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Improving PCR efficiency with inorganic pyrophosphatase

Anuj Singhal

IMPROVING PCR EFFICIENCY WITH INORGANIC PYROPHOSPHATASE

by

Anuj Singhal

A Thesis

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the degree of Master of Science
in
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of
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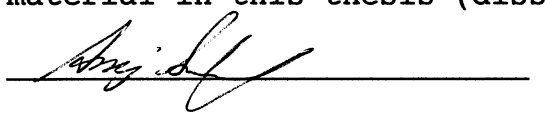

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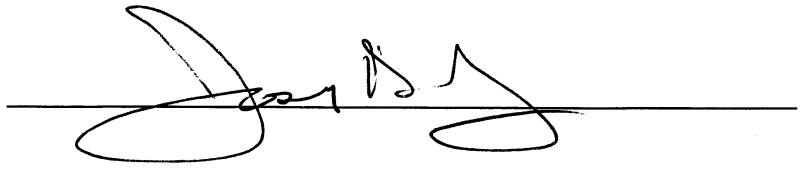
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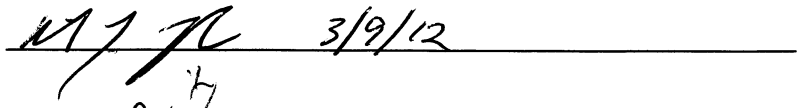
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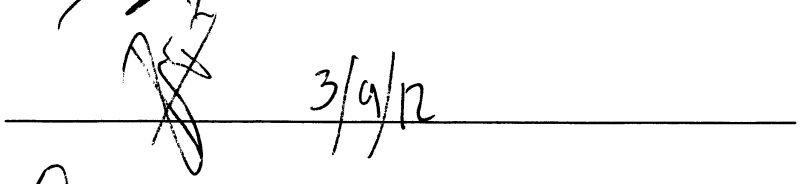
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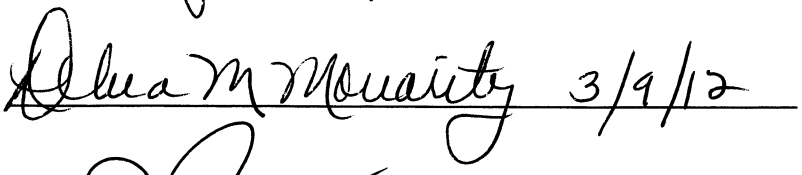
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ABSTRACT

The School of Graduate Studies
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Name of Candidate Anuj Singhal

Title Improving PCR Efficiency with Inorganic Pyrophosphatase

The recombinant Inorganic Pyrophosphatase (IPPase) enzyme from hyperthermophilic Archaea, *Thermococcus thioreducens*, was investigated for its use in improving thermal cycling reactions. In the presence of DNA polymerase B, IPPase was shown to significantly increase the rate of DNA synthesis *in vitro* and enhance the overall PCR process. The increase in efficiency was measured to be at least two fold more than those from control reactions without IPPase in terms of amplification and yield. IPPase was shown to be effective in hydrolyzing the PCR by-product, inorganic pyrophosphate, and driving the forward reaction of PCR amplification more proficiently. The strategic use of IPPase in PCR is of invaluable use in biotechnology applications.

Abstract Approval: Committee Chair

Department Chair

Graduate Dean 3/13/12

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The following study was made possible by the assistance from several individuals. My advisor and committee chair Dr. Joseph Ng helped me create, conduct, and complete this research project. Dr. Bob Zahorchak taught me many valuable skills while working together at HAIB, as well as improved my insight on this project. Dr. Roger Cruz-Vera gave me several helpful suggestions to increase the strength of my results.

I would like to thank my lab mates, Charles Wood, Jorge Barcena, Qunying Wang for all their help during my experiments. In addition my gratitude is extended to Ronny and Mana for their support with the recombinant enzyme inorganic pyrophosphatase. Thank you to my parents, brother, and sisters for all their love and support. My wife, Tanu made this thesis possible with her tremendous inspiration, love, and devotion.

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CHAPTER I

Introduction

1.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is one of the most utilized techniques in molecular biology(Bartlett and Stirling 2003). Since its conception by Kary Mullis in 1986 (Mullis, Faloona et al. 1986), researchers have applied its benefits to dozens of applications including DNA fingerprinting, sequencing, cloning, genetic testing, infectious disease detection, inheritance, evolution studies, and gene expression (Kwok, Mack et al. 1987; Boehnke, Arnheim et al. 1989; Saiki, Scharf et al. 1992; Bartlett and Stirling 2003; Quill 2008). PCR is basically the *in vitro* exponential amplification of a specific DNA segment using a thermo cycling device and the basic components of DNA replication.

PCR requires three main stages. The first stage is known as denaturation. High temperatures (94-98°C) are used to separate double stranded DNA by disrupting the hydrogen

bonds between complementary bases. The second stage is annealing where the temperature decreases (50-65°C) to allow the primers to bind to the single stranded DNA. The final stage is extension/elongation (70-80°C) where DNA polymerase binds the primed template and synthesizes DNA using dNTP's. These stages are then repeated 30-40 times as cycles until the amplification becomes exponential.

The value of PCR lies in the ability to selectively amplify a target DNA sequence away from other genetics material in an experimental fashion. Consequently, millions of copies of the targeted DNA can be used for genetic analysis and manipulation. Without this method, even the most basic tasks used in gene discovery, sequencing, or DNA fingerprinting are difficult.

1.2 Inorganic Pyrophosphatase (IPPase)

Inorganic pyrophosphatase (IPPase) is an essential enzyme in many biochemical reactions including DNA replication (Voet and Voet 2000). It catalyzes the hydrolysis of inorganic pyrophosphate (PP_i) into two molecules of orthophosphate ($2P_i$), releasing approximately 33.5KJ of crucial free energy(Voet and Voet 2000). The need for this catalysis extends to lipid, glucose, and ATP metabolism as well as complex synthesis processes such as

DNA replication, transcription, and translation (Voet and Voet 2000).

The recombinant IPPase employed for this study was isolated and purified by Hughes et al. from the hyperthermophilic Archaea, *Thermococcus thioreducens* (Hughes 2011). It has been proven to be most active between 70-85°C and stable up to 100°C (Hughes 2011). This IPPase's thermophilic activity and stability is essential for its function within the PCR reaction, where temperatures cycle between 50°C and 95°C for an extended period of time.

