dpo4 was error prone or error free depending on the on the local sequence of the adduct. When the studies were carried out with Kf⁻, extension past the G_1 adduct was inefficient, but the G_3-adducted base was extended and resulted in a two-base deletion. Studies performed with pol II⁻ showed that the G_3-adduct was extended resulting in both a -1 and -2 frameshift products.

In contrast to, the G_1 adduct was replicated in an error-free manner. These experiments were able to show that the G_3-position has a higher rate of deletions.[8]

Part B: Experimental

Unless otherwise noted, all commercially available chemicals were used as received. MADLI-TOF mass spectrometry was performed at the Vanderbilt University Mass Spectrometry Resource Center. 3-Hydroxypicolinic acid (HPA) was the matrix, and ammonium hydrogen citrate (7 mg/mL) was added to suppress sodium and potassium adducts. The purified DNA polymerases Dpo4, Kf$^-$, and pol II$^-$ were obtained from the lab of Dr. Guengerich (Vanderbilt University). dNTP solutions (100mM) were purchased from New England BioLabs. [γ-^{32}P]ATP was purchased from Perkin-Elmer (NEN). The unmodified oligonucleotides and primer nucleotides were purchased from Midland Certified Reagents. All synthesized oligonucleotides and their purification would not have been made possible without the help of Dr. Ivan Kozekov and Albena Kozekova.

Synthesis of Oligonucleotides: An Expedite 8909 DNA synthesizer (PerSeptive Biosystems) was used to synthesize the adducted nucleotides using a 1 μmol scale of the Ultramild line of phosphoramidites (phenoxyacetyl-protected dA, 4-isopropyl-phenoxyacetyl-protected dG, acetyl-protected dC, and T phosphoramidites). The solid supports were purchased from Glen Research. Manual coupling of the adduct was used to incorporate the adduct into the oligonucleotides, otherwise the manufacturer's standard synthesis protocol was used. For manual coupling, the column was removed from the synthesizer and syringes were placed at each end of the column, one of which contained

250-300 μL of the manufacturer's 1*H*-tetrazole activator solution and the other contained 250 μL of the phosphoramidite (11mg, 0.095M in anhydrous methylene chloride). The solutions were drawn through the column (1*H*-tetrazole first). This was repeated for 30 min, with several minutes between sets. The column was returned to the synthesizer and allowed the capping, oxidation and detritylation steps to occur before the remainder of the oligonucleotide could be synthesized.

High Performance Liquid Chromatography (HPLC): Analyses and purification was performed using a Beckman HPLC with a UV diode array detector (Model 166) that monitored at a wavelength of 254 nm. The software used with the HPLC was 32 Karat (Version 3.1). The oligonucleotides were purified with a sodium phosphate buffer at pH 7.0 (Solvent 1) and methanol (Solvent 2). The gradient was initially at 1% solvent 2 and was then linearly increased over 27.5 min to 35% solvent 2. After that time, the gradient was increased to 50% over 2.5 min and then followed by 5 min isocratic at 50% solvent 2. Finally, the gradient was brought back to initial conditions over a period of 5 min.

Capillary Gel Electrophoresis (CGE): Analyses of the purified oligonucleotides were carried out on a Beckman P/ACE Instrument System 5500 Series, in which the oligonucleotide was monitored at a wavelength of 260 nm. The capillaries used with the P/ACE MDQ instrument were 31.2 cm x 100 μm eCAP. The samples were applied at 10 kV and were allowed to run at 9 kV. The manufacturer's 100-R gel (for ss DNA) was used to pack the column using a Tris-borate buffer with 7.0 M urea. Modified oligonucleotides were found to be of >95% purity.

Synthesis of 5'-d(ACTC-G$^{\text{C8-CCIQ}}$-GCGCCAATCCTTACGAGCCCCC)-3'

Oligonucleotide was purified using the HPLC method to yield 29.2μmol. MALDI-TOF

MS (HPA): *m/z* calcd for (M-H) 8320.5, found 8317.4

Synthesis of 5'-d(ACTCGGC-G$^{\text{C8-CCIQ}}$-CCAATCCTTACGAGCCCCC)-3'

Oligonucleotide was purified using the HPLC method to yield 27.7 μmol. MALDI-TOF

MS (HPA): *m/z* calcd for (M-H) 8320.5, found 8317.7

Labeling and Annealing of Oligonucleotides: The given primer was labeled at
the 5'-end by T4 polynucleotide kinase (T4PNK) and buffer (T4PNK buffer), which were
obtained from New England BioLabs, and [γ-^{32}P]ATP. One nmol of primer was used
with 5 μL of T4PNK buffer (10x concentration) and 40 units of the T4PNK enzyme. [γ-^{32}P]ATP, with 12.5 μL, was added and the volume adjusted to 50 μL with water. The
reaction was allowed to react for one hour at 37 °C and then stopped by placing the
reaction in a 95 °C bath for 10 min. The reaction was purified on a Biospin column
(BioRad). Annealing a 1:1 molar ratio of the adducted or unadducted template and
primer was carried out with 50 mM Tris-HCl buffer (pH 7.8) by heating for 5 min at
95 °C and then allowing for a slow cool down to 30 °C.

Single Nucleotide Incorporation Assays: The annealed ^{32}P-labeled
oligonucleotides were extended in the presence of a single dNTP. The dNTPs (2 μL)
were added to preincubated enzyme/DNA mixtures which brought the total amount of
solution to 10 μL. The final concentrations for the assays with Dpo4, Kf⁻ and pol II⁻
were: 50 mM Tris-HCl, pH 7.8, 100 nM DNA duplex, 48 nM enzyme, 1 mM DTT, 50 μg
mL^{-1} BSA, 50 mM NaCl, and 5 mM MgCl$_2$. Dpo4 reactions were run at 37 °C for 30
min; while Kf⁻ and pol II⁻ were allowed to react for 15 min at ambient temperature. The

reactions were quenched by 70 μL of a 20 mM EDTA in 95% formamide (v/v) containing bromophenol blue and heated for 10 min at 95°C. Electrophoresis was used to separate aliquots (2-5 μL) of the reaction on a denaturing gel containing: 8.0 M urea, 16% acrylamide (w/v) (from 19:1 Acrylamide/Bisacrylamide Stabilized Solution, AccuGel, National Diagnostics), and 80 mM Tri borate buffer, pH 7.8, with 1 mM EDTA. A PhosphorImager screen (Imaging Screen K, Bio-Rad) was exposed to the gel for a minimum of 6 hours. The screen was imaged using a PhosphorImaging system (Bio-Rad, Personal Molecular Imager) and the manufacturer's Quantity One software, version 4.6.3.

Full-length Extension Assay: The annealed ^{32}P-labeled oligonucleotides were extended in the presence of all four dNTPs. The dNTPs (2 μL) were added to preincubated enzyme/DNA mixtures to bring the total amount of solution to 10 μL. The final concentrations for the assays with Dpo4, Kf$^-$, and poll II were: 50 mM Tris-HCl, pH 7.8, 100 nM DNA duplex, 48 nM enzyme, 1 mM DTT, 50 μg BSA mL^{-1}, 50 mM NaCl, and 5 mM MgCl$_2$. Dpo4 reactions were ran at 37 °C for 30 min; while Kf$^-$ and pol II$^-$ were allowed to react for 15 min at 23 °C. The reactions were quenched with 70 μL of a 20 mM EDTA in 95% formamide (v/v) containing bromophenol blue and heated for 10 min at 95°C. Electrophoresis was used to separate aliquots (2-5 μL) of the reaction on a denaturing gel containing: 8.0 M urea, 16% acrylamide (w/v) (from 19:1 Acrylamide/Bisacrylamide Stabilized Solution, AccuGel, National Diagnostics), and 80 mM Tri borate buffer, pH 7.8, with 1 mM EDTA. A PhosphorIamger screen (Imaging Screen K, Bio-Rad) was exposed to the gel for a minimum of 6 hours. The screen was imaged using a PhosphorImaging system (Bio-Rad, Personal Molecular Imager) and the manufacturer's Quantity One software, version 4.6.3

Part C: Results and Discussion

A: CCIQ

Three different oligonucleotides were synthesized, two containing the C8-CCIQ adduct at either the G_1 or the G_3 position of the *Nar*I sequence and one containing no modification at all. Once the oligonucleotides were purified, they were labeled at the 5' end with ^{32}P phosphate. The template was then annealed with the corresponding -1 primer for single nucleotide incorporation or -4 primer for nucleotide extension. The given sequences were used in order to compare the results obtained by James Stover with the C8-IQ adduct.[8] The two positions tested have significantly different properties. The G_1 position of *Nar*I sequence with the C8-IQ adduct has been found to be in a minor groove-bound conformation by Feng Wang and Michael P. Stone.[9] They also found that the G_3 position of *Nar*I with IQ adopts a base-displaced intercalated conformation.[10]

Klenow Fragment

CCIQ was shown to be a significant block to Kf$^-$. The polymerase was able to incorporate only a negligible amount of dCTP (**Figure 7: 1a, 2a**), but only at high dCTP concentrations. This was also the case when the adduct was incorporated at both the G_1 and G_3 position. This differed from the IQ results, which showed that Kf$^-$ could incorporate dCTP more efficiently. We observed incorporation of dCTP with the unmodified oligonucleotides, even with the lowest concentration in both oligonucleotides (**Figure 7: 1b, 2b**).

5'- GGG GGC TCG TAA GGA TTG GCG C
3'- CCC CCG AGC ATT CCT AAC CGC GXC ACT -5'

| A | T | G | C |

1a
(X = CCIQ₁)

0 50 100 250 μM   0 50 100 250 μM   0 50 100 250 μM   0 50 100 250 μM

1b
(X = dG)

0 1 2.5 5 μM   0 1 2.5 5 μM   0 1 2.5 5 μM   0 1 2.5 5 μM

5'- GGG GGC TCG TAA GGA TTG G
3'- CCC CCG AGC ATT CCT AAC CXC GGC ACT -5'

| A | T | G | C |

2a
(X = CCIQ₃)

0 50 100 250 μM   0 50 100 250 μM   0 50 100 250 μM   0 50 100 250 μM

2b
(X = dG)

0 1 2.5 5 μM   0 1 2.5 5 μM   0 1 2.5 5 μM   0 1 2.5 5 μM
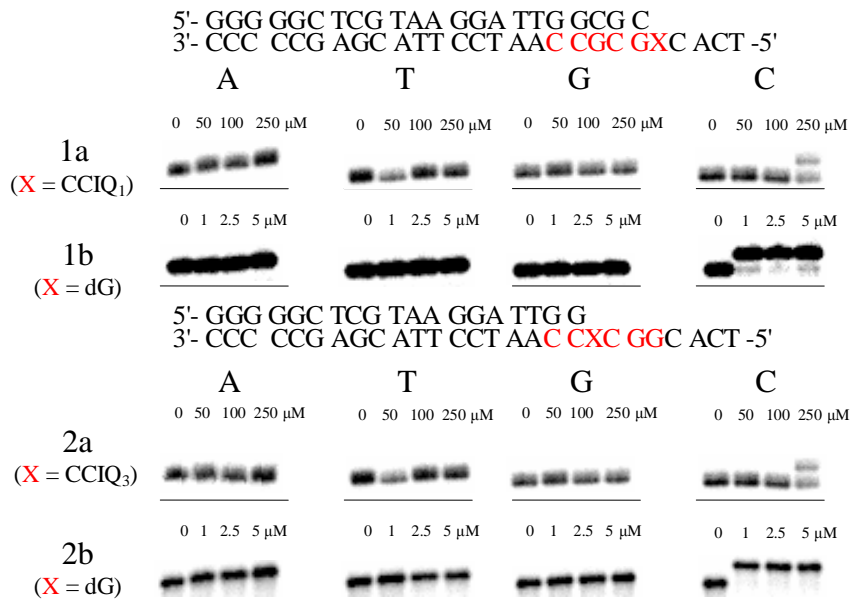
Figure 7: Single Nucleotide Incorporation by Kf⁻

The CCIQ modified oligonucleotides were annealed to -4 primers, and extension by Kf⁻ in the presence of all four dNTPs was also examined. The polymerase was determined to be unable to extend the primer up to the adduct but insertion opposite the CCIQ was completely inhibited (**Figure 8: 1a, 2a**). This result is consistent with the results observed for the single nucleotide incorporation studies, where Kf⁻ was unable to incorporate a base opposite the adduct. On the other hand, Kf⁻ was able to fully extend the primer opposite the unmodified template in both cases (**Figure 8: 1b, 2b**). When CCIQ is compared to IQ, these results show that CCIQ is a complete block to replication by Kf⁻, but the IQ adduct is bypassed and gives a modest amount of full extension products and -2 deletion product at the G_3 position.[8]

26

5'- GGG GGC TCG TAA GGA TTG G   22   25
3'- CCC CCG AGC ATT CCT AAC CGC GXC ACT -5'

    0   2.5   5   10 μM   0   0.5   1   5 μM

                                                        ← 27

                        ← 22

1a
(X = CCIQ$_1$)   1b
                                                       (X = dG)

5'- GGG GGC TCG TAA GGA T   19   22   25
3'- CCC CCG AGC ATT CCT AAC CXC GGC ACT -5'

   0   2.5   5   10 μM   0   0.5   1   5 μM

                                                        ← 27

                        ← 19

2a
(X = CCIQ$_3$)   2b
                                                       (X = dG)

Figure 8: Nucleotide Extension by Kf$^-$

Polymerase II

CCIQ was also found to be a complete block for pol II$^-$ at the G$_1$ position of the *Nar*I sequence (**Figure 9: 1a**). Pol II$^-$ efficiently incorporated dCTP opposite an unmodified G (**Figure 9: 1b**). At the G$_3$ position, pol II$^-$ incorporated both dCTP and dGTP opposite CCIQ (**Figure 9: 2a**). This differed greatly from results obtained with the C8-IQ adduct, where pol II$^-$ was only able to incorporate dGTP opposite the G$_1$ position and was able to insert dCTP and dGTP opposite the G$_3$ position, with dGTP incorporated more efficiently.[8] The unmodified oligonucleotide was able to incorporate only the correct base, dCTP (**Figure 9: 2b**). In summary, misincorporation was observed for

27

CCIQ adduct at the G_3 position, which is similar to that of the C8-IQ adduct, though the
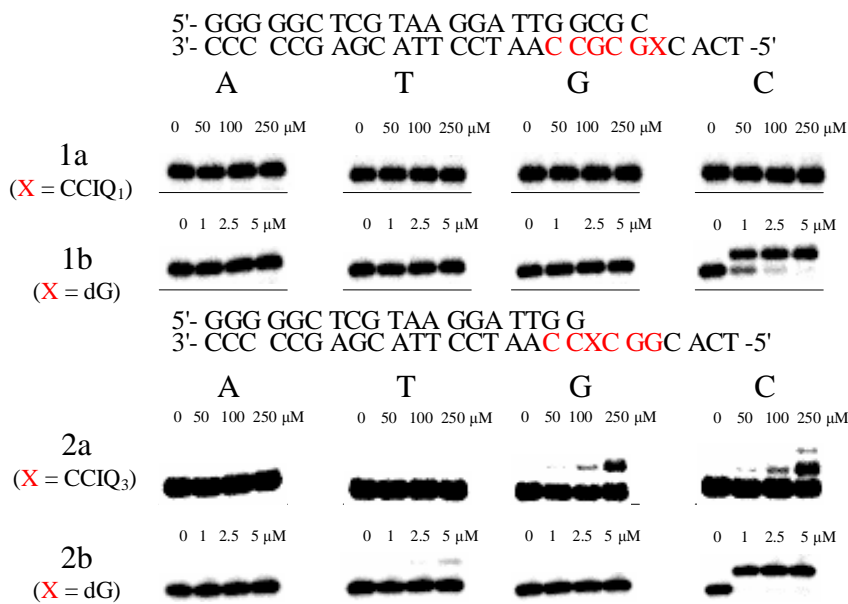
dNTP insertion appears to be less efficient.

Figure 9: Single Nucleotide Incorporation by Pol II$^-$

The results obtained from pol II$^-$ for primer extension are consistent with the

single nucleotide incorporations (**Figure 10**). Pol II$^-$ was not able to extend the primer

past the CCIQ adduct at the G_1 position (**Figure 10: 1a**), suggesting that the C8-CCIQ

adduct is a block to replication. As was the case with the CCIQ adduct at the G_3 position

(**Figure 10: 2a**), with a running start pol II$^-$ was able to incorporate up to the adduct, but

not across from or past the adduct. This differs from the single nucleotide incorporation

reactions because slight incorporation at the higher concentrations of dCTP and dGTP

was observed. The reactions were run again in which both dNTPs and time were

increased. The results confirmed the full extension reactions in which the CCIQ adduct at

the G_3 position is a block to replication (**Appendix A**). Unlike CCIQ, pol II⁻ was able to replicate the full length extended product opposite the C8-IQ adduct, with error-free bypass seen for the G_1 position and -1 and -2 products seen for the G_3 position.[8] Pol II⁻, like Kf⁻, was able to carry out full length extension for the unmodified oligonucleotides (**Figure 10: 1b, 2b**).

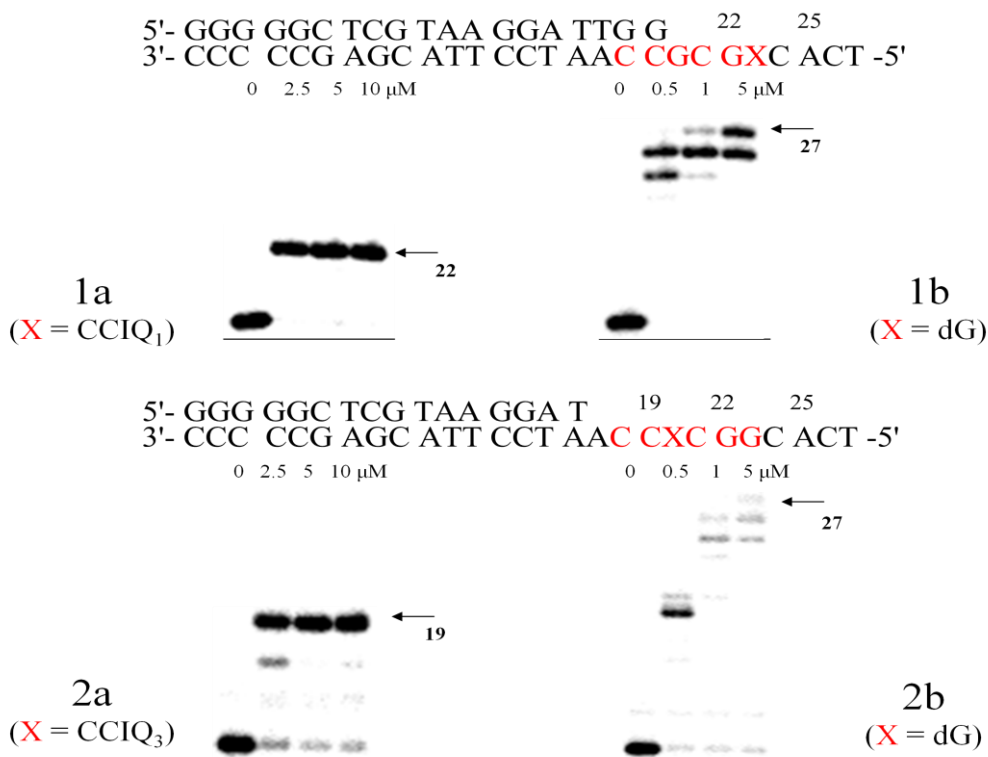**Figure 10:** Nucleotide Extension by Pol II

Dpo4

For the polymerase Dpo4, efficient incorporation of dCTP opposite the C8-CCIQ adduct at the G_1 position was observed (**Figure 11: 1a, 1b**). At the G_3 position, Dpo4 was able to incorporate dCTP opposite the C8-CCIQ adduct but with less efficiency than when the adduct was at the G_1 position (**Figure 11: 2a, 2b**). These results were also

observed in the studies with C8-IQ adduct, with the difference being that C8-CCIQ adduct at the G_3 position did not misincorporate T.[8]

5'- GGG GGC TCG TAA GGA TTG GCG C
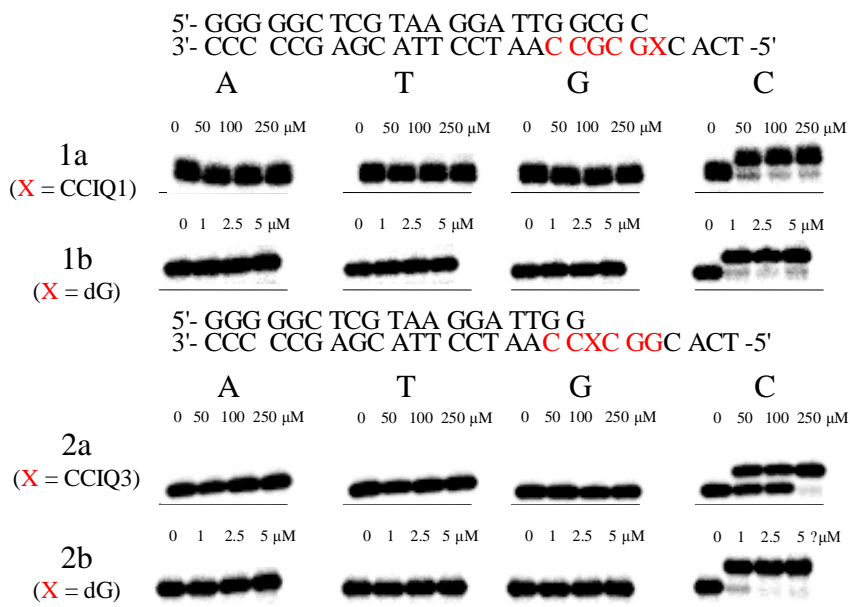3'- CCC CCG AGC ATT CCT AAC CGC GXC ACT -5'

Figure 11: Single Nucleotide Incorporation by Dpo4

In the full extension reactions with the recessed primers, it was observed that Dpo4 was able to incorporate opposite the C8-CCIQ adduct but there was a pause just before the adduct, and extension past the adduct was not observed for either position (**Figure 12: 1a, 2a**). The incorporation opposite the adduct is in accordance with the results from our single nucleotide incorporation studies. Once more, like the other polymerases, the unmodified oligonucleotide was fully extended by Dpo4 (**Figure 12: 1b, 2b**). In contrast to the present results, Dpo4 was able to extend the C8-IQ adduct at the G_3 position to give -1 and -2 deletion products.[8]
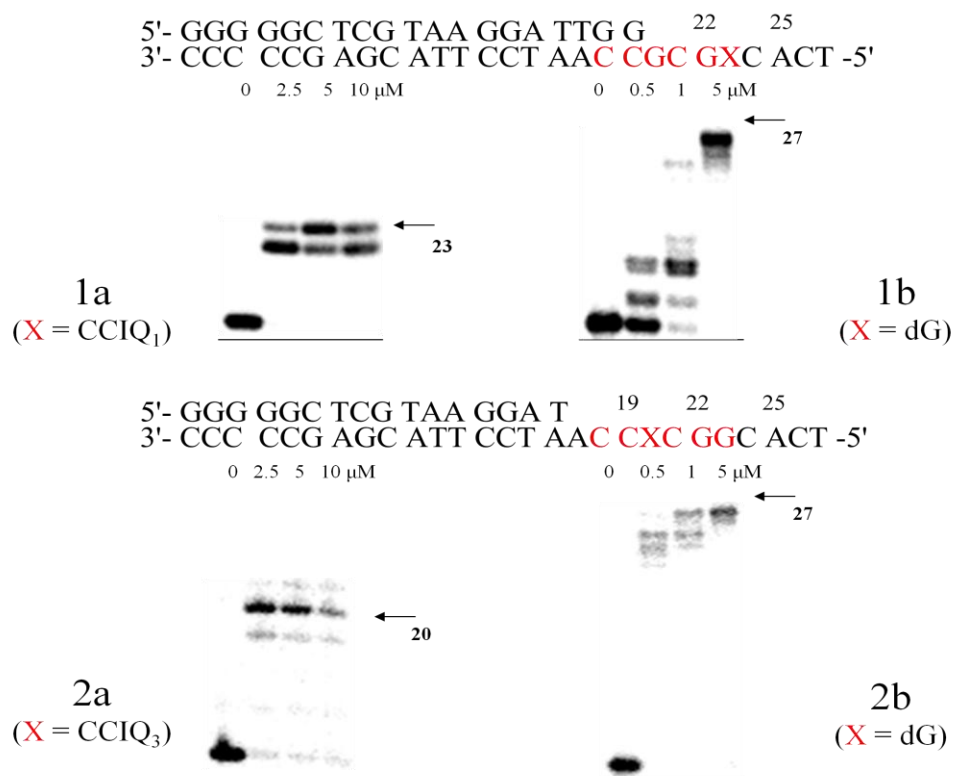
5'- GGG GGC TCG TAA GGA TTG G $\quad$ 22 $\quad$ 25
3'- CCC CCG AGC ATT CCT AAC CGC GXC ACT -5'

0   2.5   5   10 μM   0   0.5   1   5 μM

← 27

← 23

1a
(X = CCIQ$_1$)

1b
(X = dG)

5'- GGG GGC TCG TAA GGA T $\quad$ 19 $\quad$ 22 $\quad$ 25
3'- CCC CCG AGC ATT CCT AAC CXC GGC ACT -5'

0   2.5   5   10 μM   0   0.5   1   5 μM

← 27

← 20

2a
(X = CCIQ$_3$)

2b
(X = dG)

Figure 12: Nucleotide Extension by Dpo4

Part D: Conclusion

The dietary mutagen IQ has been well studied in the Rizzo lab, as well as others, but the analogue CCIQ has not been. The C8-CCIQ adduct was placed in a sequence containing the frameshift prone *Nar*I sequence that was previously studied with the C8-IQ adduct. Two positions were studied in the *Nar*I sequence, the G$_1$ and G$_3$ positions. Replicative studies were then carried out with recessed primers for both single nucleotide incorporation and full length extension. The three polymerases used for the *in vitro* replication studies were Kf⁻, pol II⁻, and Dpo4.

Single nucleotide incorporation assays showed that Kf⁻ was blocked by the C8-CCIQ adduct at both positions. Pol II⁻ was also blocked when the C8-CCIQ adduct at

the G_1 position; but pol II⁻ was able to incorporate both a dCTP and dGTP with reasonable efficiencies opposite the adduct when at the G_3 position of the *Nar*I sequence. Unlike the other polymerases, Dpo4 was able to insert opposite the CCIQ adduct at both positions of the *Nar*I sequence and no mis-incorporation was seen.

Full-length extension reactions were also examined for the C8-CCIQ adduct at both positions of the *Nar*I sequences with the three polymerases. At the G_1 and G_3 positions, the C8-CCIQ adduct blocked insertion opposite the adduct and extension past the adducts was fully inhibited with Kf⁻ and pol II⁻. These results are in line with the results obtained by the single nucleotide incorporation reactions, except for the G_3 position of the C8-CCIQ adduct with pol II⁻. We expected to see at least minor incorporation opposite the adduct. Increasing the concentration of dNTPs and length of the reaction time resulted in low levels of incorporation opposite the adduct, but no further extension (**Appendix** A). Unlike these two polymerases, Dpo4 was able to incorporate opposite C8-CCIQ adduct at both positions but extension past the adduct was blocked. Increasing the reaction time and concentration of dNTPs resulted in low levels of extension past CCIQ, but given the extreme conditions for the reaction, it is believed that CCIQ is a serve block to replication with Dpo4 (**Appendix A**).

As noted earlier, an Ames assay showed CCIQ to be much less mutagenic than IQ.[4] Our studies suggest the C8-CCIQ adduct is a strong block for replication for both Kf⁻, pol II⁻, and Dpo4. Thus, the lack of revertents observed in the Ames assay could be due to lethality rather than a lack of mutagenicity.

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CHAPTER III

1,N^2-DEOXYGUANOSINE ADDUCTS

Part A: Introduction

Another DNA adduct of interest is γ-hydroxy-1,N^2-propano-2'-deoxyguanosine (γ- HOPdG, **3**), which is formed from both endogenous and exogenous sources. The source for this adduct is the α,β-unsaturated aldehyde acrolein. Acrolein is believed to be produced endogenously through lipid peroxidation.[1,2] Biological studies of acrolein in human cells showed that it causes mutations and has tumor-initiating activity.[3,4]

In previous *in vitro* DNA polymerase assays, **3** was studied with the human DNA polymerases η, ι, and κ, but not in frameshift prone DNA sequences.[5,6] In these previous studies, pol η was shown to incorporate the correct base C opposite **3**. Additionally, when extension assays were performed with a primer that was recessed by nine bases, it was shown that pol η could incorporate opposite **3** as well as extend past it.[5] It was shown that pol ι was able to incorporate the correct base C and the incorrect base T opposite **3**. However, pol ι was not able to further extend past **3**. Pol κ, on the other hand, was not able to incorporate a nucleotide opposite **3**. When the study was performed with a combination of pols ι and κ, insertion opposite the adduct and extension was observed.[6]
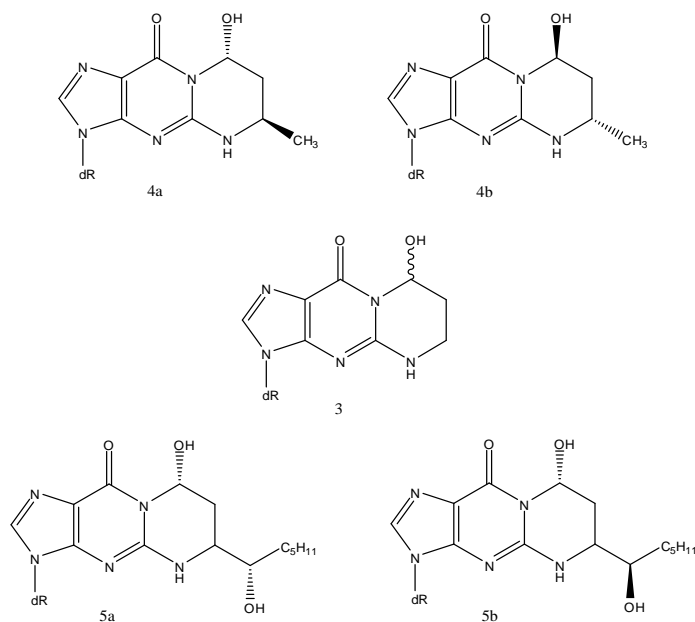
Figure 13: Adducts of Acrolein and Crotonaldehyde

Two adducts that are closely related to **3** are the isomers of crotonaldehyde, *R*-and *S*-α-CH₃-γ-OH-1,N²-propano-2'-deoxyguanosine (**4a** and **4b**). This α,β-unsaturated aldehyde has been shown to induce tumors in rodent livers and in human lymphoblasts.[7,8] Like **3**, **4a** and **4b** can result from exogenous and endogenous sources. Lipid peroxidation is a likely endogenous source for **3**, while exogenous sources can be tobacco smoke and *N*-nitrosopyrrolidine.[9-10] While these two adducts have not been studied with regards to *in vitro* DNA polymerase assays, the related adducts of 4-hydroxynonenal (HNE), 11*R* and 11*S*-trans-4-hydroxy-2-nonenal adducts of dG (**5a** and **5b**), have been examined.[11]

The DNA polymerase extension assay studies of **5a** and **5b** were done with pol ι and pol κ, and the results were similar to **3**. The first nucleotide extension studies with a recessed primer showed that pol ι is able to incorporate opposite both isomers but is not able to extend past the adducts. Unlike pol ι, pol κ was not able to incorporate a dNTP

opposite the adducts. For a second set of studies, the primer was not recessed, but had a C opposite the adduct. In this case pol ι could not extend past the adduct for either of the isomers. However, pol κ was able to extend from a primer terminus in which C was paired opposite adducts **5a** and **5b** with reasonable efficiency; although, it could not insert a dNTP opposite the adduct.[12]

The interest of our research is twofold. The first goal is to study the effects of specific adducts on the replication of DNA by Y-family polymerases. The second goal is to study these adducts in frameshift prone sequences. These studies were done through *in vitro* DNA polymerase extension assays.   The adducts were conducted as previous studies, i.e. **3**, **4a**, and **4b** were studied with pol η, pol ι, and pol κ. Through these studies we were able to indicated compounds which may be frameshift inducers.

Part B: Experimental

Unless otherwise noted, all commercially available chemicals were used as received. MADLI-TOF mass spectrometry was performed at the Vanderbilt University Mass Spectrometry Resource Center. A 3-hydroxypicolinic acid (HPA) matrix was used, and ammonium hydrogen citrate (7 mg/mL) was added to suppress sodium and potassium adducts. Purified enzymes pol η, pol ι, and pol κ were purchased from Enzymax. dNTP solutions (100mM) were purchased from New England BioLabs.   [γ-^{32}P]ATP was purchased from Perkin-Elmer (NEN).   The unmodified oligonucleotides and primer nucleotides were purchased from Midland Certified Reagents.   All synthesized oligonucleotides and their purification were prepared by Dr. Ivan Kozekov and Albena Kozekova.

Synthesis of Oligonucleotides: An Expedite 8909 DNA synthesizer (PerSeptive Biosystems) was used to synthesize the adducted oligonucleotides on a 1 μmol scale, using the Ultramild line of phosphoramidites (phenoxyacetyl-protected dA, 4-isopropyl-phenoxyacetyl-protected dG, acetyl-protected dC, and T phosphoramidites). The solid supports were purchased from Glen Research. The manufacturer's standard synthesis protocol was used.[13,14]

High Performance Liquid Chromatography (HPLC): Analyses and purification were performed using a Waters HPLC with a dual UV wavelength detector (Model 2487) that monitored at a wavelength of 254 nm. The software used with the HPLC was Empower 1. The oligonucleotides were purified with a 0.1M ammonium formate buffer at pH 3.5 (Solvent 1) and acetonitrile (Solvent 2). The gradient was initially at 1% solvent 2 and was then linearly increased over 15 min to 8% solvent 2. The gradient was isocratic for 10 min at 8% solvent 2; after which time the gradient was increased to 99% solvent 2 over a period of 2 min. The gradient was then isocratic for 3 min at 99% solvent 2. The gradient was decreased to 1% solvent 2 over a period of 2 min and isocratic for the final 3 min at 1% solvent 2.

Capillary Gel Electrophoresis (CGE): Analyses of the purified oligonucleotides were carried out on a Beckman P/ACE Instrument System 5500 Series, in which the oligonucleotide was monitored at a wavelength of 260 nm. The capillaries used with the P/ACE MDQ instrument were 31.2 cm x 100 μm eCAP. The samples were applied at 10 kV and were allowed to run at 9 kV. The manufacturer's 100-R gel (for ss DNA) was used to pack the column using a Tris-borate buffer with 7.0 M urea. Modified oligonucleotides were found to be of >95% purity.

Synthesis of 5'-d(CCCGGGG-G$^{\gamma\text{-HOPdG}}$-AAGCCTGCTCATGAGCCCCC)-3'

Oligonucleotide was purified using the Method II given above to yield 9.3 μmol. MALDI-TOF MS (HPA): *m/z* calcd for (M-H) 8588.4, found 8588.6

Synthesis of 5'-d(CCCGGGG-G$^{R\text{-croto}}$-AAGCCTGCTCATGAGCCCCC)-3'

Oligonucleotide was purified using the method given above to yield 22.2 μmol. MALDI-TOF MS (HPA): *m/z* calcd for (M-H) 8602.4, found 8603.0

Synthesis of 5'-d(CCCGGGG-G$^{S\text{-croto}}$-AAGCCTGCTCATGAGCCCCC)-3'

Oligonucleotide was purified using the method given above to yield 21.2 μmol. MALDI-TOF MS (HPA): *m/z* calcd for (M-H) 8602.4, found 8603.3

Labeling and Annealing of Oligonucleotides: The given primer was labeled at the 5'-end by T4 polynucleotide kinase (T4PNK) and T4PNK buffer, which were obtained from New England BioLabs, using [γ-^{32}P]ATP. A concentration 0.5 nmol of primer was used with 2.5 μL of T4PNK buffer (10x concentration) and 20 units of the T4PNK enzyme. [γ-^{32}P]ATP, with 6.25 μL, was added and the volume adjusted to 8.85 μL with water. The reaction was allowed to react for one hour at 37 °C and then stopped by placing the reaction in a 95 °C bath for 10 min. The reaction was purified on a Biospin column (BioRad). Annealing of the template (adducted or unadducted) and primer, using a 1:1 molar ratio, was carried out with 50 mM Tris-HCl buffer (pH 7.8) by heating for 2 min at 95 °C and then allowing to slowly cool to 30 °C.

Single Nucleotide Incorporation Assays: The ^{32}P-labeled oligonucleotides, that have been annealed, were extended in the presence of single dNTPs. The dNTPs (2 μL) were added to preincubated enzyme/DNA mixtures which brought the total amount of solution to 10 μL. The final concentrations for the assays with pol η, κ, and ι were: 25

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mM Tris-HCl, pH 7.8, 10 nM DNA duplex, 4 nM enzyme, 5 mM DTT, 0.1 mg mL^{-1} BSA, and 5 mM MgCl$_2$. Pol η, κ, and ι were allowed to react for 30 min at ambient temperature. The reactions were quenched by 20 μL of a 20 mM EDTA in 95% formamide (v/v) containing bromophenol blue and heated for 10 min at 95°C. Electrophoresis was used to separate aliquots (5-8 μL) of the reaction on a denaturing gel containing: 8.0 M urea, 16% acrylamide (w/v) (from 19:1 Acrylamide/Bisacrylamide Stabilized Solution, AccuGel, National Diagnostics), and 80 mM Tri borate buffer, pH 7.8, with 1 mM EDTA. A PhosphorImager screen (Imaging Screen K, Bio-Rad) was exposed to the gel for a minimum of 6 hours. The screen was imaged using a PhosphorImaging system (Bio-Rad, Personal Molecular Imager) and the manufacturer's Quantity One software, version 4.6.3.