1.3 DNA Replication and PCR Similarites

DNA replication occurs when DNA polymerase catalyzes the addition of a nucleotide base to a DNA strand. The addition occurs via a phosphoryl transfer reaction in which a deoxynucleoside monophosphate (dNMP) is added to the free hydroxyl group of the 3′ end of the DNA strand from a deoxynucleoside triphosphate (dNTP) molecule, releasing inorganic pyrophosphate (PP_i) as a byproduct (Voet and Voet 2000)(Fig. 1.1). The PP_i is hydrolyzed by IPPase, which releases free energy into the reaction system. This free energy is coupled with the less thermodynamically favorable DNA synthesis reaction to drive it forward at a faster rate

(Voet and Voet 2000). The functional activity of IPPase is therefore very attractive for PCR enhancement.

PCR models itself after *in vivo* DNA replication using the basic components: DNA polymerase, dNTP's, primers, DNA template, and a thermocycler to provide strand separation. However, IPPase's role *in vivo* has been largely ignored in the *in vitro* DNA replication reaction. It is reasonable to hypothesize that IPPase will prove to be beneficial in driving the *in vitro* DNA synthesis reaction forward in the same way as occurs during DNA replication in a living cell.

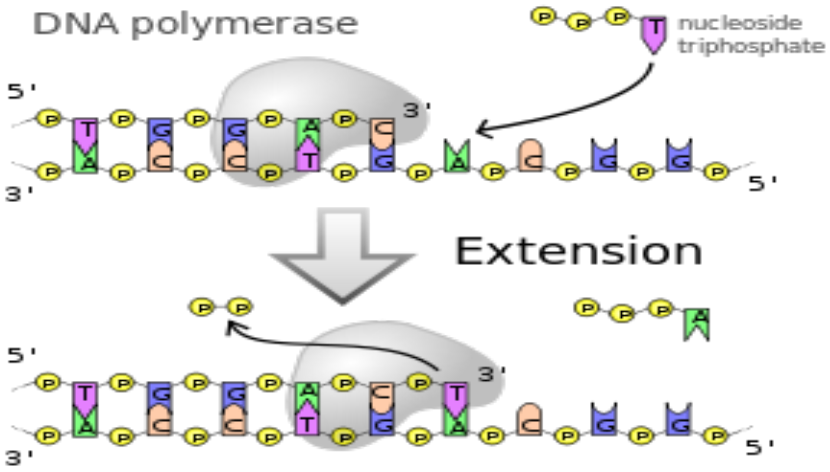


Figure 1.1 DNA synthesis releasing PP_i as a byproduct.

1.4 PCR Efficiency

The PCR reaction is not 100% efficient. It has been shown that PCR only occurs at the expected exponential rate

for a limited amount of time, gradually slowing and finally reaching a plateau (Freeman, Walker et al. 1999). PCR reactions show a predictable trend in DNA amplification. Early cycles of amplification gradually become exponential, followed by a quasi linear phase, and then by a plateau phase in the last cycles. This is easily visualized during real-time PCR (Freeman, Walker et al. 1999). There are a multitude of explanations for the plateau phase including degradation of template, primers, polymerase, dNTP's by the high temperatures of the PCR reaction. In addition, a limiting amount of polymerase, primers, or dNTP's could contribute to the plateau effect. Degradation of product by polymerase exonuclease activity, inhibition by accumulation of PCR products, and finally inhibition by PP_i accumulation are also possible factors (Saiki, Gelfand et al. 1988; Mullis 1991; Heid, Stevens et al. 1996). It has been suggested by some that exhausting levels of DNA polymerase, dNTP's, or primers have a very negligible effect on PCR efficiency (Suzuki and Giovannoni 1996). This assertion has turned more focus onto inhibiting factors such as PP_i and product accumulation. The final plateau of amplification is likely caused by a combination of all these factors (Mullis 1991).

The accumulation of PP_i could be slowing the reaction from the early stages and also having a more dramatic effect as the levels increase exponentially in later cycles. IPPase could then improve the overall efficiency of the PCR reaction as well as the maximum yield per reaction.

1.5 Objectives

IPPase plays a vital role in DNA synthesis *in vivo* (Voet and Voet 2000), and thus may improve the *in vitro* reaction. Previous studies by Park et al. have shown some enhancement of the PCR reaction with IPPase present (Park, Lee et al.). There is also evidence of PCR enhancement with thermostable enzymes that remove inhibitory nucleotides (Dabrowski and Kiaer Ahring 2003; Cho, Lee et al. 2007; Kim, Ryu et al. 2008).

The object of this study is to show that IPPase can indeed increase the efficiency of PCR reactions by hydrolyzing PP_i in the reaction. Qualitative results will show increasing PCR product by increasing the amounts of IPPase in the reaction, finding an optimal concentration for PCR enhancement by IPPase. IPPase assay results will confirm IPPase activity during the PCR reaction by measuring orthophosphate (P_i) levels in the reactions. Also, a DNA quantitation assay will be employed to measure,

for the first time, the degree of enhancement of DNA synthesis and amplification with IPPase present, as well as the effects of PP_i on the reactions.

CHAPTER II

MATERIALS AND METHODS

2.1 Inorganic Pyrophosphatase (IPPase)

Inorganic pyrophosphatase (IPPase) from *Thermococcus thioreducens* was isolated and purified by Hughes (Hughes 2011). The enzyme was stored at 4°C in lyophilized form with 1 mg enzyme in a 2 ml tube. Reconstitution was done by adding 1 ml deionized sterile H_20 (1 mg/ml), and then further diluted into a working solution by a 1:200 dilution (5 μg/ml) into 1X Rainbow buffer (described later).

2.2 Polymerase Chain Reaction (PCR)

PCR Reactions were carried out with polymerase B, which was purified and characterized by Marsic et al. (Marsic, Flaman et al. 2008). The reaction mixture contained 10X Rainbow buffer, 10 mM dNTP's, 10 μm primers, DNA template, polymerase B (170 ng/ml), and sterile water. The reaction used a Pet3A plasmid with a cloned gene as a template and primers producing a 756 bp pair product. The 10X Rainbow buffer consists of 100 mM Tris-HCl pH 8.25, 600

mM KCl, 20 mM $MgCl_2$, and 1% Triton X-10(Marsic, Flaman et al. 2008). The thermocycler was set to: initial denaturation, 5 min at 95°C; 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55-60°C, 30 sec-1 min extension at 72°C, and final extension of 7 min at 72°C unless otherwise indicated.