Full-length Extension Assay: The ^{32}P-labeled oligonucleotides (after annealing) were extended in the presence of all four dNTPs. The dNTPs (2 μL) were added to preincubated enzyme/DNA mixtures which brought the total amount of solution to 10 μL. The final concentrations for the assays with pol η, κ, and ι were: 25 mM Tris-HCl, pH 7.8, 10 nM DNA duplex, 4 nM enzyme, 5 mM DTT, 0.1 mg mL^{-1} BSA, and 5 mM MgCl$_2$. Pol η, κ, ι, and a mixture of pol κ and ι were allowed to react for 30 min at ambient temperature. The reactions were quenched by 20 μL of a 20 mM EDTA in 95% formamide (v/v) containing bromophenol blue and heated for 10 min at 95°C. Electrophoresis was used to separate aliquots (5-8 μL) of the reaction on a denaturing gel containing: 8.0 M urea, 16% acrylamide (w/v) (from 19:1 Acrylamide/Bisacrylamide Stabilized Solution, AccuGel, National Diagnostics), and 80 mM Tri borate buffer, pH 7.8, with 1 mM EDTA. A PhosphorImager screen (Imaging Screen K, Bio-Rad) was

exposed to the gel for a minimum of 6 hours. The screen was imaged using a PhosphorImaging system (Bio-Rad, Personal Molecular Imager) and the manufacturer's Quantity One software, version 4.6.3.

Sequencing of Full-Length Extension: The full-length extension assays were performed at 37 °C with 25mM Tris-HCl, pH 7.8, 100 nM DNA duplex with a biotinated primer, 40 nM enzyme, 5 mM DTT, 0.1 µg mL^{-1} BSA, 5 mM MgCl$_2$, and 50 µM of each dNTP. The enzymes used were pol ι, pol κ, and a combination of pol ι and κ. The reaction was allowed to run overnight, then 0.5 mL of Streptavidin Sepharose™ beads, (GE Healthcare) which had been centrifuged and washed subsequently with phosphate buffer (2 x 500 µL), was added and rinsed with phosphate buffer (3 x 200 µL). The resulting suspension was allowed to shake for 2 hours in a rotation shaker. The beads were then washed with water (2 x 400 µL). Hydrolysis by UDG was completed in a 500 µL solution at 37 °C with 20 units UDG, 50 mM Tris-HCl, 1mM EDTA, and 1 mM DTT for 4 hours. The beads were then washed with water (2 x 400 µL). The DNA is hydrolyzed using 0.25 M piperidine in a 95 °C bath for 1 hour. The liquid was collected and the beads were washed with water (2 x 350 µL). All fractions were combined, filtered, and lyophilized. The resulting product was dissolved in water (60 µL). A mixture of 20 µL of the solution and 0.007 A260 of 5'-pTG TGT GTG G, which is used as an internal standard, were used for MS analysis on a Waters Acquity UPLC contected to a Finnigan LTQ mass spectrometer with an Acquity UPLC C18 column at the Vanderbilt University Mass Spectrometry Research Center. The UPLC conditions started with the column being warmed to 55 °C and consisted of Solvent 1, 10 mM NH$_4$CH$_3$CO$_2$ in 2% CH$_3$CN, and Solvent 2, 10 mM NH$_4$CH$_3$CO$_2$ in 95% CH$_3$CN, with a

gradient initially at 0% for Solvent 2, with a flow rate of 150 µL. The gradient for Solvent 2 increased to 3% over 3 minutes, at which time the gradient became linear for 1.5 minutes in order to reach 20%. Solvent 2 was then increased to 100% in 0.5 minutes and then isocractic for Solvent 2 for 0.5 minutes. The gradient was then reduced to 0% of Solvent 2 in 1 minute, at which time Solvent 2 was isocratic for 3 minutes. The samples were injected by an auto-sampler. The conditions for the LTQ consisted of the source voltage at 4 kV, source current 100 µA, auxiliary gas flow at 20, sweep gas flow at 5, sheath gas flow at 34, capillary voltage at -49 V, cappilarry temperature at 350 °C, and tube lens voltage at -90 V. The MS/MS conditions consisted of normalized collision energy at 35%, activation Q 0.250, and activation time of 30 ms. The product ion spectra were acquired for a range of 345-2000 *m/z*. The ions chosen for MS/MS went through a CID analysis and the CID products that showed promise for being an oligonucleotide sequence were compared to data from the Mono Oligo Mass Calculator, Mass Spectrometry Group of Medicinal Chemistry at the University of Utah (http://library.med/utah.edu/masspec).

Part C: Results and Discussion

Section I: Polymerase By-pass Assays

Four different oligonucleotides were synthesized, one each containing the dG adducts of acrolein, *R*-crotonaldehyde, *S*-crotonaldehyde, and one with no modified base. The adducts were incorporated at the beginning of a reiterated sequence of four G's. The template was then annealed to the corresponding -1 primer for single nucleotide

incorporation or a -4 primer for full-length extension assays. The primer strands were then labeled at the 5'-end with ^{32}P. Single nucleotide incorporation and extension reactions were then examined using the human DNA polymerases η, κ, and ι.

Polymerase η

The single nucleotide incorporation of a reiterated sequence of G's produces an extended product (**Figure 14**). While for the most part dATP, dTTP, and dGTP were incorporated opposite the adducts, likely due to high concentration of dNTP. All adducts allowed for some incorporation and extension, but not as efficiently as the unmodified oligonucleotide. Of the three adducts studied, the acrolein and *R*-crotonaldehyde adducts showed considerably better incorporation efficiency than the *S*-crotonaldehyde adduct (**Figure 14: 1b, 1c, 1d**). Of note is that the adducts showed a +1 extension, but because the unmodified oligonucleotide gave this same product (**Figure 14: 1a**), the result is likely to be from a blunt end insertion at the end of the sequence.

5'- GGG GGC TCA TGA GCA GGC TT ^{22} ^{25} ^{28}
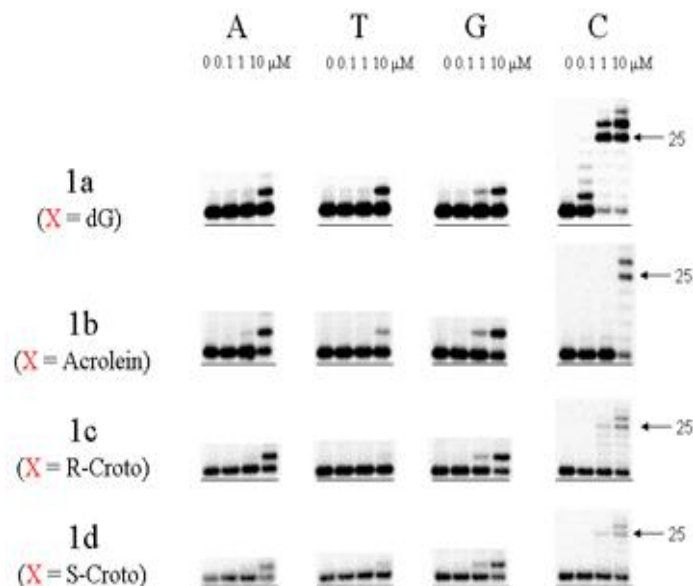3'- CCC CCG AGT ACT CGT CCG AAX GGG GCC C -5'

Figure 14: Single Nucleotide Incorporation by Pol η

Pol η and all four dNTPs were used for full-length extension reactions. Pol η was able to complete the extension past the acrolein adduct (**Figure 15: 1b**). The fully extended insertion product was a +1 product; while this could be interpreted as a frameshift insertion, examination of the unmodified oligonucleotide also showed a +1 extension product (**Figure 15: 1a**), indicating that an insertion is not likely and probably the result of blunt end insertion. Pause sites were observed for the *R* isomer of crotonaldehyde up to the adduct site (**Figure 15: 1c**). However, pol η was able to bypass and extend to a +1 product. Again, this would not seem to be a frameshift insertion due to the unmodified oligonucleotide being extended to the same length product. The *S*-Crotonaldehyde adduct appeared to be the most significant block of the three adducts studied to replication by pol η (**Figure 15: 1d**). The +1 extension product was less

visible with the *S*-crotonaldehyde adduct than by the other adducts, although full-length
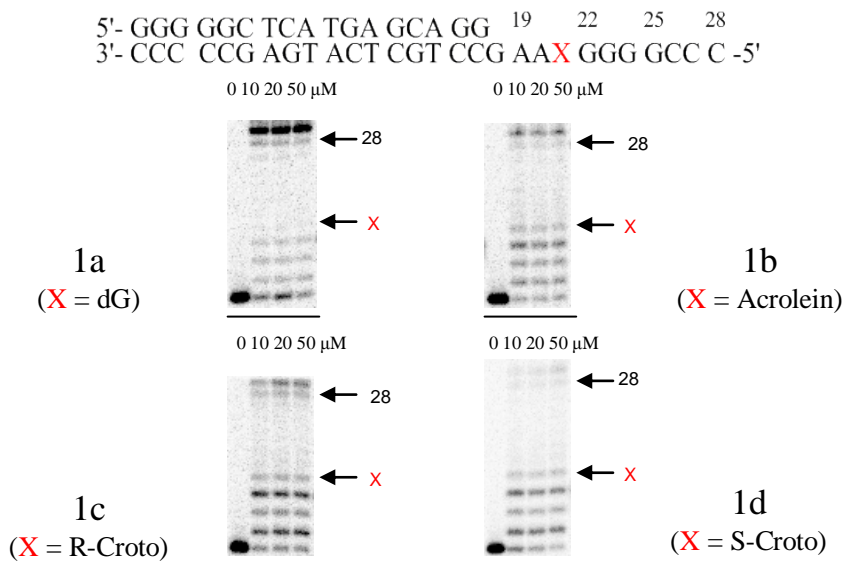
products could be observed.

5'- GGG GGC TCA TGA GCA GG   19   22   25   28
3'- CCC CCG AGT ACT CGT CCG AAX GGG GCC C -5'

0 10 20 50 µM   0 10 20 50 µM

← 28   ← 28

← X   ← X

1a   1b
(X = dG)   (X = Acrolein)

0 10 20 50 µM   0 10 20 50 µM

← 28   ← 28

← X   ← X

1c   1d
(X = R-Croto)   (X = S-Croto)

Figure 15: Nucleotide Extension by Pol η with All Four dNTPs

Polymerase Kappa

As with pol η, pol κ was able to incorporate multiple dCTPs with the unmodified

oligonucleotide, albeit with reduced efficiency (**Figure 16: 1a**). This same incorporation

was observed up to the G_5 position with the acrolein adduct (**Figure 16: 1b**), with similar

efficiency as the unmodified.   The *R*-crotonaldehyde adduct mis-incorporated a dGTP

opposite the adduct (**Figure 16: 1c**), but no incorporation of dCTP was seen. Insertion of

dGTP was observed opposite the *S*-crotonaldehyde adduct, but at a drastically reduced

efficiency as compared to the *R*-crotonaldehyde adduct, and there was no dCTP
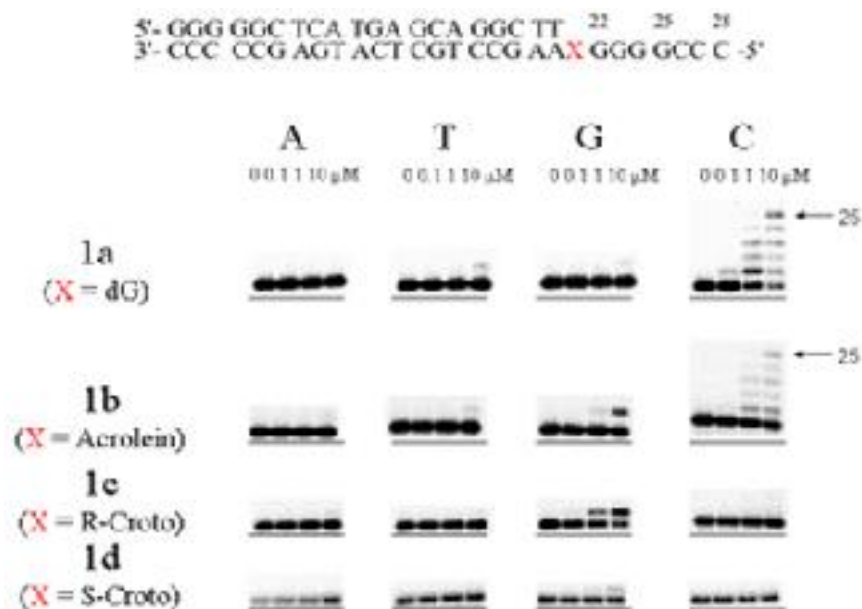
incorporation (**Figure 16: 1d**).

Figure 16: Single Nucleotide Incorporation by Pol κ

Pol κ appeared to be less efficient than pol η at extension (**Figure 17**). Specifically, the unmodified oligonucleotide required a higher concentration of dNTP in order to reach the fully extended product (**Figure 17: 1a**). When pol κ was used to replicate past the acrolein adduct, the adduct was a significant block to full extension, although a -1 extension product was observed, which may suggest a possible one base deletion product (**Figure 17: 1b**). Extension past the *R*-crotonaldehyde adduct appeared also to be very inefficient with pol κ (**Figure 17: 1c**). Pol κ was able to insert opposite the adduct at higher dNTP concentrations, but further extension was inefficient. Even at the high dNTP concentration further extension was largely inhibited, and only low levels of fully extended products were observed (**Figure 17: 1d**). With the *S* isomer of the crotonaldehyde adduct, Pol κ was severly blocked. The polymerase was only able to incorporate opposite the adduct but not extend, even at high concentration of dNTP.
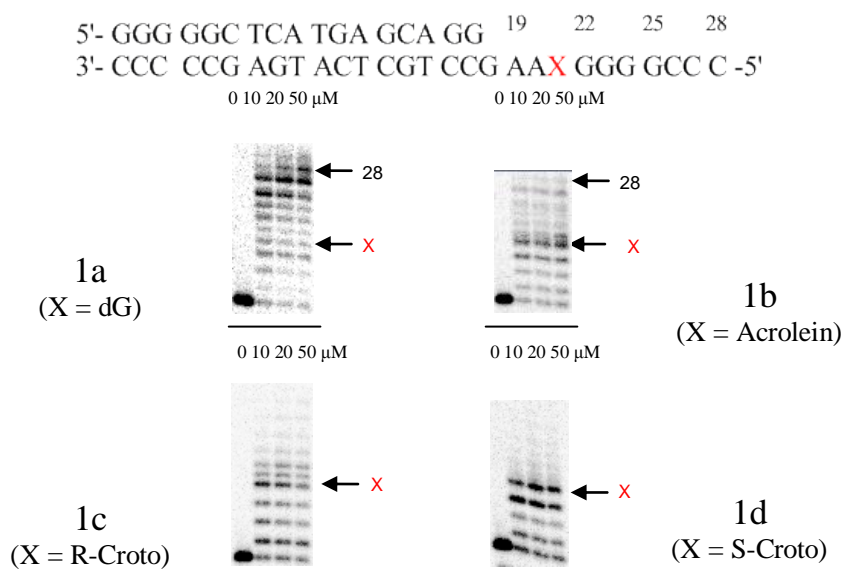
5'- GGG GGC TCA TGA GCA GG ^{19} ^{22} ^{25} ^{28}
3'- CCC CCG AGT ACT CGT CCG AAX GGG GCC C -5'

Figure 17: Nucleotide Extension by Pol κ with All Four dNTPs

Polymerase Iota

Pol ι is known to be an inefficient polymerase, but one that can incorporate a dNTP opposite a myriad of adducts (**Figure 18: 1a**). In the unmodified sequence, pol ι was able to extend the full G repeat sequence. The acrolein adduct showed that pol ι was only able to extend to the G_5 position, but a considerable amount of product remained with just one dCTP incorporated (**Figure 18: 1b**). In regards to the crotonaldehyde adducts, pol ι was not able to extend past either adduct, but did incorporate dCTP and dTTP opposite the adduct (**Figure 18: 1c, 1d**). This is not surprising given that studies have shown pol ι to work in tandem with pol κ, where pol ι incorporates opposite the adduct and pol κ extends past it. As for the mis-incorporation, we know that pol ι has a higher mis-incorporation rates in which dTTP is incorporated with almost equal efficiency as dCTP.
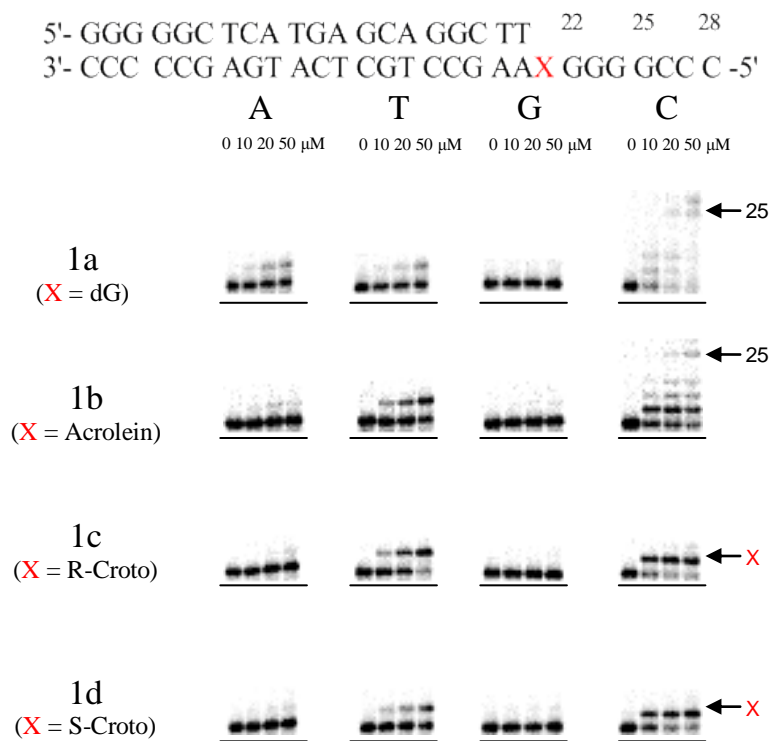
47