2.3 PCR Product Enhancement Gel

IPPase was added to 50 µl PCR reactions, with 60 sec extension cycles, in varying volumes. The reactions with IPPase contained IPPase at 5 µg/ml and volumes of: 0.05 µl, 0.1 µl, 0.2 µl, 0.5 µl, 1 µl, corresponding to 15, 30, 60, 150, and 300 pmol IPPase. The gel was stained with ethidium bromide and visualized with a UV transilluminator. The photograph was analyzed using ImageJ densitometry software (http://rsbweb.nih.gov).

2.4 IPPase activity assay on PCR reactions

The activity of IPPase was measured based on the assays of Park et al. and Sigma Aldrich (Park, Lee et al. ; Horder 1972). A pyrophosphate (PP_i) reagent was made with 0.223 g sodium pyrophosphate (Fisher, St. Louis, MO #S390-500) mixed into 50 ml water. This reagent was used to test IPPase activity after PCR was completed with IPPase present. The colorimetric reagents were made fresh on the day of the experiment, 0.5 g of ammonium molybdate

(Mallinckrodt, St. Louis, MO, #3420) was added to 5 ml of 10 N H_2SO_4. Tausky-Shorr (Horder 1972) reagent (reagent E) was also made the same day from l ml of the previous reagent, 0.5 g ferrous sulfate (Matheson Coleman and Bell, USA #CB391), and 9 ml water. A phosphate (Potassium phosphate mono basic, Mallinckrodt #AR ACS) standard curve was obtained from dilutions of a known standard (van Alebeek, Keltjens et al. 1994). The standard was diluted from 0.65 μmol/ml P_i to 0.065, 0.13, 0.2, 0.26, and 0.33 μmol solutions in water to make a 1 ml solution and then adding 1 ml of the final reagent, incubating for 5 min, followed by spectrophotometric analysis at 660 nm wavelength. The PCR reactions were performed as described above with 60 sec extension cycles, in 50 μl volumes, with or without 60 pmol IPPase. 10 μl of the PCR samples were added to 90 μl water and 100 μl reagent E, and measured after 5 min. The amount of P_i was quantified by using the standard curve produced. The amounts of P_i were compared for reactions with no IPPase and no polymerase B, with IPPase and no polymerase and PP_i added after PCR, no IPPase with polymerase and IPPase added after PCR, and PCR with and without and IPPase.

2.5 DNA Synthesis Assay

The amount of DNA synthesized over time was measured by adaptation of an assay for DNA polymerase activity used by Seville et al. and Marsic et al. (Seville, West et al. 1996; Marsic, Flaman et al. 2008). A DNA quantitation assay (#Q33120) from Invitrogen Life Sciences (USA) measures double-stranded (ds) DNA by a fluorometric reagent (Quant-iT) was used. The synthesis of double stranded DNA from single stranded primed template was set up in a reaction. The primed template was prepared as follows, 2 μl of M13mp18 ssDNA (New England Biolabs N4040S) at 250 ug/ml was added to 98 μl water along with 1 μl of 100 μM UPlong primer (Marsic, Flaman et al. 2008), and incubated at 70°C for 5 min then allowed to reach room temperature over 15-25 min and frozen at -20°C for further use. The DNA synthesis (extension) reaction was set up by adding 13 μl sterile deionized water, 2 μl 10mM dNTP's, 1.8 μl 10x buffer, 1.2 μl primed-template, and 0.2 μl IPPase. The reaction mixture was place in a dry heating block at 74°C for 1 min before starting the reaction by adding 2 μl polymerase B. The reactions were stopped at precise time points by adding 2μl 5mM EDTA and placing on ice. The samples were measured by fluorometer (PerSeptive Biosystems) with excitation and

emission wavelengths of 485 and 525 nm respectively, by adding 200 μl Quant-iT reagent and 10 μl of the synthesis reaction per well of a 96-well plate, and waiting approximately 5 min for the solution to stabilize.

2.6 Rate of DNA Synthesis Reaction

The synthesis reaction was set up as described above, and stopped at precisely 5 min. The reaction was repeated three times with and without IPPase. Each experiment contained a negative control, which did not have polymerase B, and thus had no DNA synthesis. The reading for the negative control was subtracted from the readings with polymerase B to measure only the new dsDNA synthesized.

2.7 PCR Reaction Quantitation

50 μl PCR reactions were setup identically to the IPPase PCR described above with or without 60 pmol IPPase. The reactions were stopped at 15 cycles and 30 cycles with 30 sec extension times. 5 μl Of the PCR reaction was added to 200 μl Quant-iT reagent and measured as previously.

2.8 PP_i Inhibition Reaction

The synthesis reaction was set up as before. PP_i was added to the reactions at 0.01, 5, 10, 20, 50 μmol concentrations to measure the inhibition effect. 60 pmol IPPase was added to show restoration of synthesis.

2.9 Error Bar Calculation

The error bars were calculated in the same way for each experiment. The experiments were repeated three times, and the mean was calculated. The data points in the figures represent the mean values. The error bars represent the standard deviation from the mean.

CHAPTER III

RESULTS

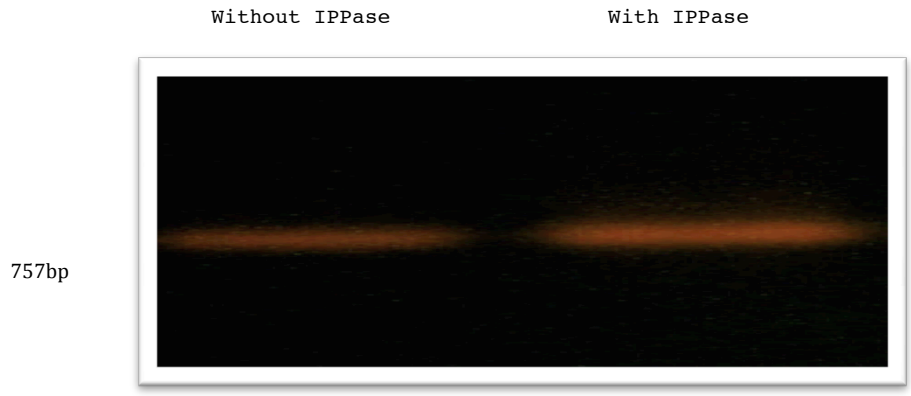
3.1 IPPase Enhancement Gel

IPPase was hypothesized to improve the yield of PCR by removing PP_i and driving the reaction forward. The improvement of obtaining PCR products becomes more evident after many cycles of PCR in which exponentially increasing amounts of PP_i are being created. This increased PCR product should be visible by gel electrophoresis followed by ethidium bromide staining. The intensity of the PCR product bands, visualized by a UV transilluminator, should be greater as the amount of product increases. A PCR reaction with IPPase present should in theory, have a brighter product band than a PCR reaction without IPPase.

Two PCR reactions were set up to test this hypothesis. One reaction contained all the normal components of a PCR reaction, while the other also had IPPase included. The IPPase was diluted to the working solution used previously when doing an IPPase activity assay, and added at half the

volume to polymerase volume. The polymerase used was polymerase B. Both enzymes come from *T. thioreducens* and thus may be best suited to work in conjunction with each other as they do in the organism itself. A 1% agarose gel carefully stained with ethidium bromide showed a brighter more intense product band for the IPPase-included reaction (Fig. 3.1a).

Upon this confirmation, an experiment was set up with the goal of finding the maximum enhancement level with IPPase. Increasing concentrations of IPPase were added to PCR reactions to show increasing product band intensity until a maximum was reached at which point the intensity would remain the same or decrease due to some type of inhibition. A result was produced and reproduced on four occasions using five concentrations of IPPase ranging from 15 pmol to 300 pmol (Fig. 3.1b). There was a consistent trend from 0-60 pmol of IPPase that showed increasing product. The 150 and 300 pmol concentrations of IPPase either stayed about the same as 60pmol or decreased slightly in product band intensity (considering all results), giving the indication that 60 pmol would be the optimal concentration for further experiments.



(a)

Figure 3.1a. PCR Products obtained with and without IPPase.

(b)

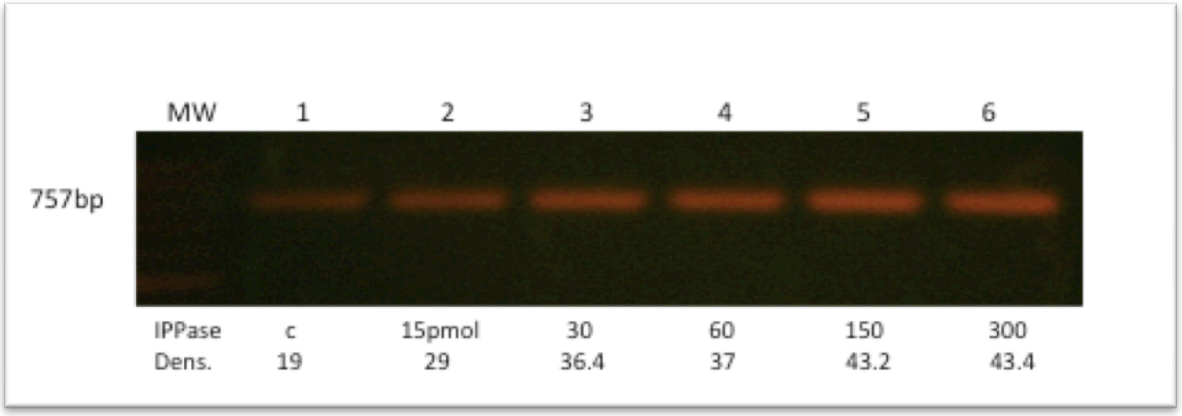


Fig. 3.1b. PCR products obtained with increasing amounts of IPPase 60-300 pmol. Densitometry numbers correlate to an increase in products.

3.2 IPPase Activity Within the PCR Reaction

The gels gave a strong indication that IPPase was indeed causing the increase in PCR product observed, but proof of IPPase activity would provide additional confirmation (Fig. 3.2). A negative control was set up without polymerase but with IPPase present in the PCR reaction. This result provided a baseline for the amount of PP_i and P_i in the reaction without any DNA synthesis occurring. The negative control indicated there was ortho phosphate present in the reaction. Another control was set up and not placed in the thermocycler to see if phosphate hydrolysis was taking place during the heating and cooling during PCR. The results showed about one third the P_i was present before thermocycling. So the presence of P_i in the negative control can be attributed largely to hydrolysis by high temperature, likely from the dNTP's. The smaller than expected increase in P_i of the test reaction can be attributed to this unexpected hydrolysis that is unrelated to the DNA synthesis reaction.

The next reaction was set up the same way but PP_i was added after the PCR was complete, this would test for remaining IPPase activity after the high temperature cycling of PCR. The result showed a large increase in P_i meaning the IPPase was able to hydrolyze the added PP_i after

enduring the PCR reaction. This activity took place at 75°C for 5 min. Next a PCR reaction was carried out normally and IPPase was added afterwards, and incubated for 5 min at 75°C to test if IPPase could hydrolyze the PP_i produced during PCR into P_i. The result showed a significant amount of PP_i was produced and converted to P_i. Finally the two test reactions were compared, one normal PCR reaction and one PCR reaction with IPPase. The control PCR showed 0.11 μmol of P_i, similar to the negative control since the PP_i was not hydrolyzed by IPPase. The PCR reaction with IPPase showed 0.31 μmol of P_i, similar to the result when IPPase was added after the reaction. These results showed that the IPPase is active and converting PP_i to P_i during the PCR reactions, producing a 288% increase in P_i when present. These results are similar with those obtained by Park et al., which also showed significant IPPase activity in a similar reaction (Park, Lee et al.). The indicated hydrolysis of PP_i into P_i by IPPase produces free energy and likely pushes the PCR reaction forward increasing the amount of PCR product.