Figure 18: Single Nucleotide Incorporation by Pol ι

In the unmodified oligonucleotide, it is not a surprise that this polymerase struggles to reach the fully extended product (**Figure 19: 1a**). As stated earlier, pol ι is known for its ability to incorporate opposite a wide range of adducts, thus while all three adducts were blocks to some extent, incorporation opposite the adducts was still achieved (**Figured 1b, 1c, 1d**). These results are in accord with the results obtained from the single nucleotide incorporation results.
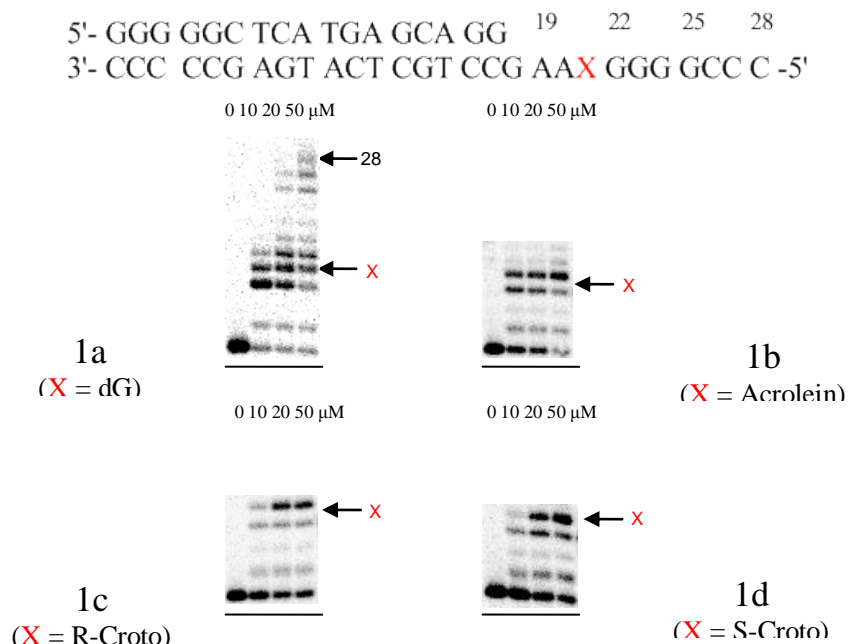
5'- GGG GGC TCA TGA GCA GG 19 22 25 28
3'- CCC CCG AGT ACT CGT CCG AAX GGG GCC C -5'

1a
(X = dG)

1b
(X = Acrolein)

1c
(X = R-Croto)

1d
(X = S-Croto)

Figure 19: Nucleotide Extension by Pol ɩ with All Four dNTPs

Polymerases Iota and Kappa

As noted earlier, it has been observed that pol ɩ and κ can work together to bypass and extend damaged DNA. We observed that pol ɩ can incorporate opposite all three of our adducts and then pol κ can complete the extension. Given this, nucleotide extension reactions were examined with equal concentrations of pol ɩ and κ present (**Figured 20**). As expected, the results suggest that the bypass and extension of the adducts are slightly more efficient, but this must be confirmed with quantification. This could be the result of the polymerases handing off replication, thus requiring more time to complete the full length replication.
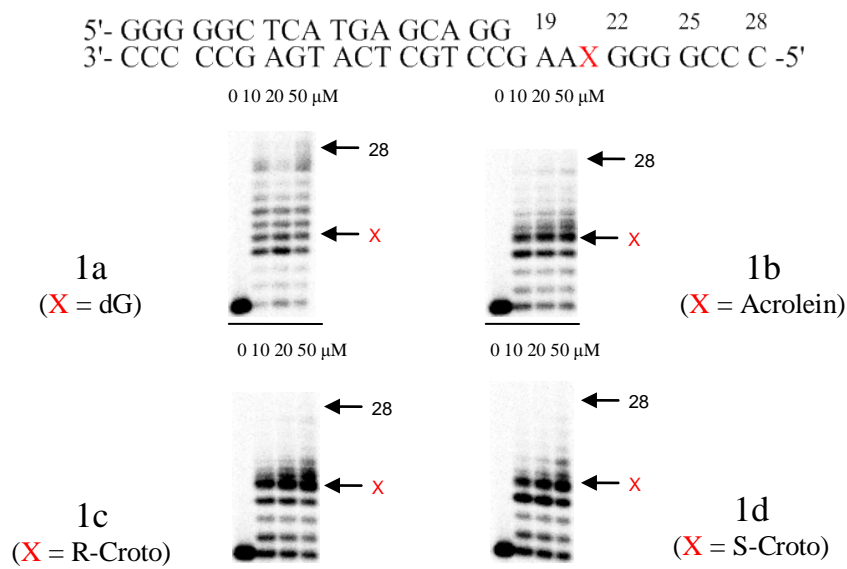
49

5'- GGG GGC TCA TGA GCA GG ^{19} ^{22} ^{25} ^{28}
3'- CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C -5'

Figure 20: Nucleotide Extension by Pol ι and κ

Section II: Polymerase By-pass Sequencing

Four different oligonucleotides were synthesized, containing the acrolein , *R*-crotonaldehyde, and *S*-crotonaldehyde adducts of dG, and an unmodified dG. The adducts were inserted at the prior to a reiterated sequence of four G's. After the oligonucleotides were purified, they were annealed to a -1 complementary primer that contained a biotin unit at the 5'end, which allows for separation of the extended oligonucleotide. A run of 10 T's was used as a spacer between the biotin moiety and the primer sequence. A schematic of the procedure is available in **Figure 21**. The products greater than 5% relative intensity were considered for sequencing and quantification.
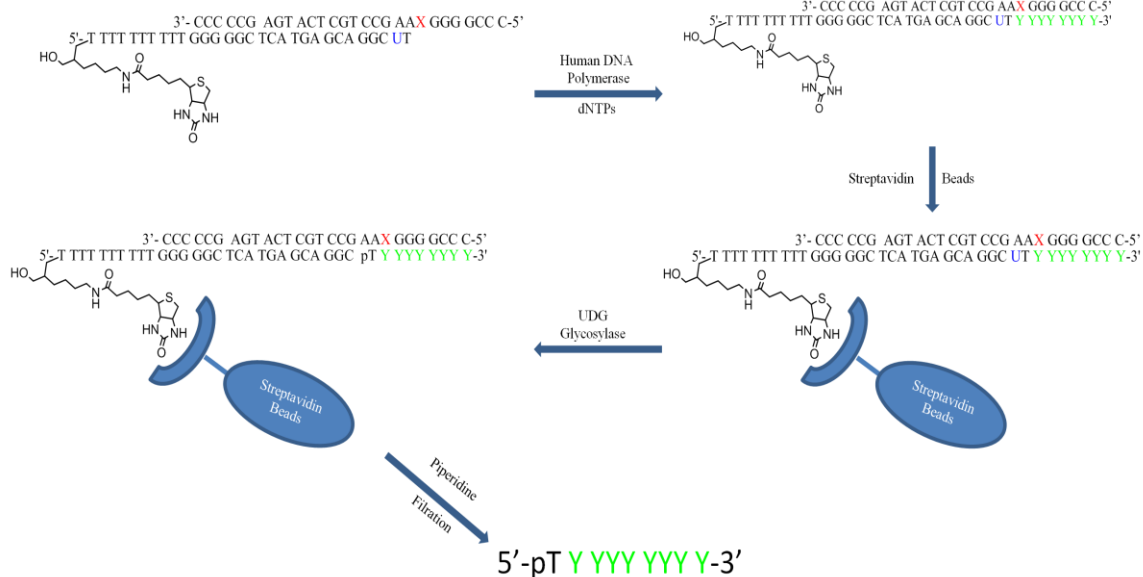
Figure 21: Extended Nucleotide Collection

Polymerase η

The nucleotide extension reaction was completed using pol η and all four dNTPs in order to study the replication of the adducted oligonucleotide (**Figure 22**). The major product from the extension of the unmodified oligonucleotide was a result of error free bypass and extension (**Figure 23**, *m/z* = 917) with the yield of 54%. The peak at *m/z*= 917.4 represents the [M-3H] ion.

3'- CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'₂₂   ₂₅   ₂₈
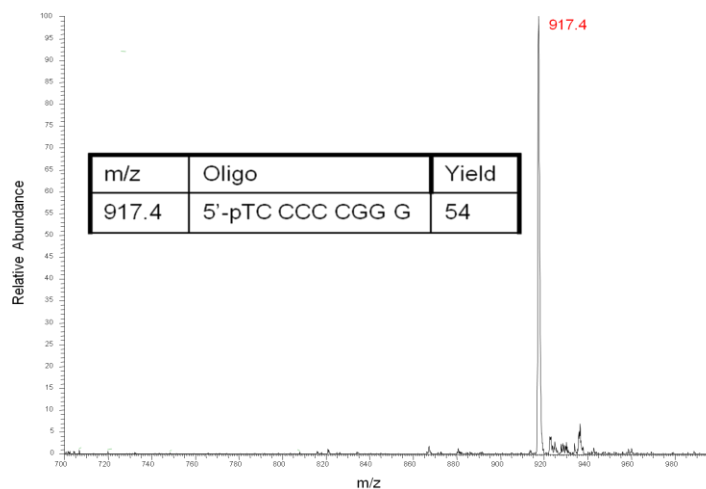
3'- CCC CCG AGT ACT CGT CCG AAX GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ $_{25}$ $_{28}$

m/z	Oligo	Yield
917.4	5'-pTC CCC CGG G	54

Figure 22: MS of the Product from Pol η Extenstion ($X = dG$)

5'-pTC CCC CGG G

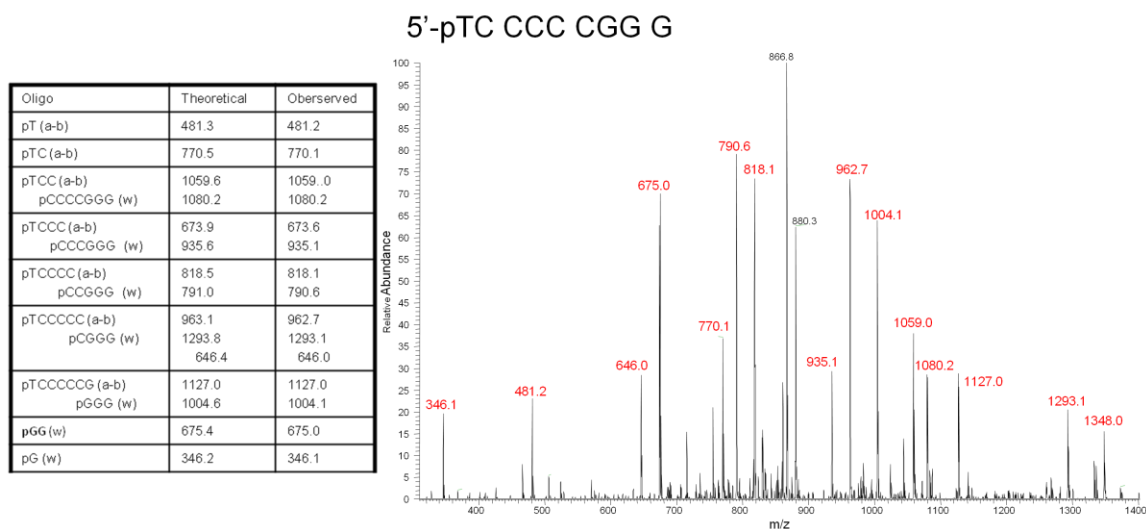| Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.2
pTC (a-b)	770.5	770.1
pTCC (a-b)	1059.6	1059..0
pCCCCGGG (w)	1080.2	1080.2
pTCCC (a-b)	673.9	673.6
pCCCGGG (w)	935.6	935.1
pTCCCC (a-b)	818.5	818.1
pCCGGG (w)	791.0	790.6
pTCCCCC (a-b)	963.1	962.7
pCGGG (w)	1293.8	1293.1
	646.4	646.0
pTCCCCCG (a-b)	1127.0	1127.0
pGGG (w)	1004.6	1004.1
pGG (w)	675.4	675.0
pG (w)	346.2	346.1

Figure 23: MS/MS of *m/z* = 917 Product

When the acrolein adduct was examined (**Figure 24**), the major product resulted

from error free extension with a 20% yield (*m/z* = 917). Two products observed arose

from misincorporation of dATP (*m/z* = 926) with 2.2% yield and the mis-incorporation of

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dGTP ($m/z = 931$), in 3.2% yield; no frameshift products were observed with the acrolein adduct and pol η (**Figure 25**).
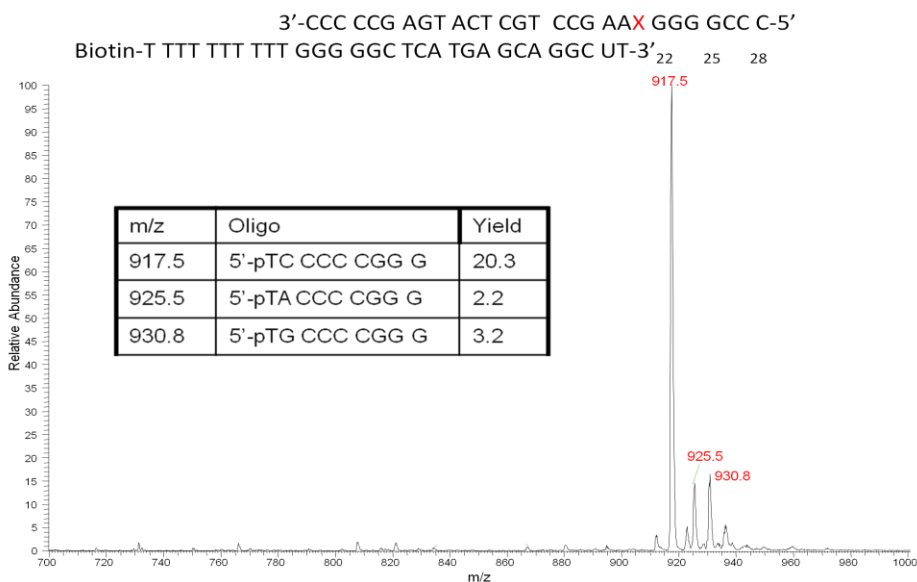
3'-CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ $_{25}$ $_{28}$

m/z	Oligo	Yield
917.5	5'-pTC CCC CGG G	20.3
925.5	5'-pTA CCC CGG G	2.2
930.8	5'-pTG CCC CGG G	3.2

Figure 24: MS of the Products from Pol η Extension (X = Acrolein)

5'-pTG CCC CGG G

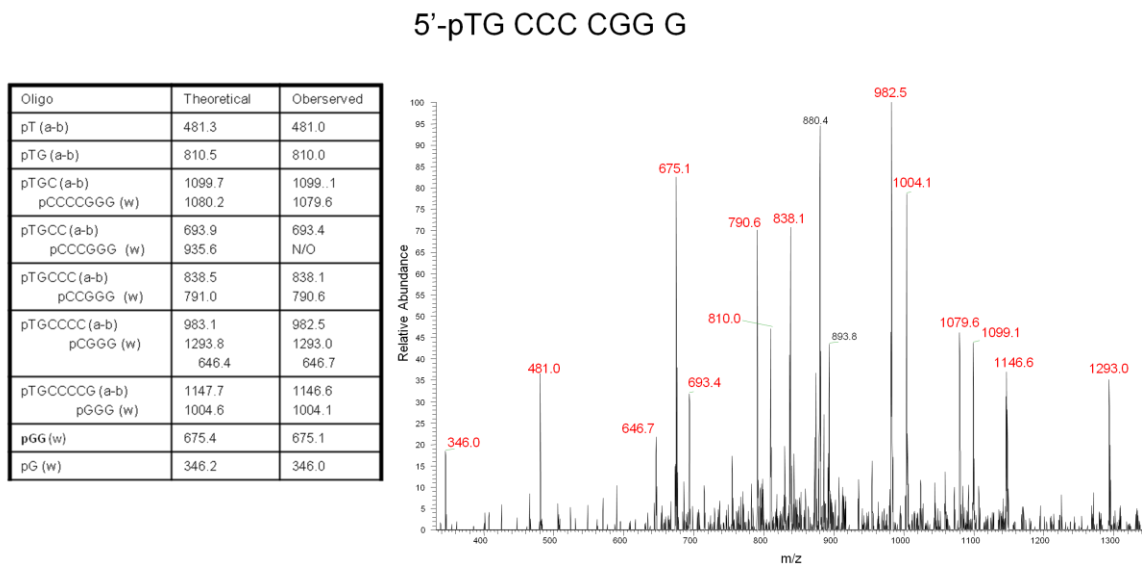| Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.0
pTG (a-b)	810.5	810.0
pTGC (a-b)	1099.7	1099..1
pCCCCGGG (w)	1080.2	1079.6
pTGCC (a-b)	693.9	693.4
pCCCGGG (w)	935.6	N/O
pTGCCC (a-b)	838.5	838.1
pCCGGG (w)	791.0	790.6
pTGCCCC (a-b)	983.1	982.5
pCGGG (w)	1293.8	1293.0
	646.4	646.7
pTGCCCCG (a-b)	1147.7	1146.6
pGGG (w)	1004.6	1004.1
pGG (w)	675.4	675.1
pG (w)	346.2	346.0

Figure 25: MS/MS of *m/z* 931 Product

The *R*-crotonaldehyde adduct produced a more interesting array of products (**Figure 26**). The error-free product was not the most abundant and was obtained, in only 1.6% yield; the most abundant product observed (7.6% yield) resulted from a deletion of a G (5'-TA CCC GGG-3', $m/z= 829$). This deletion product resulted from the initial mis-incorporation of dATP opposite the adduct followed by the deletion of a G to give a -1 frameshift product. An expected one-base deletion product was observed in our mass spectrum ($m/z = 821$) in 2% yield (**Figure 27**). Two additional non-frameshift, but error-prone products were observed in which dATP ($m/z = 926$) and dGTP ($m/z = 931$) were mis-incorporated in 0.9% and 3.0% yield, respectively.
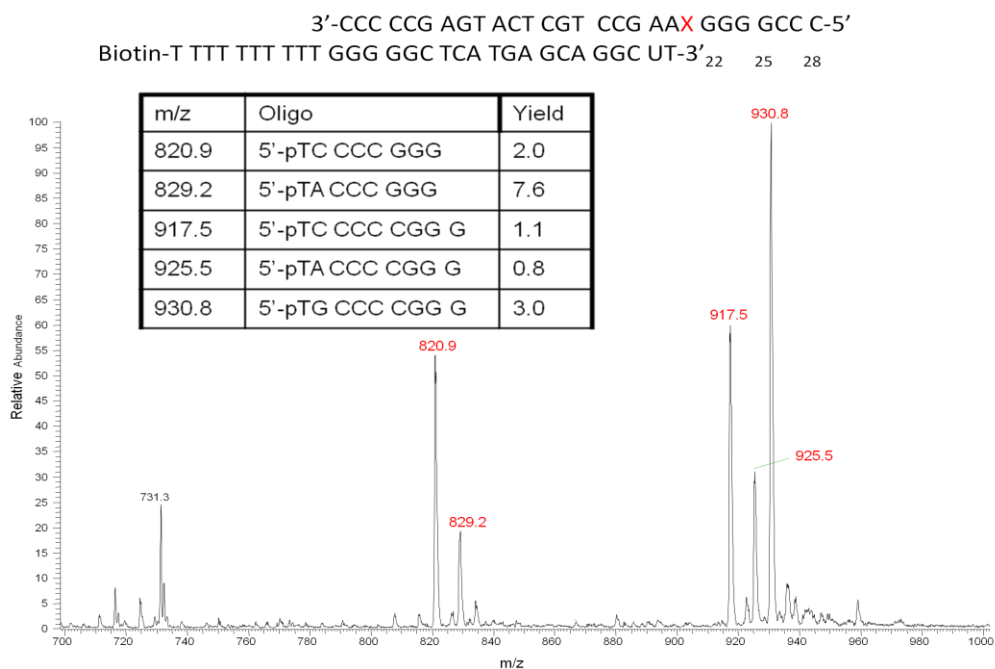