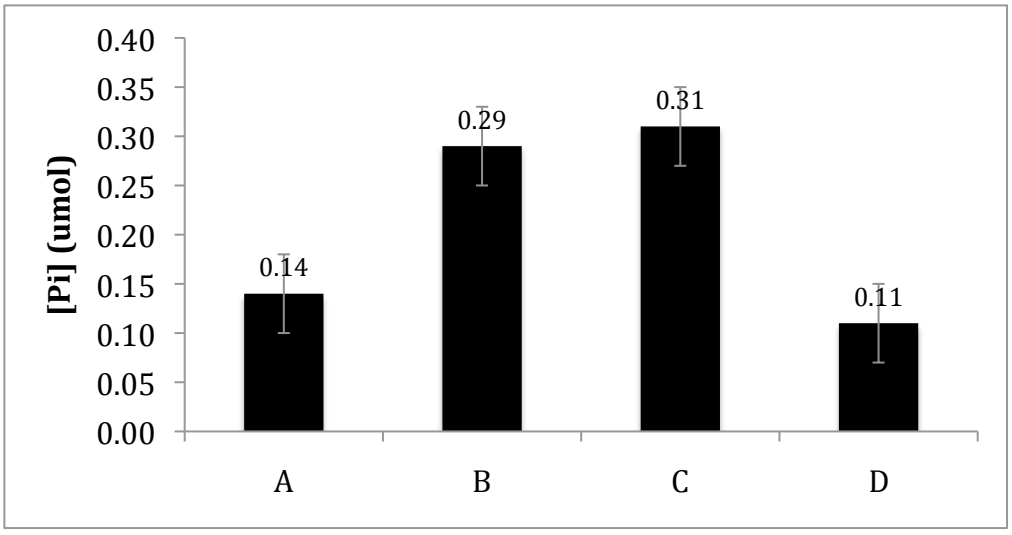


Figure 3.2 PCR IPPase activity assay results. Negative control (A) gives a baseline of P_i, to compare the positive control, test, and control reactions to (B,C,D).

3.3 Quantitation of PCR Products

The Quant-It DNA quantitation kit (Invitrogen) was used to measure and compare amounts of DNA in PCR reactions with and without IPPase present. A negative control was set up containing all PCR components except polymerase, which served as the baseline and was subtracted from readings with polymerase present. PCR amplification was conducted for 15 cycles and 30 cycles with and without IPPase present. It was hypothesized that after 15 cycles less exponential amplification would have occurred compared to 30 cycles. The initial cycles of PCR remain linear,

whereas the later cycles should be mostly exponential amplification, so the 30-cycle PCR reactions would have exponentially larger amounts of synthesis and PP_i production allowing for exponentially larger amounts of hydrolysis by IPPase to occur compared to the 15 cycle reactions. The results show a definite increase with IPPase even after only 15 cycles (21%), but a much more profound effect in the 30 cycle reactions (61%) as predicted (Fig. 3.3). The fact that a greater enhancement of PCR is seen after more cycles have taken place supports the theory that IPPase's hydrolysis of PP_i is the enhancing factor. If there were some other mechanism that did not depend directly on by-products of DNA synthesis, then the enhancement would be consistent through out the cycles of the PCR reaction.

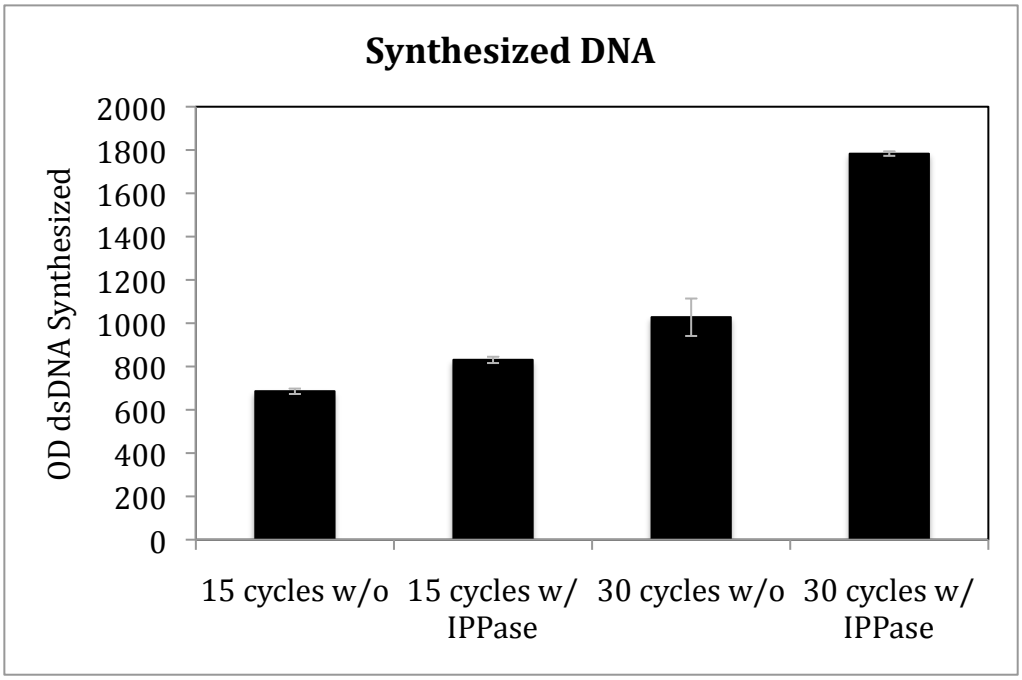


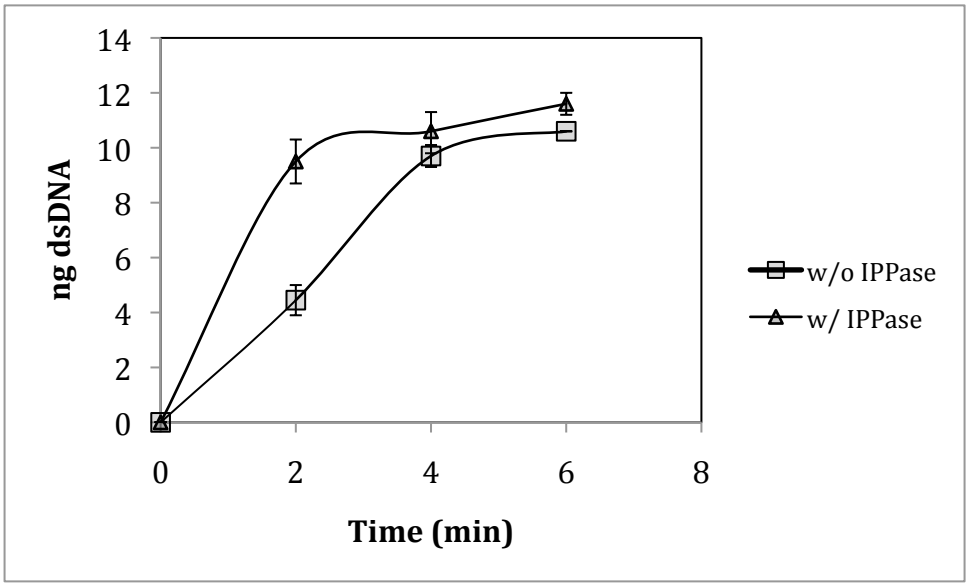
Figure 3.3 DNA Quantitation Assay comparison of PCR after 15 and 30 cycles, with and without IPPase present in the reaction.

3.4 DNA Synthesis Assay

In order to test in more detail IPPase's effect on DNA synthesis *in vitro*, a simple DNA extension reaction was set up and quantitated. An assay used commonly for testing DNA polymerase activity (Marsic, Flaman et al. 2008), was used to compare the rate of DNA synthesis with and without IPPase. The single stranded DNA template and the measurement of purely double stranded DNA by the quantitation reagent allow for a precise quantitation of the amount of DNA synthesized in a given amount of time. IPPase's proposed effect of driving the DNA synthesis

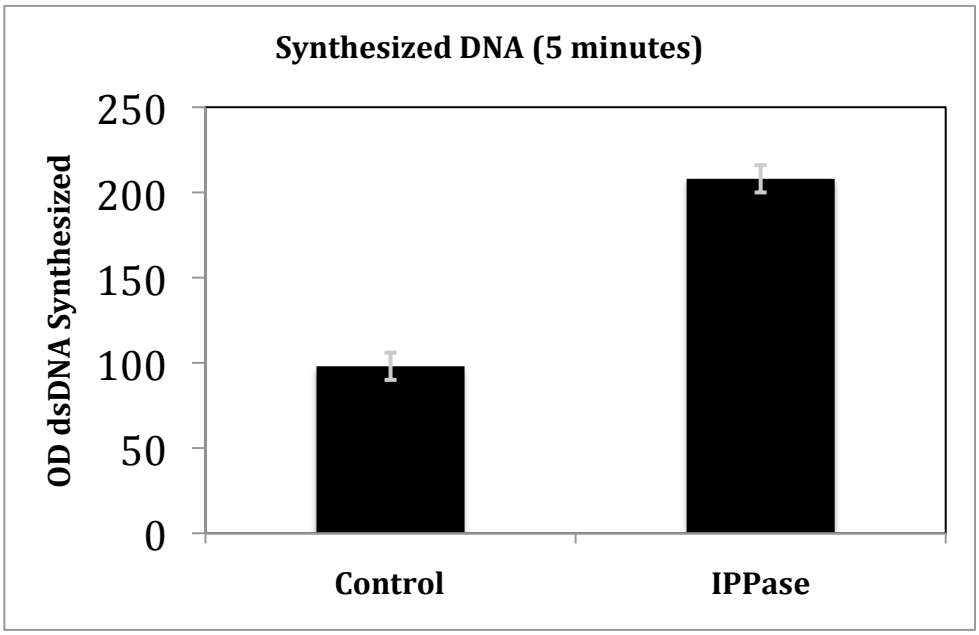
reaction forward would presumably increase the actual rate of DNA synthesis. The degree of increase and the timing of such were of interest in this experiment.

It was observed around 2 min into the reaction that IPPase was allowing for almost a 2-fold increase of DNA synthesis compared to the control (Fig. 3.4a). It was difficult to get accurate measurements during this short time period, so the amount of polymerase was decreased from approximately 4 μl to 2 μl allowing for a reaction rate that produced a consistent and significant result at the five-minute mark with IPPase (Fig. 3.4b). The results indicate that during the early stages of a DNA synthesis reaction when the most template is available to be extended by DNA polymerase, IPPase has a substantial effect by doubling the rate of synthesis.



(A)

Figure 3.4a. Plot of DNA synthesis over six min with (triangle) and without (square) IPPase present.



(B)

Figure 3.4b. Comparison of the amount of DNA synthesized after five min with and without IPPase.

3.5 PP_i Inhibition

If the DNA synthesis reaction that releases PP_i is driven forward when IPPase hydrolyzes the PP_i produced, then adding excessive PP_i to the reaction system should slow the reaction by pushing the favorability in the opposite direction. Measuring the amount of DNA synthesized with excessive PP_i present would reveal the effect.

A large amount of PP_i (50 μmol) was added initially to test for inhibition. Nearly all (84%) DNA synthesis was halted confirming the expected result (Fig. 3.5). Subsequent reactions showed a proportional trend of inhibition with the varying amounts of PP_i added. IPPase was then added to reactions with PP_i to test for a restorative effect, expected as IPPase hydrolyzed the PP_i, which was inhibiting the reaction. The IPPase restored some synthesis to the reactions with 0.01, 5, 10 μmol PP_i added. The reactions containing 20 and 50 μmol PP_i reactions apparently had too much PP_i for IPPase to overcome in the concentration being added. The results are consistent with the inhibition/restoration effects expected under the proposed model.