Figure 26: MS of the Products from Pol η Extension (X = *R*-Crotonaldehyde)
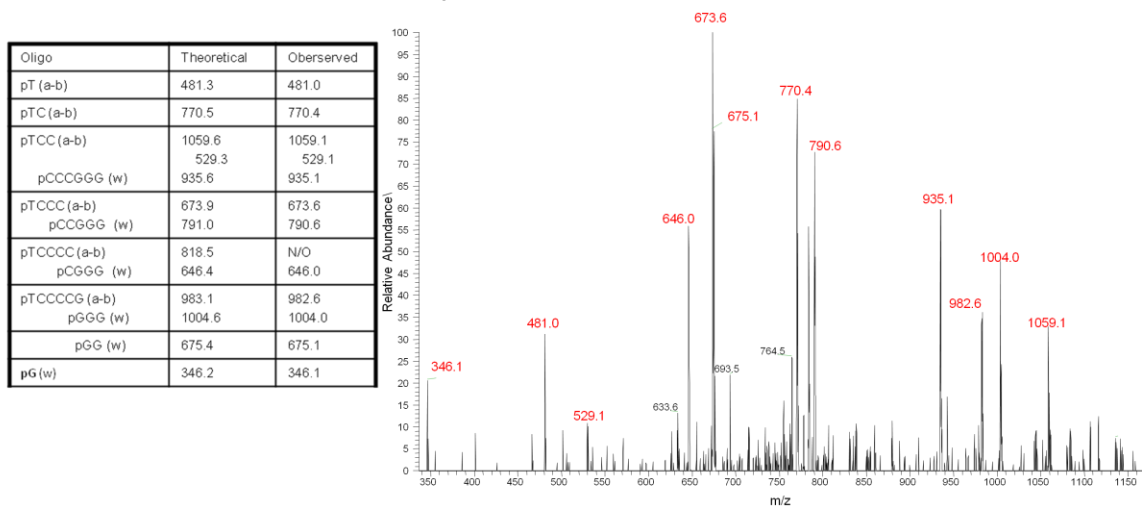
5'-pTC CCC GGG

Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.0
pTC (a-b)	770.5	770.4
pTCC (a-b)	1059.6	1059.1
	529.3	529.1
pCCCGGG (w)	935.6	935.1
pTCCC (a-b)	673.9	673.6
pCCGGG (w)	791.0	790.6
pTCCCC (a-b)	818.5	N/O
pCGGG (w)	646.4	646.0
pTCCCCG (a-b)	983.1	982.6
pGGG (w)	1004.6	1004.0
pGG (w)	675.4	675.1
pG (w)	346.2	346.1

Figure 27: MS/MS of *m/z* 821 Product

The MS spectrum for the *S*-crotonaldehyde adduct and pol η revealed similar products as the *R* isomer, but the distribution of products was greater (**Figure 28**). The three most abundant products from the extension reaction had roughly the same yield. These resulted from the correct incorporation of dCTP (*m/z* = 917) in 2.3%, the mis-incorporation of dGTP (*m/z* = 931) in 3.0%, and a deletion of a G (*m/z* = 821) in 2.5% yield. Another product that was observed was the mis-incorporation of dATP (m/z = 925) followed by error-free extension with a 1.8% yield (**Figure 29**). The *S*-crotonaldehyde adduct produced a myriad of product types that were relatively evenly distributed, which is in contrast with the *R*-crotonaldehyde adduct.

3'-CCC CCG AGT ACT CGT CCG AAX GGG GCC C-5'
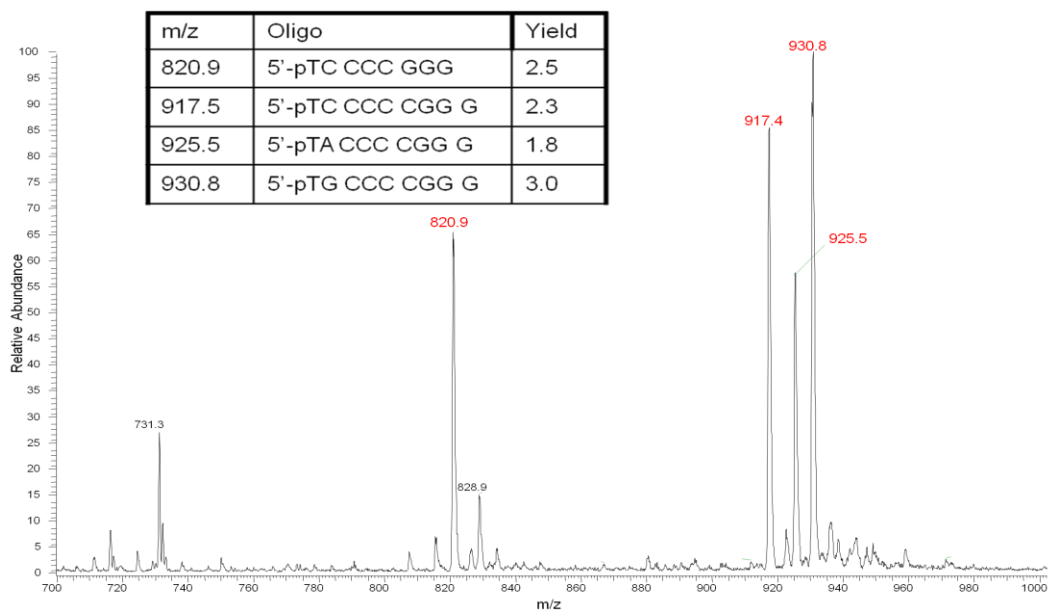Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'₂₂   ₂₅   ₂₈

m/z	Oligo	Yield
820.9	5'-pTC CCC GGG	2.5
917.5	5'-pTC CCC CGG G	2.3
925.5	5'-pTA CCC CGG G	1.8
930.8	5'-pTG CCC CGG G	3.0

Figure 28: MS of the Products from Pol η Extension (X = *S*-Crotonaldehyde)

5'-pTA CCC CGG G

Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.0
pTA(a-b)	794.5	794.1
pTAC (a-b)	1083.7	1083.0
pCCCCGGG (w)	1080.2	1078.5
pTACC (a-b)	1372.9	1371.9
	685.9	685.5
pCCCGGG (w)	935.6	935.1
pTACCC (a-b)	830.5	838.1
pCCGGG (w)	791.0	790.7
pTACCCC (a-b)	975.1	974.6
pCGGG (w)	1293.8	1292.8
	646.4	646.2
pTACCCCG (a-b)	1139.7	1138.9
pGGG (w)	1004.6	1003.9
pGG (w)	675.4	675.1
pG (w)	346.2	345.9
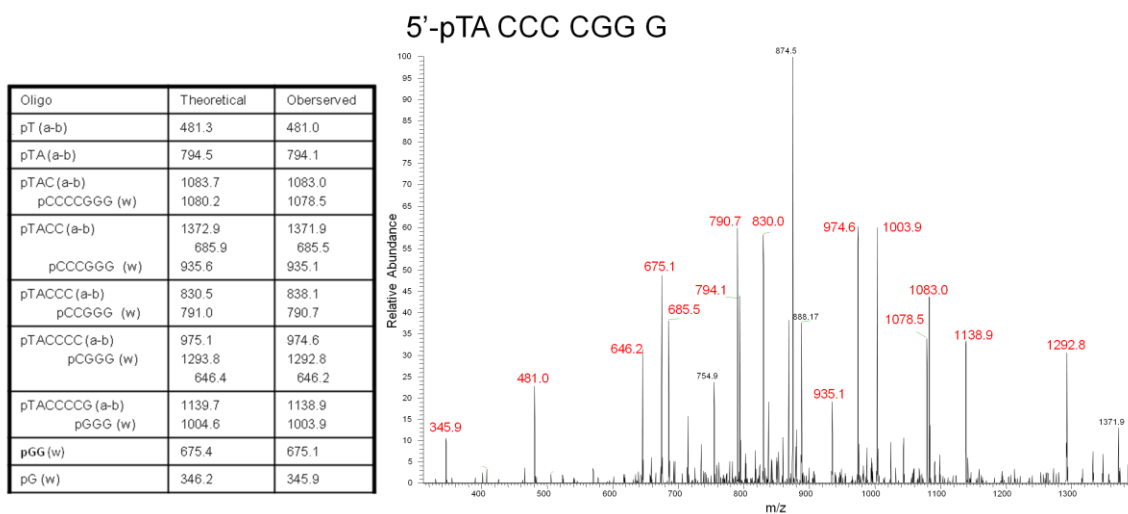
Figure 29: MS/MS of *m/z* 925

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Polymerase κ

The extension reactions with pol κ afforded a simpler distribution of products than pol η (**Figure 30**). The major product observed was the truncated oligonucleotide (*m/z* = 808) in 25% yield (**Figure 31**). This oligonucleotide is missing the last G, but this is not an uncommon extension product and is not seen as an error-prone product. The other observed product was the fully extended, error-free product (*m/z* = 917) in 2.8% yield.
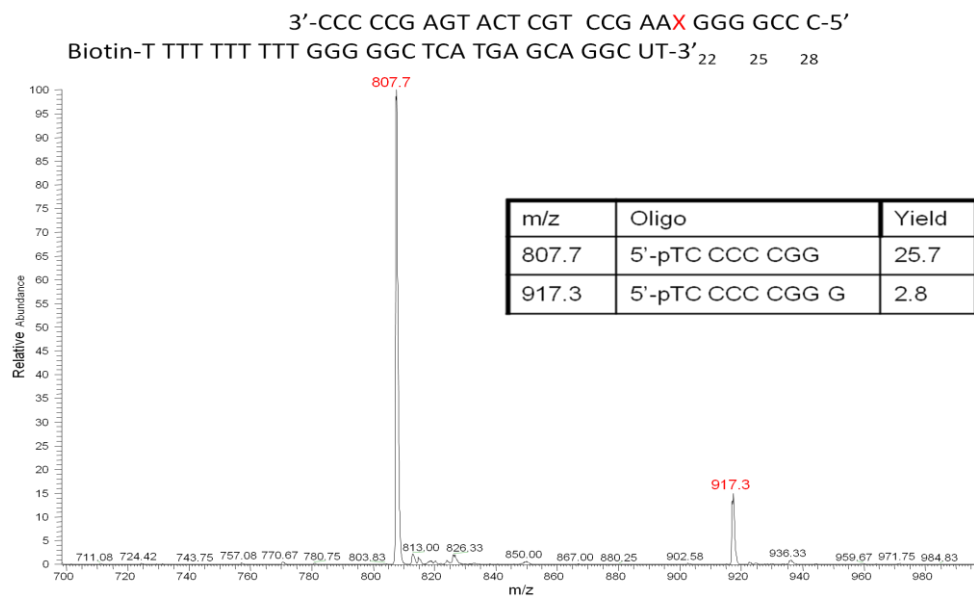

Figure 30: MS of the Products from Pol κ Extension (X = dG)
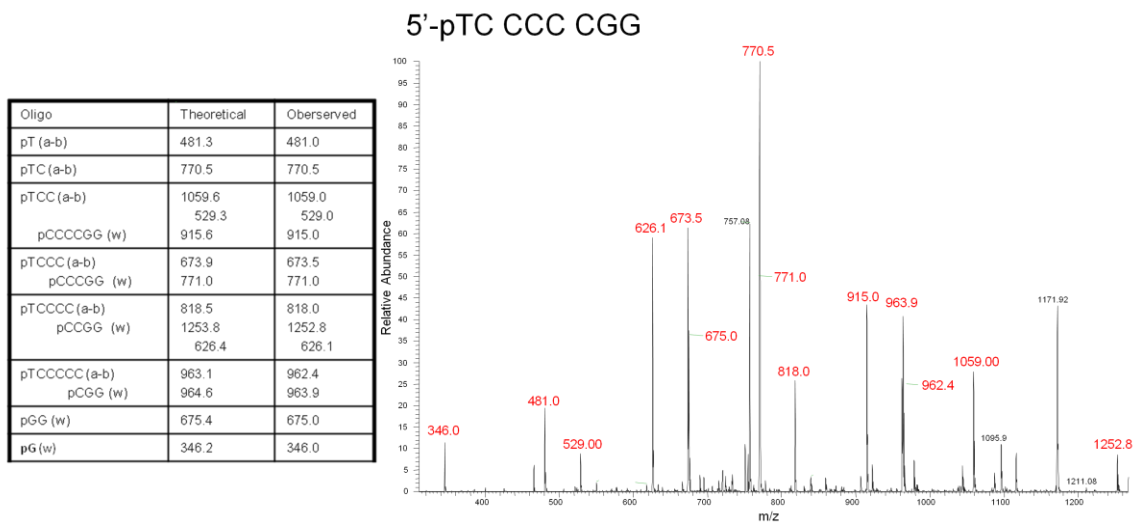
Figure 31: MS/MS of *m/z* 808

When the nucleotide extension reaction was examined with the acrolein adduct, four products were observed (**Figure 32**). Those with error-free products are the truncated oligonucleotide (*m/z* = 808) and the fully extended oligonucleotide (*m/z* = 917), which totals to roughly 24% yield. The error-prone product resulted from the mis-incorporation of dGTP followed by error-free extension. The two products differed by the presence of the 3'-terminal G (*m/z* = 821 vs. 931, **Figure 33**).

3'-CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ $_{25}$ $_{28}$

m/z	Oligo	Yield
807.5	5'-pTC CCC CGG	17.9
820.7	5'-pTG CCC CGG	3.8
917.3	5'-pTC CCC CGG G	5.9
930.8	5'-pTG CCC CGG G	1.2
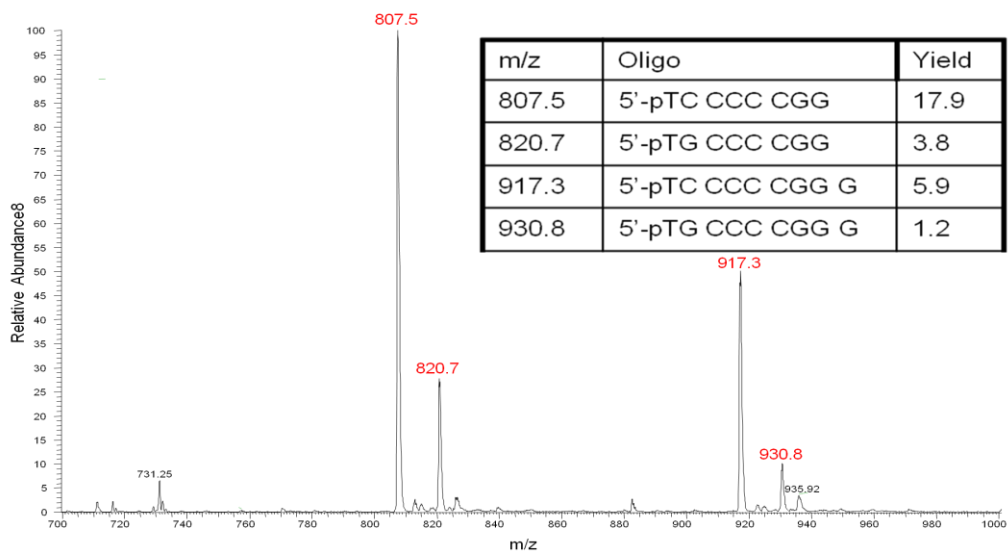
Figure 32: MS of Products from Pol κ Extension (X = Acrolein)

5'-pTG CCC CGG

Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.1
pTG (a-b)	810.1	810.0
pTGC (a-b)	549.1	549.0
pCCCCGG (w)	915.1	915.0
pTGCC (a-b)	693.6	693.6
pCCCGG (w)	771.0	770.6
pTGCCC (a-b)	838.1	838.0
pCCGG (w)	1253.2	1252.9
	626.1	626.0
pCGG (w)	964.2	964.5
pGG (w)	675.4	675.0
pG (w)	346.2	346.1
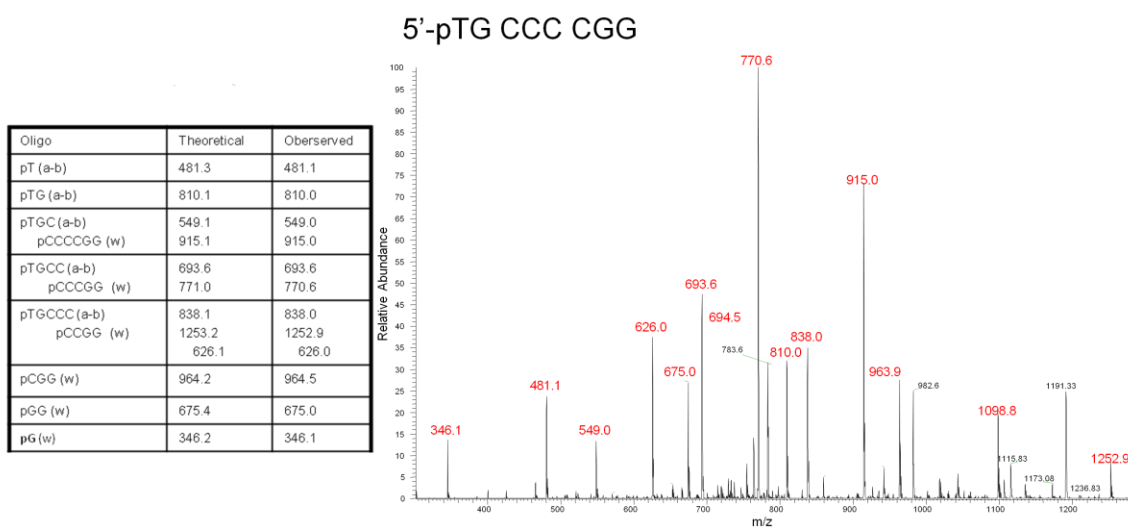
Figure 33: MS/MS of *m/z* 821

The R-crotonaldehyde adduct produced products that were difficult to elucidate the sequences (**Figure 34**). The product in greatest abundance (19% yield) was the mis-incorporation of dGTP followed by error-free extension, but missing the 3'-terminal G

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(m/z = 821). A related error-free product (m/z = 808) was observed at 2%. The product

with the second highest abundance (m/z = 931) in 2% yield was the fully extended

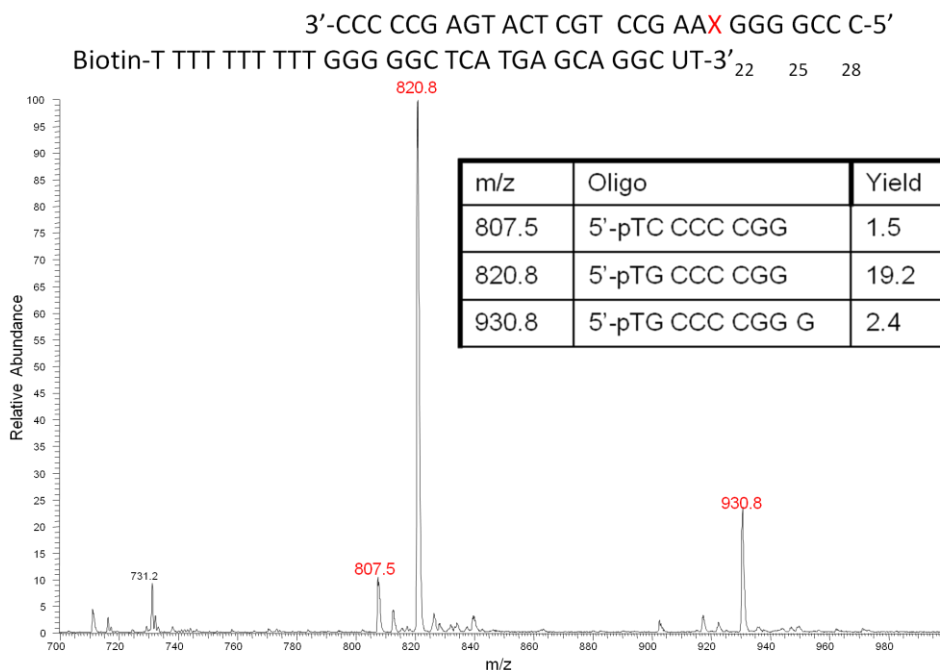sequence after a mis-incorporation of dGTP opposite the adduct.