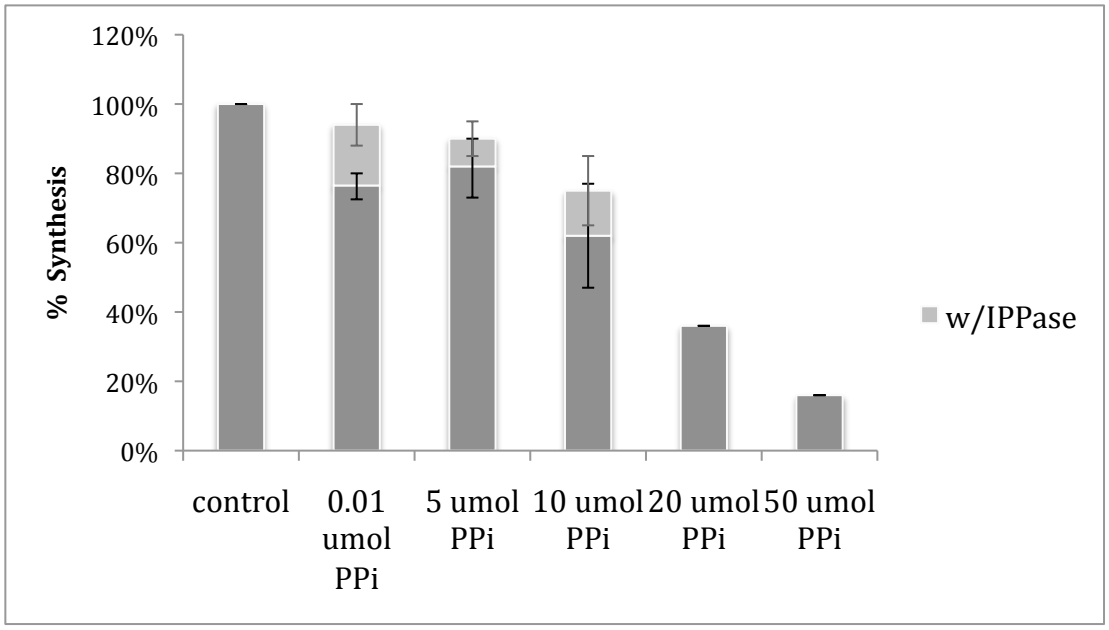


Figure 3.5 Effect of PP_i on DNA synthesis reaction, and restorative effect of IPPase.

CHAPTER IV

DISCUSSION

4.1 Degree and Timing of Enhancement by IPPase in PCR and DNA Synthesis Reaction

Initially several synthesis reactions were set up from 5-60 min in length. It was determined that most of the synthesis was taking place in the first 5 min, and that the most dramatic effect of IPPase was observed during this time. During the 2-5 min range in the reaction process there was little unextended template remaining in the IPPase reaction because of its enhancement, allowing the control reaction to continue to extend and catch up to the IPPase reaction from 2-5 min (Fig. 3.4a). This conclusion coincides with the previously shown increase of IPPase's PCR enhancement in the later cycles when there are more template-primer molecules present to be affected.

The plateau of the synthesis reaction was seen after 2 min with IPPase present, and after 4 min without IPPase. This plateau took place after about 10 ng of dsDNA (15 pmol) had been synthesized, which corresponds with the

complete extension of the 5 ng of ssDNA (15 pmol) template in each reaction. The plateau seen is thus explained by the complete extension of the template, similar to results obtained by Marsic et al. when characterizing polymerase B (Marsic, Flaman et al. 2008). The fast rate of synthesis by polymerase B was also seen by Marsic et al, who calculated a rate of 96 nucleotides per second using a similar assay (Marsic, Flaman et al. 2008). The continued synthesis observed after 5 min is an anomaly seen by others when using the same quantitation kit and set up (Marsic, Flaman et al. 2008). One possible explanation for synthesis after the complete extension of the template could be a rolling-circle type of replication, which allows DNA polymerase to continue to synthesize DNA from a circular template (Nelson, Cai et al. 2002). There is evidence of a DNA polymerase with strand displacement activity performing rolling circle replication *in vitro* (Nelson, Cai et al. 2002).

The degree of enhancement during PCR proved to be substantial but less than the 100% increase seen during the DNA synthesis reaction. There are several considerations to be made. The 100% increase only occurred at a certain time point, after some additional time the control reaction gradually caught up to the IPPase reaction. The PCR

reaction is a more complex set up due to the cyclical and exponential nature of the synthesis. In the early stages the reaction without IPPase performs near to maximum efficiency leaving little room for enhancement by IPPase, thus the minimal increase in product (21%). Even though the IPPase reaction may be faster, the control may still be fast enough to complete extension during the given extension time. At some point, (after many cycles) the concentration of PP_i has increased to a level that inhibits the reaction enough to decrease the extension substantially, but at that point there are likely many other inhibiting factors (excess template, inhibitory nucleotides, etc) that IPPase does not affect. The IPPase does have an increased effect (61%) in the later cycles of PCR, but not at the same level (100%) seen during simple synthesis due to other inhibiting factors and the PCR process as a whole.

4.2 Possible Mechanisms for Increased Efficiency of PCR

The increase in speed of DNA synthesis by IPPase enhancement does not by itself account for an increase in PCR product. If the PCR was running at 100% efficiency for example a faster synthesis would only mean that each extension cycle could be shorter, but the amount of product made would not change.

In the less than perfect PCR reaction, an increase in speed could increase the product under certain conditions. There is a limited amount of time for extension of primers during PCR, after which denaturation starts again separating the strands. Perhaps the reaction may slow to a point where some of the primed templates are incompletely extended (Fig. 4.1). The incomplete template is then unable to be primed in the next cycle allowing less amplification. The IPPase could reduce incomplete extension and thus increase amplification products.

The increasing amount of template may lead to polymerase becoming limiting. In this case some primed templates would be unextended due to a shortage of polymerase. However, with IPPase increasing the rate, it is possible that one polymerase molecule can finish extension fast enough to extend another primed template during a single extension cycle, thus increasing the product (Fig. 4.2).

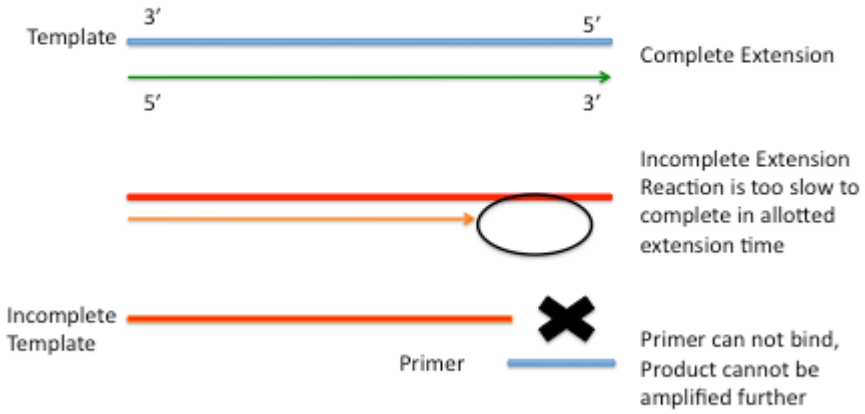


Figure 4.1 Incomplete Extension caused by slower reaction leads to less PCR product.