3'-CCC CCG AGT ACT CGT CCG AAX GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ $_{25}$ $_{28}$

m/z	Oligo	Yield
807.5	5'-pTC CCC CGG	1.5
820.8	5'-pTG CCC CGG	19.2
930.8	5'-pTG CCC CGG G	2.4

Figure 34: MS of Products from Pol κ Extension (X = *R*-Crotonaldehyde)

The pol κ extension of the *S*-crotonaldehyde adduct had four products that were

identifiable (**Figure 35**). The most abundant product, which accounted for 9% yield,

resulted from error-free bypass and extension (m/z 808 and 917) and differed by the

presence of the 3'-terminal G. The second most abundant product resulted from mis-

incorporation of dGTP (m/z = 821) and accounted for 6%. The -1 frameshift product

(711) was observed at 4% yield (**Figure 36**).

3'-CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3' 22 25 28

m/z	Oligo	Yield
711.1	5'pTC CCC GG	4.4
807.7	5'-pTC CCC CGG	7.5
821.0	5'-pTG CCC CGG	5.8
917.3	5'-pTC CCC CGG G	0.94
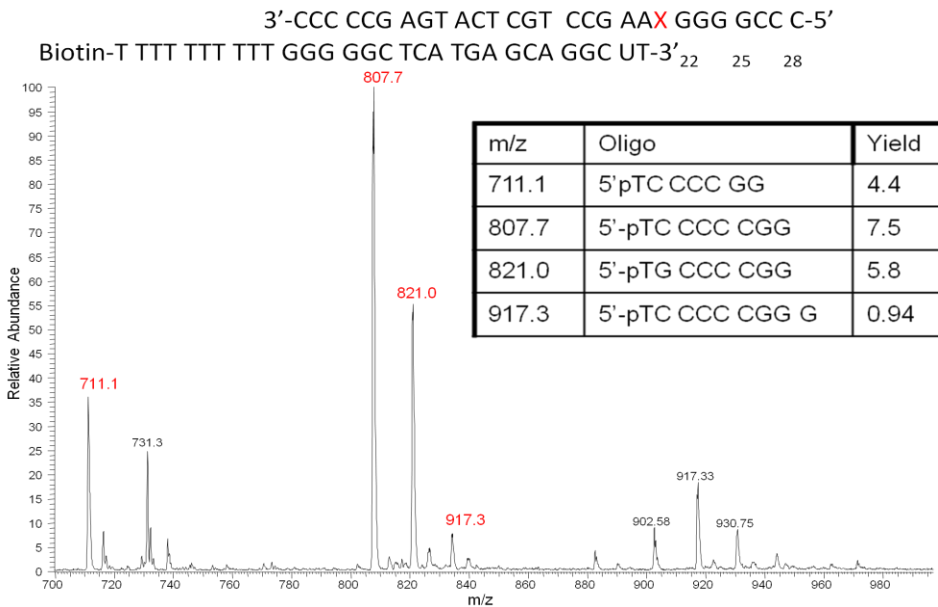
Figure 35: MS of Products from Pol κ Extension (*S*-Crotonaldehyde)

5'-pTC CCC GG

Oligo	Theoretical	Oberserved
pT (a-b)	481.3	481.5
pTC (a-b)	770.5	817.9
pTCC (a-b)	529.3	529.0
pTCCC (a-b)	673.9	673.9
pCCCGG (w)	771.0	770.4
pCCGG (w)	626.1	626.0
pCGG (w)	964.6	963.9
pGG (w)	675.4	675.0
pG (w)	346.2	346.0
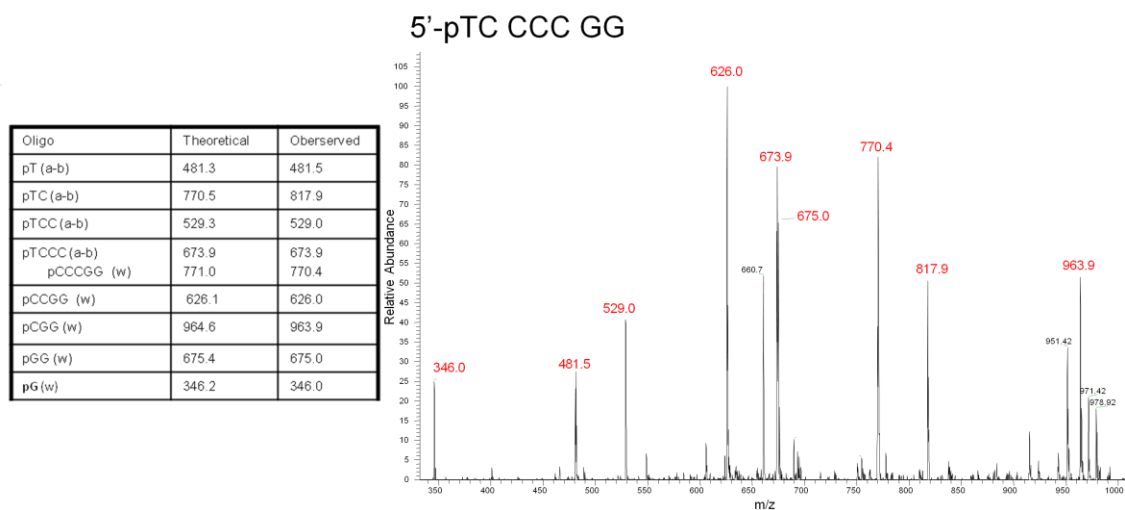
Figure 36: MS/MS of *m/z* 711

Polymerases ι and κ

As noted previously, pol ι and κ has been observed to work sequentially to bypass and extend past a DNA adduct. We observed that pol ι had considerable difficulty

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replicating past our adducts; we therefore decided to examine the bypass with pol ι and κ. The first oligonucleotide examined was the unadducted sequence (**Figure 37**). The two most abundant products resulted from error-free replication and differed by the presence of a 3'-terminal G. Together both oligonucleotides (m/z = 808 and 917) account for a 25% yield. The third product (m/z = 904) was not identified. Not surprisingly this potential collaboration between these polymerases worked well for the unadducted template oligonucleotide.

3'-CCC CCG AGT ACT CGT CCG AA**X** GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ $_{25}$ $_{28}$

m/z	Oligo	Yield
807.8	5'-pTC CCC CGG	15.0
917.4	5'-pTC CCC CGG G	9.9
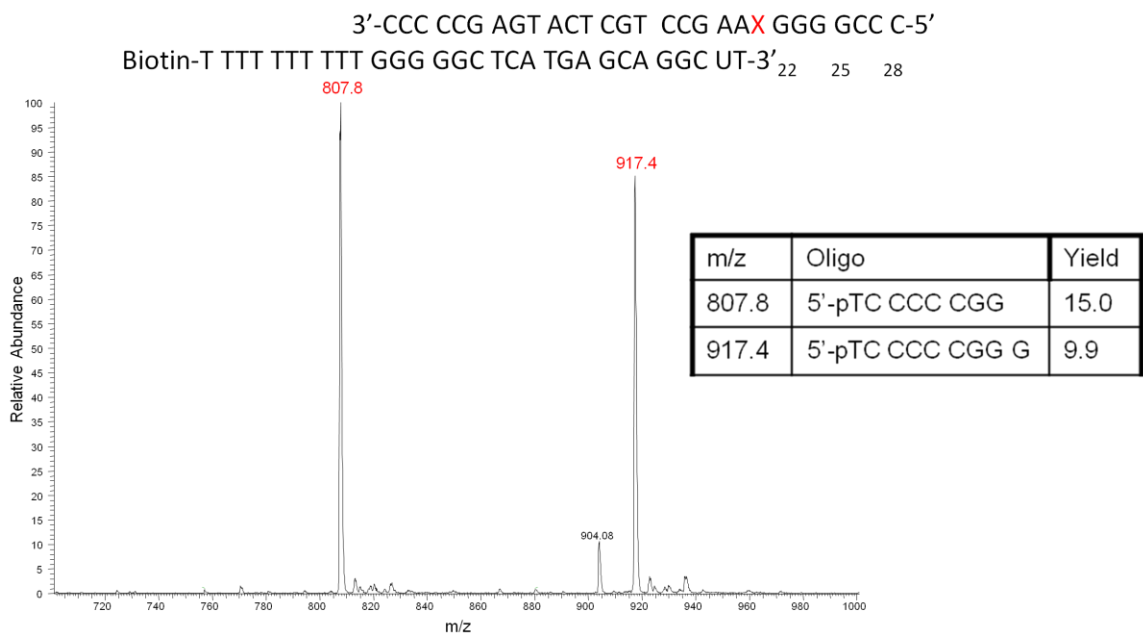
Figure 37: MS of Products from Pol ι+κ Extension (X = dG)

On the other hand, more products were observed when the acrolein adduct was replicated by the combination of pol κ and ι (**Figure 38**). As seen with the unadducted oligonucleotide, error free products at m/z = 808 and 918 accounted for 7% yield. The

most abundant product observed was the misincorporation of dCTP at the 3'-end of the sequence (m/z = 904) at 4 %. None of the products identified resulted from a frameshift.

3'-CCC CCG AGT ACT CGT CCG AAX GGG GCC C-5'
Biotin-T TTT TTT TTT GGG GGC TCA TGA GCA GGC UT-3'$_{22}$ 25   28

m/z	Oligo	Yield
807.6	5'-pTC CCC CGG	2.9
904.2	5'-pTC CCC CGG C	3.6
917.5	5'-pTC CCC CGG G	3.9
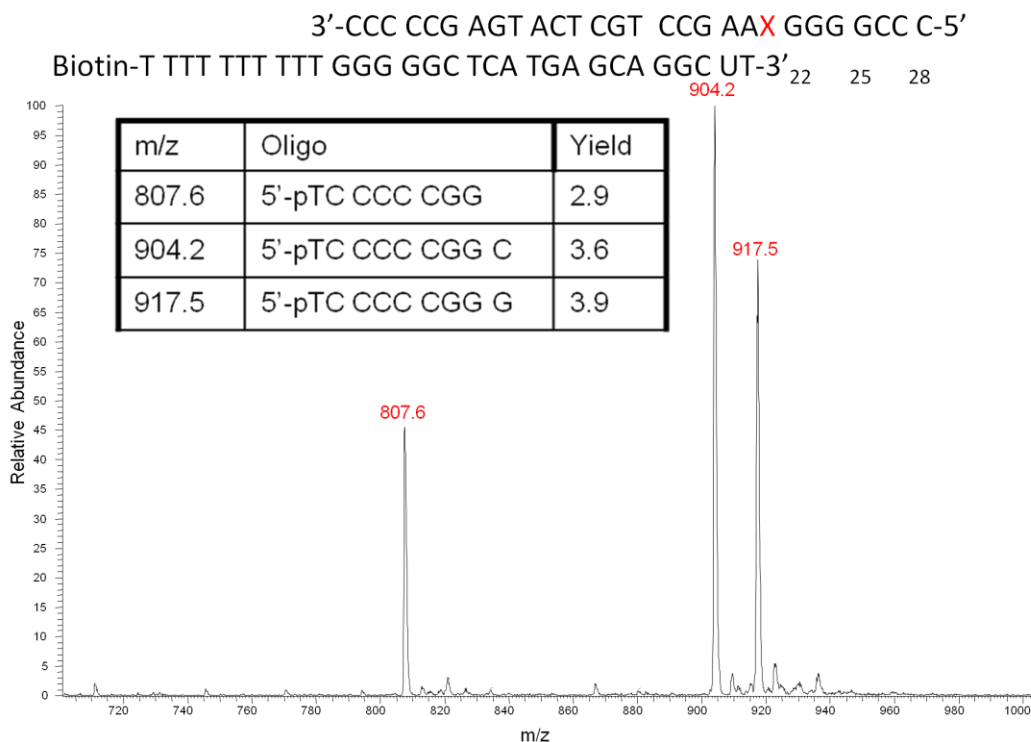
Figure 38: MS of Products from Pol ι+κ Extension (X = Acrolein)

We next looked at the *R* isomer of the crotonaldehyde adduct and saw a wider distribution of products when compared to the acrolein adduct (**Figure 39**). A frameshift product was observed (m/z = 821). The frameshift product could not be quantified because a truncated mis-incorporation of dG was also present. The UPLC column was unable to separate both products with the m/z of 821, thus inhibiting the quantification of the two products. Other products that were observed result from error-free bypass and extension (m/z 808 and 918) in roughly 2% yield. In lesser yield was a product arising

from a misincorporation dCTP at the 3'-end of the oligonucleotide (m/z = 904) in 0.5%

yield (**Figure 40**).
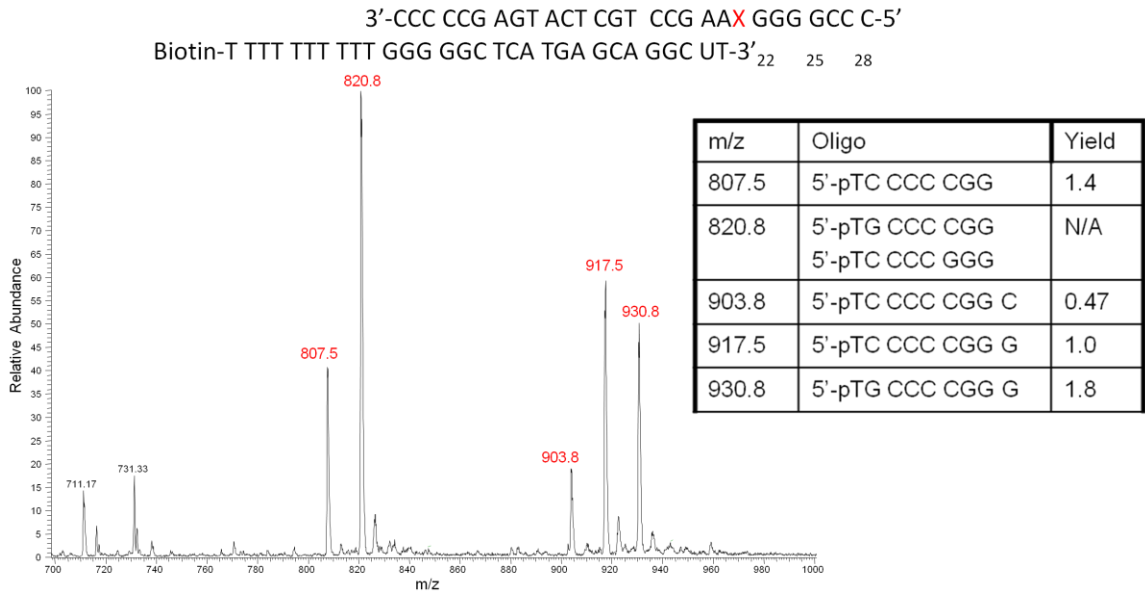
Figure 39: MS of Products from Pol ι+κ Extension (X = *R*-Crotonaldehyde)
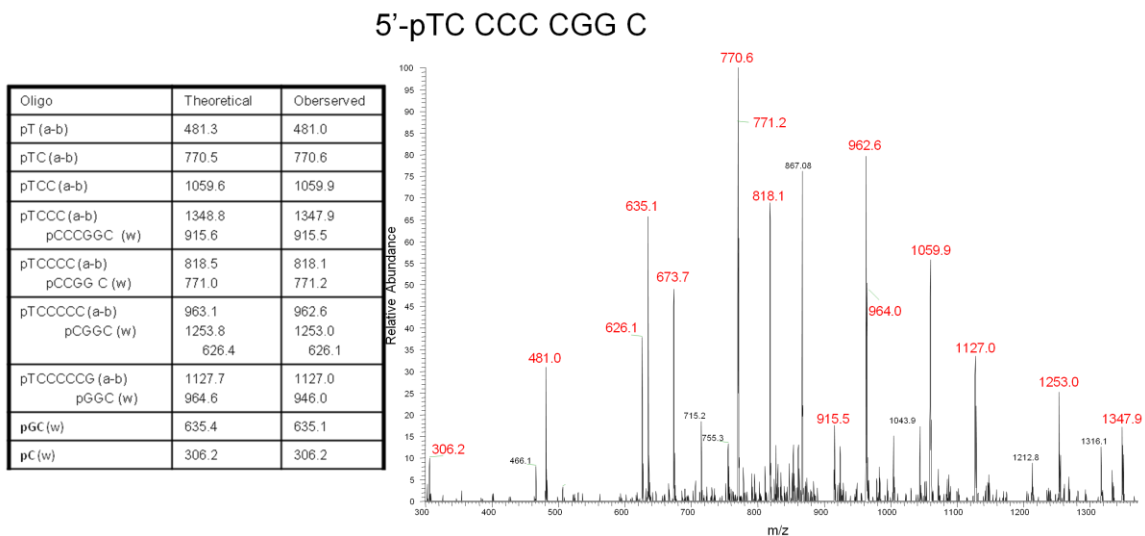
Figure 40: MS/MS of *m/z* 904

Finally, the *S* isomer of the crotonaldehyde adduct was examined (**Figure 41**). We found the error-free products at *m/z* = 808 and 904 accounted for almost 3% yield. Additionally, the -1 frameshift products (*m/z* = 711 and 821) were observed in roughly 4% combined yield and includes a product with a truncated 3'-end (*m/z* = 711), and the fully extended frameshift oligonucleotide (*m/z* = 821).
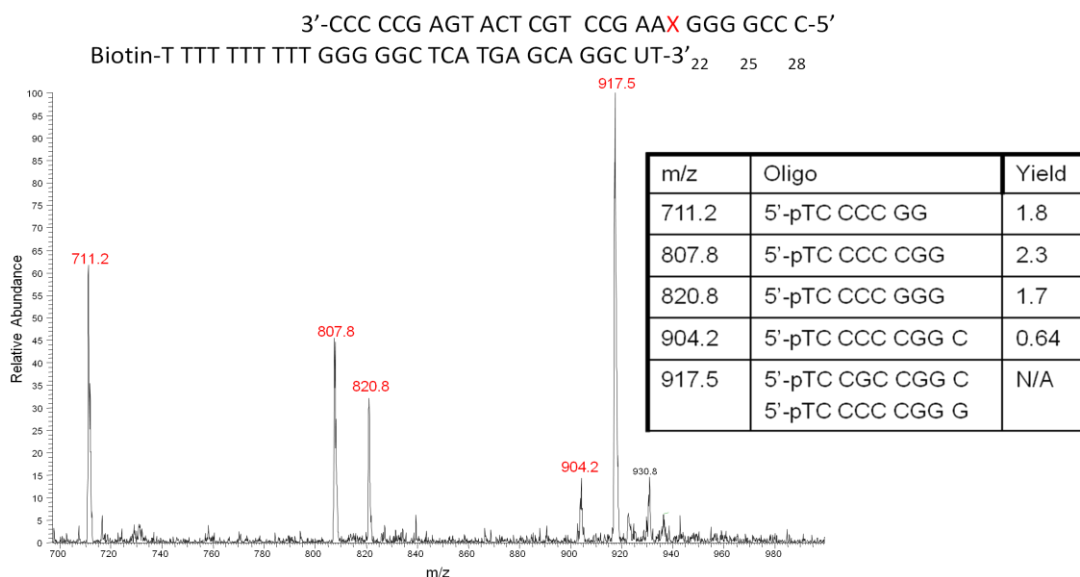
Figure 41: MS of Product from Pol ɩ+κ Extension (X = *S*-Crotonaldehyde)

Part D: Conclusion

The γ-HOPdG adduct derived from acrolein and the two isomers of the crotonaldehyde adduct, *R*- and *S*-α-CH$_3$-γ-OH-1,N^2-propano-2'-deoxyguanosine, were examined in a frameshift prone reiterated sequence of G's. Replication studies were then carried out with recessed primers using both single nucleotide incorporation and full-length extension reactions with human Y-family DNA polymerases. Full-length extension products were then identified using tandem mass spectroscopy.

Single nucleotide incorporation resulted in multiple incorporations of dCTPs, which is not surprising given that the template was a reiterated G sequence. With pol η, both the acrolein and *R*-crotonaldehyde adducts were able to incorporate the same dNTPs as the unmodified sequence. The *S*-crotonaldehyde adduct was a stronger block in the single nucleotide incorporation and extension assays. Pol κ yielded different incorporation results, with mis-incorporation of dGTP observed with the *R*-crotonaldehyde adduct, but extension past the mis-incorporation was interested. The S-crotonaldehyde adducts was a strong block to full-length extension. With pol ι, we observed that extension was inefficient. This is in agreement with other studies that have shown pol ι to be an excellent polymerase for incorporation opposite an adduct.[6]

Full-length extension of the templates was completed with all four dNTPs present. Pol η was able to replicate past all adducts, but with a wide range of efficiencies. This is evident by the reduction in extended products seen from the most efficient, dG, to the least efficient, the *S*-crotonaldehyde adduct. When pol κ was examined we found similar results to that of pol η, in that the extension products were observed but that the yield was reduced further by each subsequent adduct, with the *S*-crotonaldehyde adduct having the greatest ability to block replication. Even though pol ι was observed to inhibit extension of the template from the single nucleotide experiment, the nucleotide extension reactions were examined. As indicated in the single nucleotide experiment, pol ι is able to insert opposite the adducts, but extension past the adducts is inhibited. As noted early, it has been shown that pol ι and κ can work in tandem to replicate through adducted bases; nucleotide extension was therefore examined with both pol ι and pol κ. Minimal

extension was seen with this combination of polymerases; however, extension products were observed.

Extension assays are extremely useful in determining an adduct's effect on a polymerase for replication, but the results give us little information on the actual products formed. Because of this, identification of the extension products is desirable. This was accomplished using a modified primer with biotin, uracil, and 10 T's. Once the extended products were isolated, MS and MS/MS were used to identify and quantify the extension products. Our quantification of the products confirmed our extension assays where the unmodified oligonucleotides gave higher yields of product formation the adducted templates. The total yield for bypass extension was highest for the acrolein adduct, and lowest for the S-crotonaldehyde adduct. When the sequences of the extension products were determined, the acrolein adduct was found not to induce a deletion with any of the polymerases examined. Replication of the *R*-crotonaldehyde adduct by pol η resulted in a majority of frameshift products with 66% of the products having been either a mis-incorporation of dATP or correct incorporation of dCTP followed by a -1 frameshift. No frameshift products were observed with pol κ and the frameshift product could not be determined for the combination of pol ι and κ. For the *S*-crotonaldehyde adduct, the combination of pol ι and κ produced the greatest percentage of frameshift products with 54% of the products being the truncated and full frameshift product. Pol η and pol κ had roughly the same percentage of frameshift products, pol η producing 26% of the full frameshift product and pol κ producing 24% of the truncated frameshift product. Again, it must be stressed that the actual yield of these products were low, having no greater than

7.6% chemical yield, and were in line with our nucleotide extension assays but that the percentage of the products was significant.

In these sets of experiments we were able to show the difference in the polymerases' ability to incorporate and extend past three different adducts. Frameshift products were seen, but only with the two isomers of the crotonaldehyde adduct and not the acrolein adduct. Since the two isomers had differences in their ability to induce and type of frameshift mutations, NMR studies of the adduct in our sequence will hopeful indicate the role the stereochemistry of the isomers have on nucleotide pairing. The sequences of the extension products were determined by a method developed in our lab. This procedure has opened a new avenue to studying and indentifying frameshift inducers in oligonucleotides.

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Appendix A:

Polymerase Assay for Chapter II

3'-CCC CCG AGC ATT CCT AAC CXC GGC TCA-5'
5'-GGG GGC TCG TAA GGA T₁₇ ₁₉ ₂₂ ₂₅

Kf   pol II⁻   Dpo4 @ 37°C
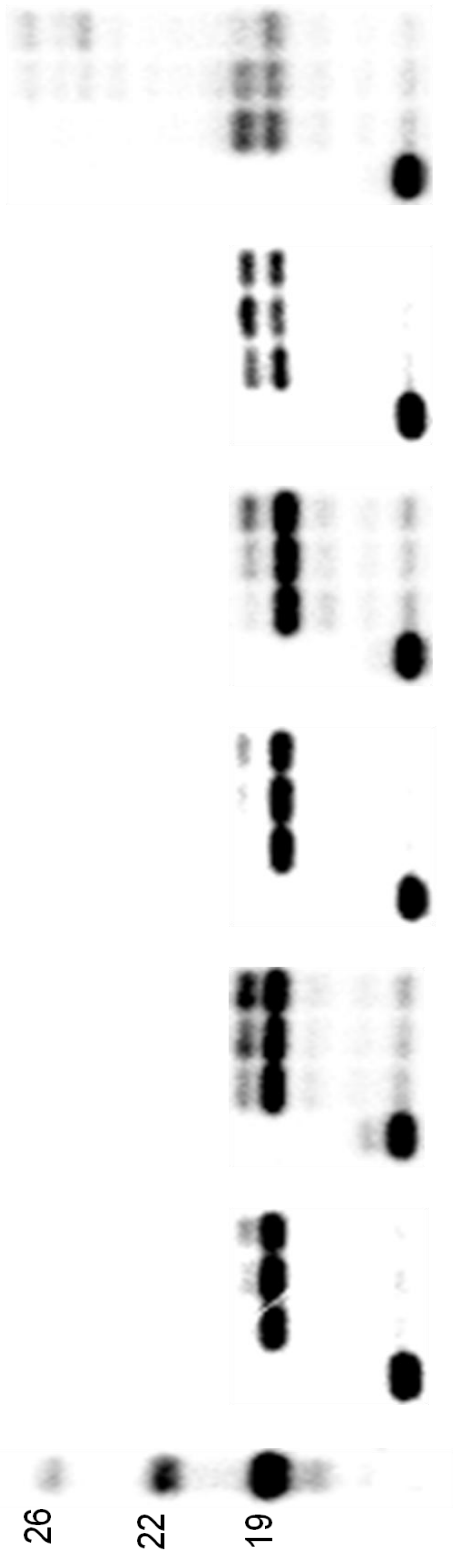
Nucleotide Extension of the G₃ Position with Increased dNTP and Time

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Appendix B:

MS/ MS for Chapter III

5'-pTA CCC GGG
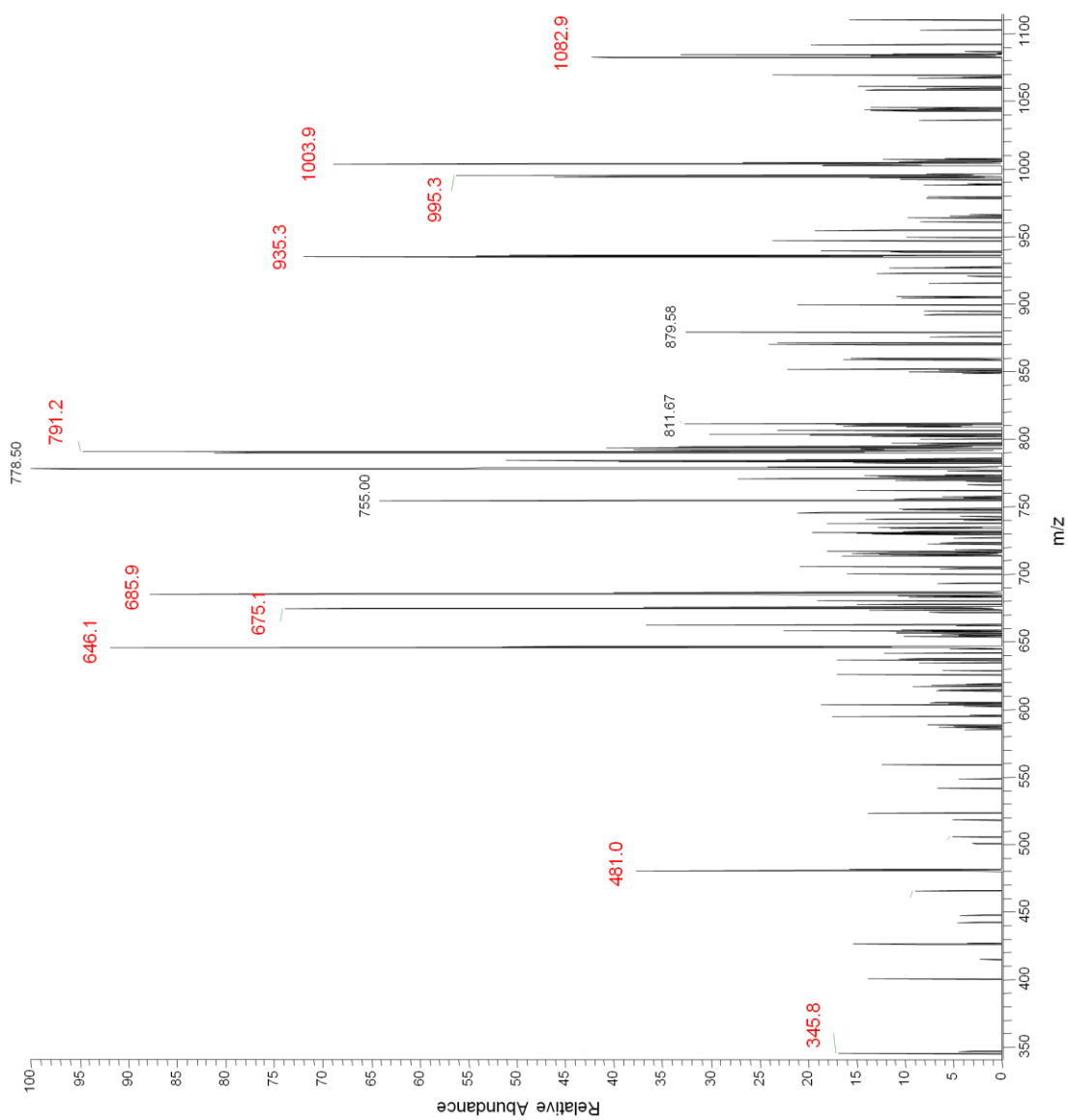
Oligo	Theoretical	Observed
pT (a-b)	481.3	481.0
pTA (a-b)	794.5	N/O
pTAC (a-b) pCCCGGG (w)	1083.7 935.6	1082.9 935.3
pTACC (a-b) pCCGGG (w)	685.9 791.0	685.9 791.2
pTACCC (a-b) pCGGG (w)	830.5 646.4	N/O 646.1
pTACCCC (a-b) pGGG (w)	995.1 1004.6	995.3 1003.9
pGG (w)	675.4	675.1
pG (w)	346.2	345.8

MS/MS of m/z 829 Product

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5'-pTG CCC GG

Oligo	Theoretical	Observed
pT (a-b)	481.3	481.0
pTG (a-b) pCCCGG (w)	810.5 915.6	810.0 915.1
pTGC (a-b) pCCCGG (w)	1099.7 771.0	1099.0 771.2
pTGCC (a-b) pCCGG (w)	693.9 1253.8 626.4	693.6 1253.1 626.1
pTGCCC (a-b) pCGG (w)	838.5 964.6	838.1 N/O
pTGCCCG (a-b) pGGG (w)	983.1 675.4	982.6 675.1
pG (w)	346.2	346.1

5'-pTC CCG GG

Oligo	Theoretical	Observed
pT (a-b)	481.3	481.0
pTG (a-b) pCCCGG (w)	770.5 935.6	770.6 935.1
pTGC (a-b) pCCCGG (w)	1059.6 791.0	N/O N/O
pTGCC (a-b) pCCGG (w)	673.9 646.4	673.5 1253.1
pTGCCC (a-b) pCGG (w)	818.5 1004.6	N/O 1004.2
pTGCCCG (a-b) pGGG (w)	983.1 675.4	982.6 675.1
pG (w)	346.2	346.1

MS/MS of m/z 821 Products

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Appendix C:

Concentration Curves for Chapter III

Concentration Curve for Extension Product m/z 711

The chart is titled "Concentration Curve (5'-pTC CCC GG)" with x-axis "Amount Ratio [Olgio/Standard, OD's]" and y-axis "TIC Ratio (Oliigo/Standard)". The trendline equation is $y = 0.5089x$ and $R^2 = 0.997$.
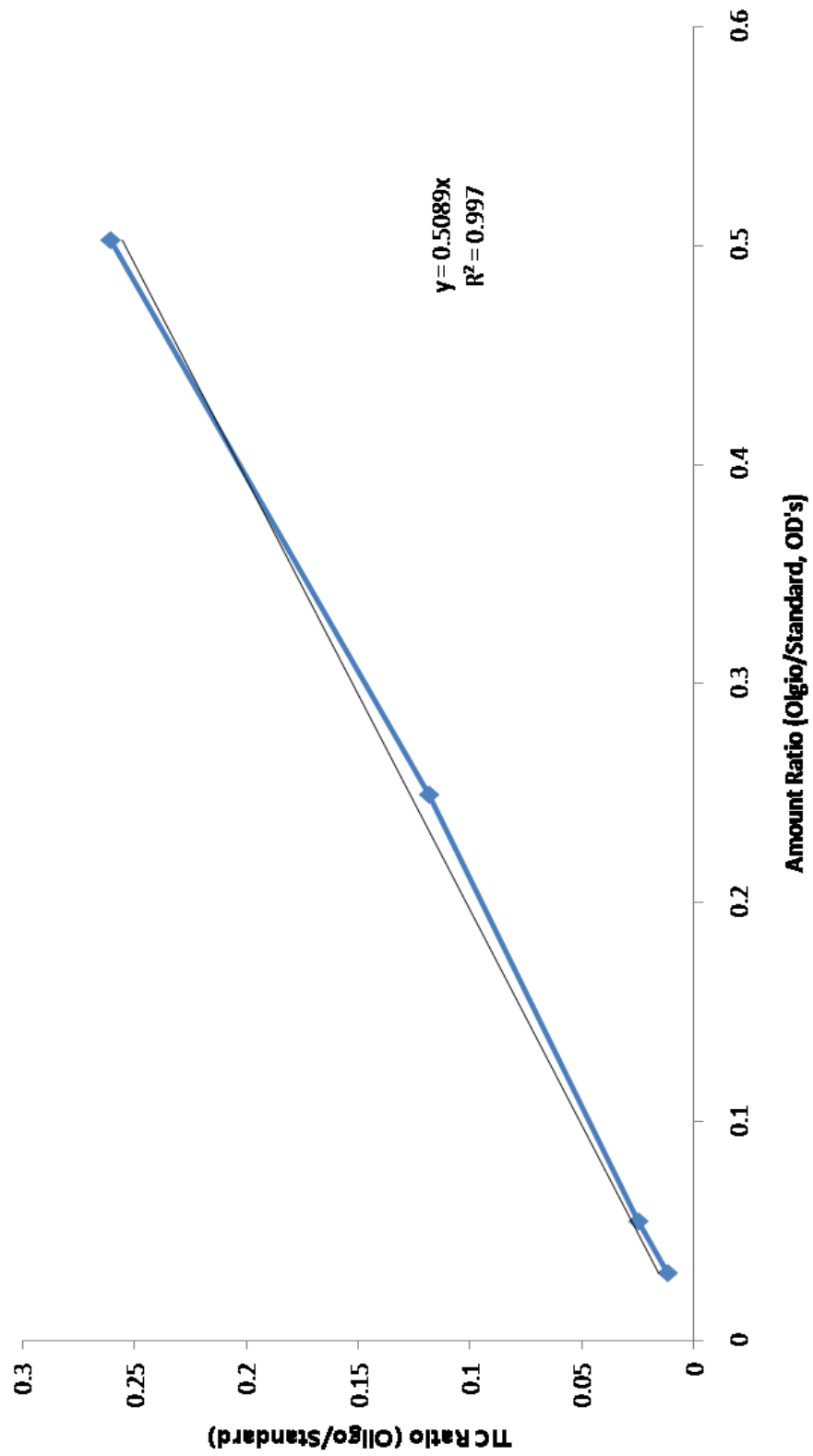
Calibration Curve (5'p-TC CCC CGG)

$y = 1.3186x$
$R^2 = 0.9932$

TIC Ratio (Olgio/Standard)

Amount Ration (Oligo/Stndard, OD's)
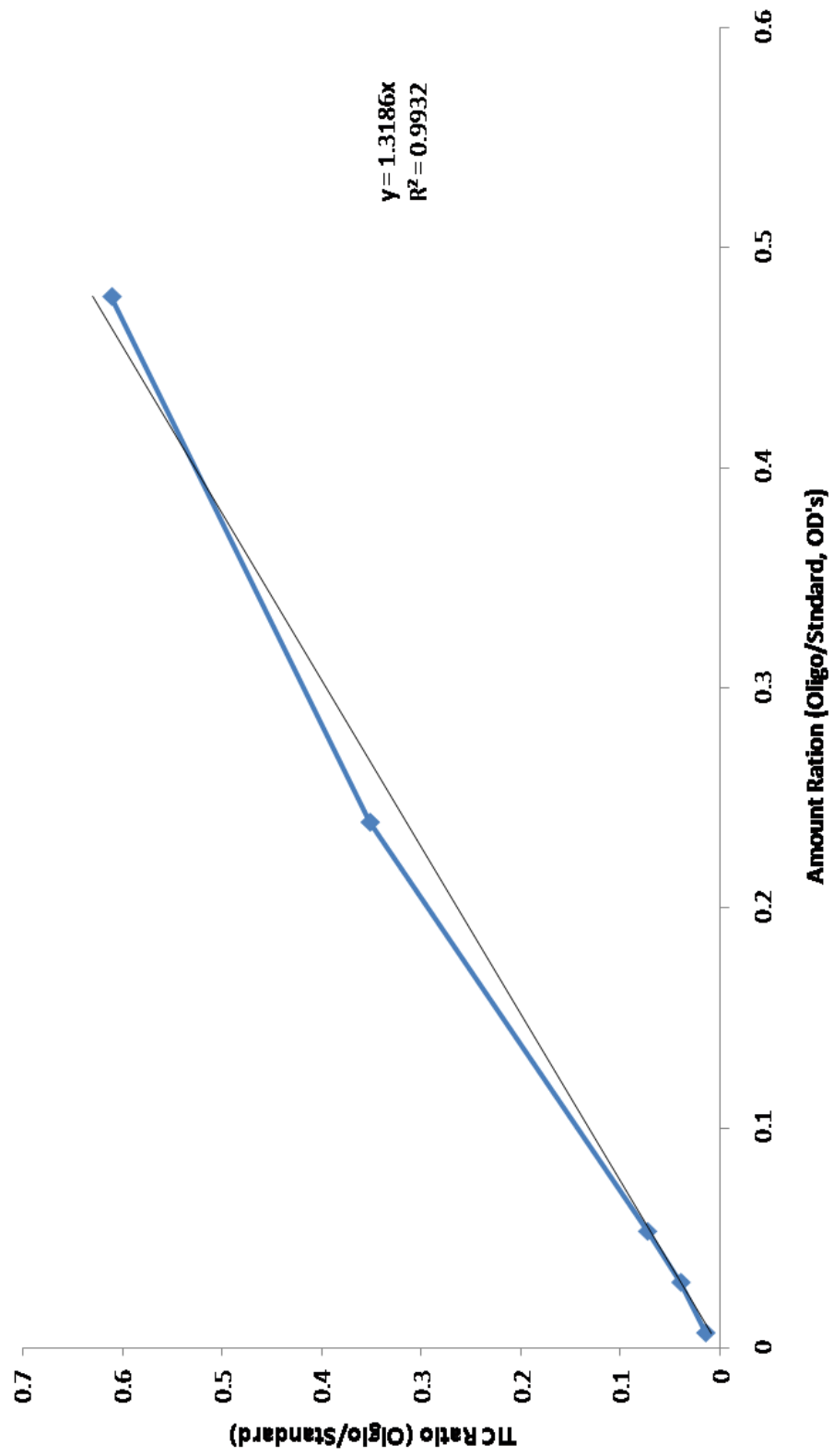
Concentration Curve for Extension Product m/z 808

Concentration Curve for Extension Product m/z 821

Concentration Curve for Extension Product m/z 821
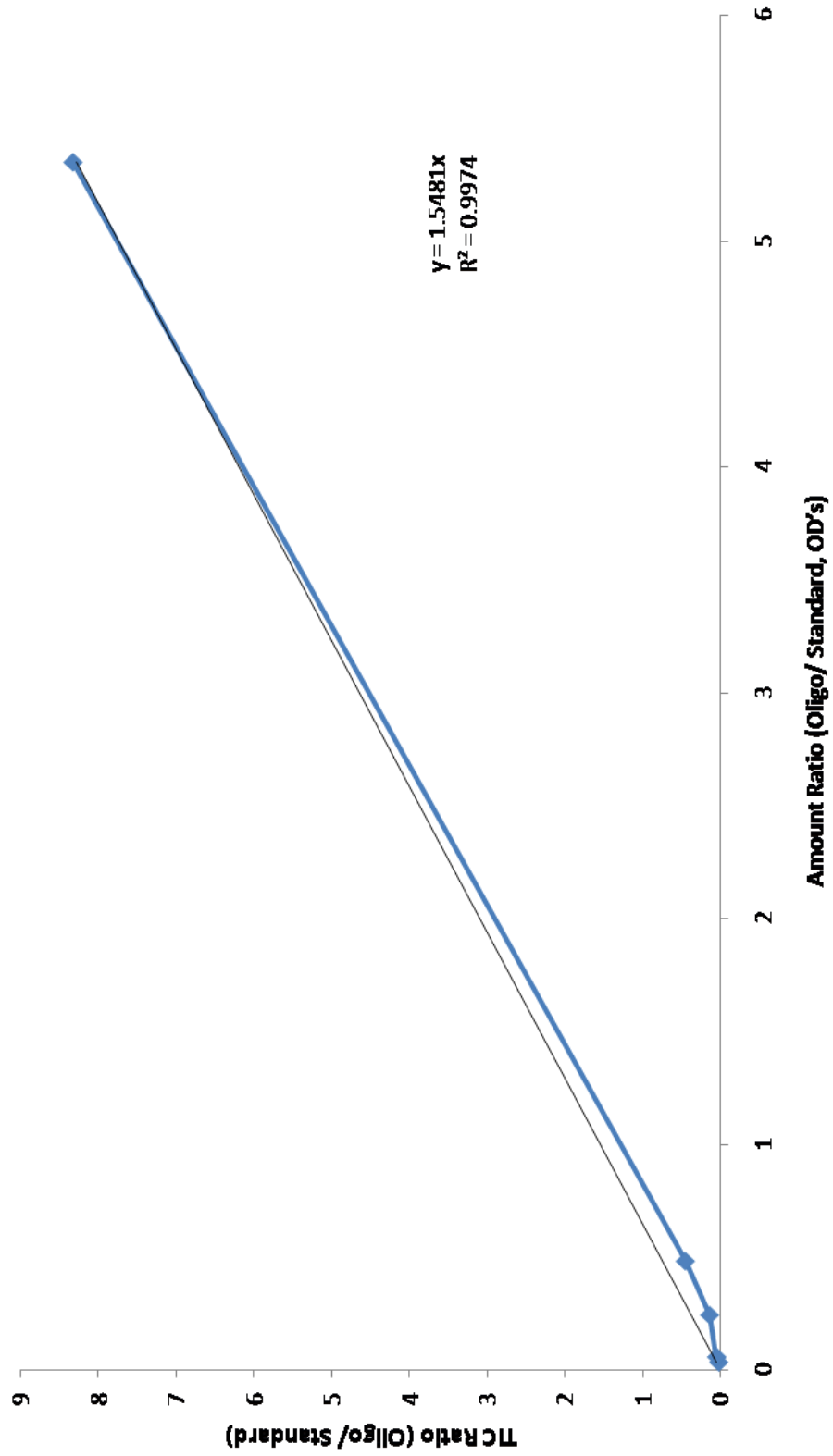
Concentration Curve for Extension Product m/z 829
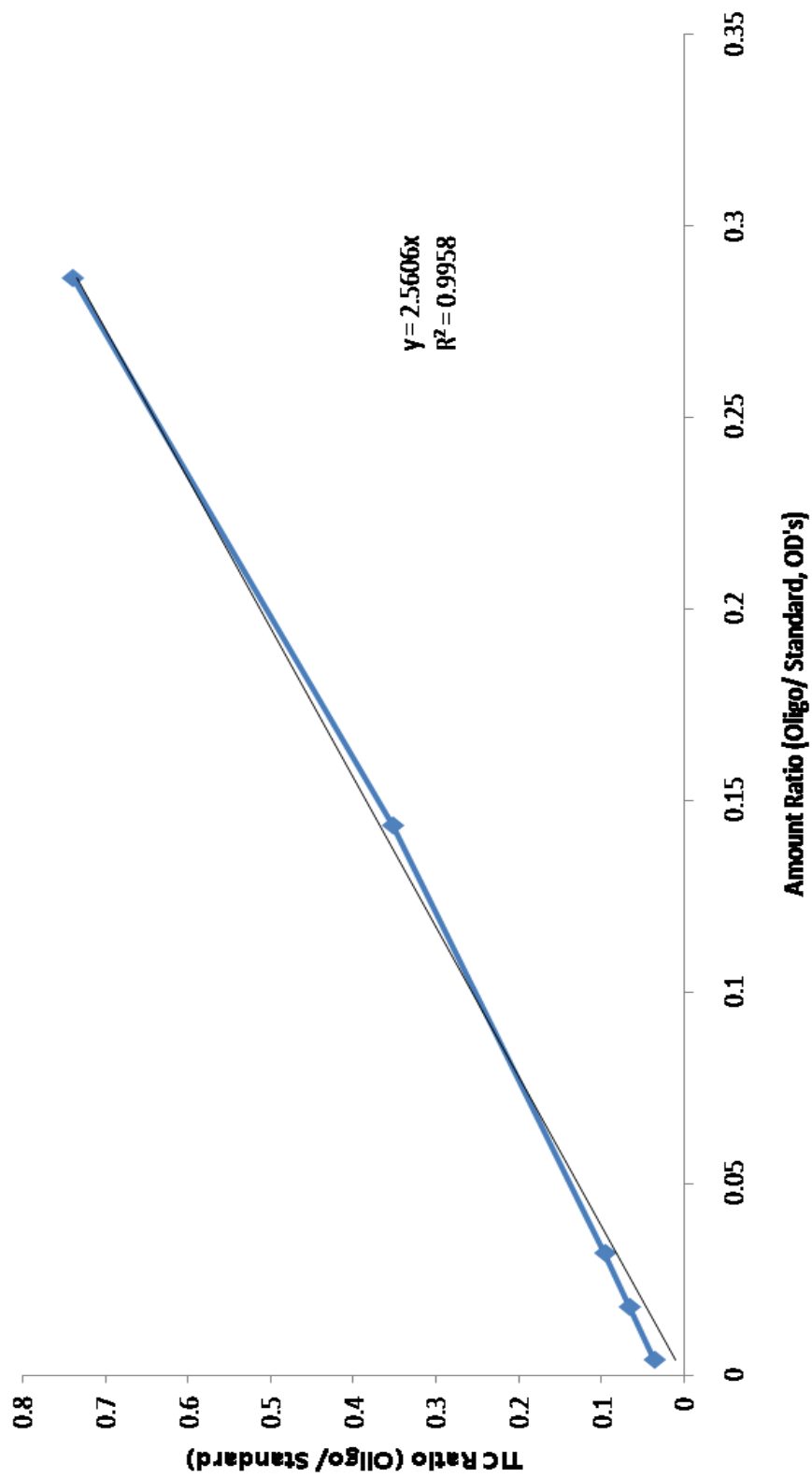
Concentration Curve for Extension Product m/z 904
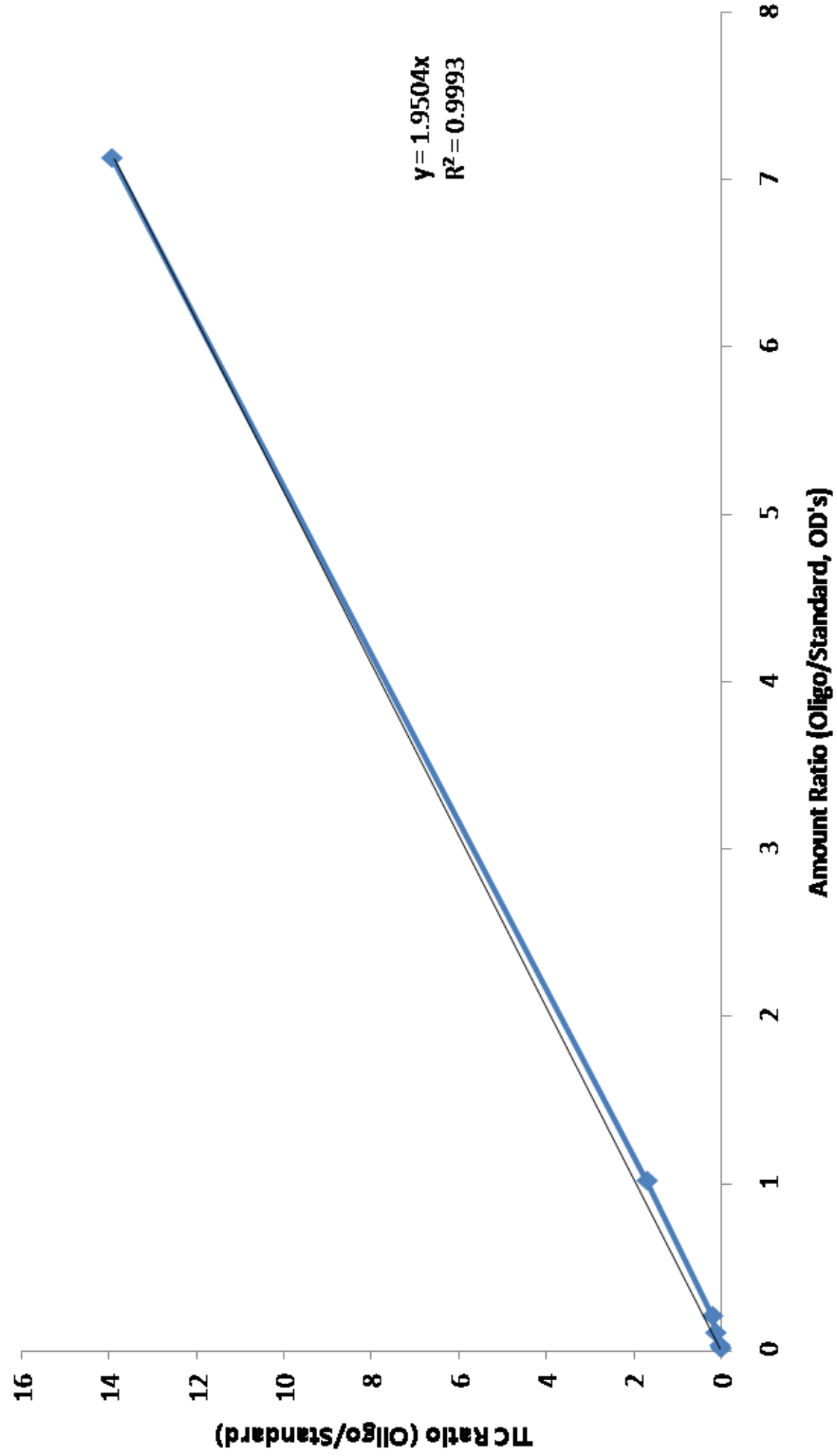
Concentration Curve for Extension Product m/z 917
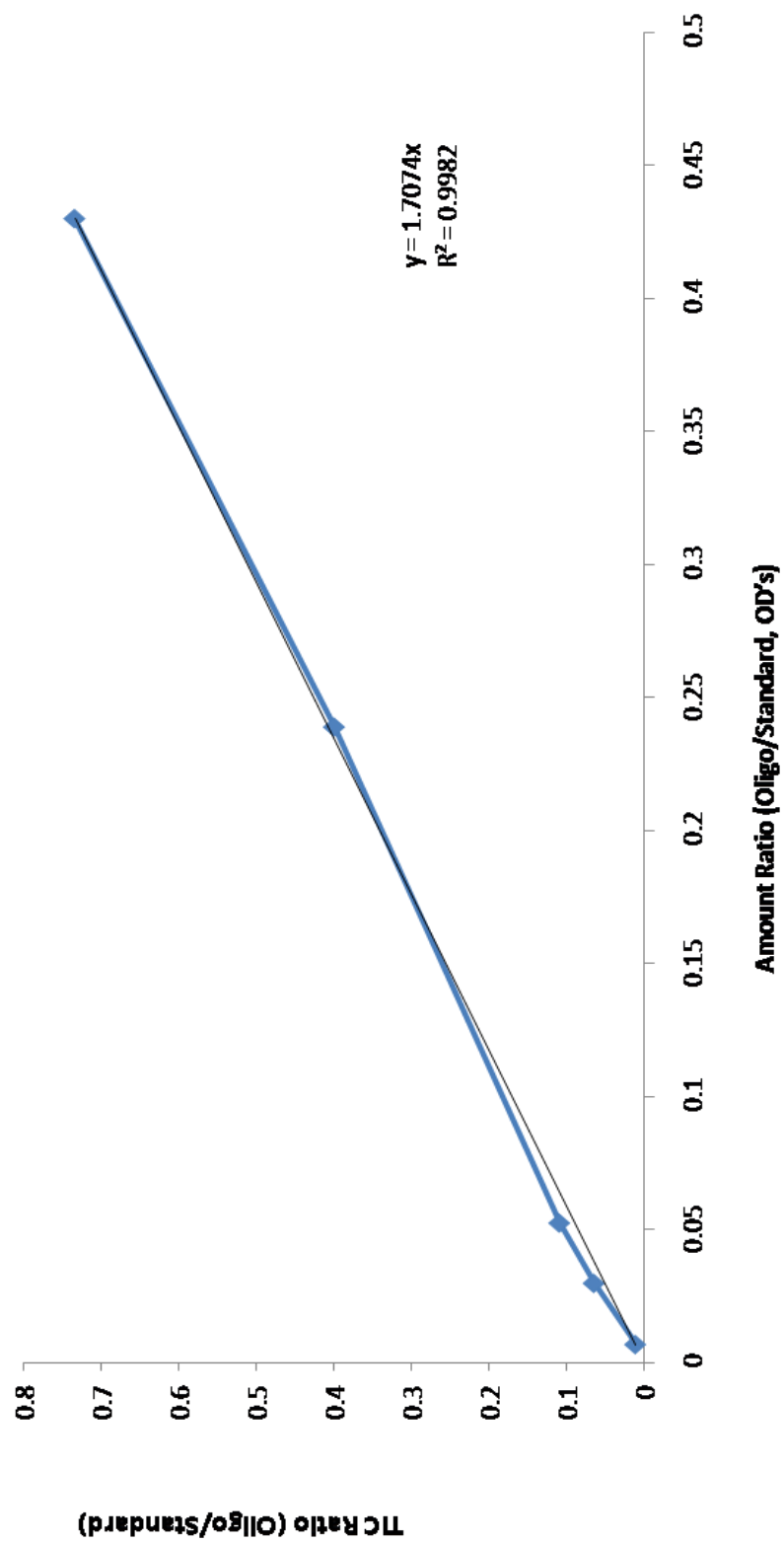
Concentration Curve for Extension Product m/z 925

Concentration Curve for Extension Product m/z 931