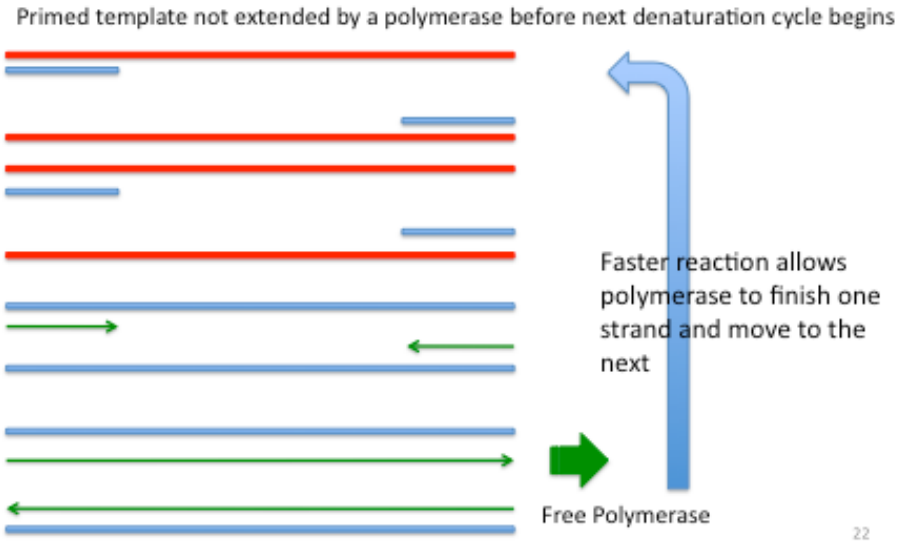


Figure 4.2 Polymerase extending multiple primers per cycle during a faster PCR reaction.

4.3 Applications of IPPase

There are many inherent benefits with making a time consuming process more efficient. In most cases increased efficiency saves time, which saves money. PCR takes a significant amount of time to complete, and is often the bottleneck-step in many processes. Thermocyclers are getting faster, but there is still time associated with changing the temperature three times per cycle. A common PCR program allows 5 min of initial denaturation and final extension, 30 sec for each step, which means 90 sec (1.5 min per cycle). Thirty cycles would take 55 min not including the time between steps, which increases the total time significantly. In general, PCR reactions take greater than 2 hours to complete. Preparation of DNA samples and setting up the PCR reaction can be done in a matter of minutes. Visualizing the PCR product via gel electrophoresis can be done in less than 30 min, so the 2-3 hours spent waiting for the PCR reaction to complete can be quite costly. In the classroom students often set up the PCR reaction in one period, and are unable to visualize the results until the next meeting, when their comprehension of the whole process diminishes greatly. Adding IPPase to the PCR reaction could allow for completion in less than one hour and same-class analysis.

Real-time quantitative PCR (RT-PCR) is another prime application for IPPase. The purpose of RT-PCR is to quantify gene expression levels via cDNA. Mathematical analysis has shown that accurate measurements can only be taken during cycles of exponential amplification (Freeman, Walker et al. 1999). RT-PCR plots show that this type of amplification takes place for only a limited number of cycles before the amplification slows and finally stops forming a plateau (Fig. 4.3)(Freeman, Walker et al. 1999). IPPase's ability to increase PCR efficiency, likely will delay the plateau effect, thus enlarging the window for accurate measurements to be taken.

Forensic science could benefit from IPPase as well. Crime scenes often have very limited DNA amounts, so IPPase could allow for PCR amplification of minute DNA samples. The faster results could also aid investigations. Other possible applications of IPPase involve mutagenesis, cloning, gene synthesis, and diagnostics.

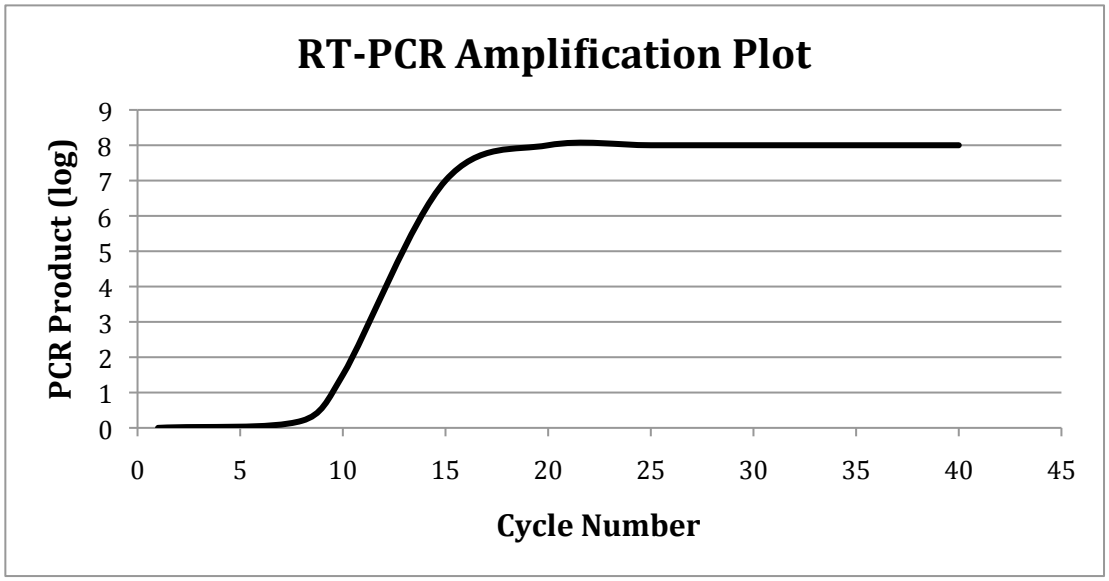


Figure 4.3 RT-PCR plot illustrating the plateau phase.

4.4 Further Study

IPPase's effect on DNA polymerase processivity is one promising area of further study. Processivity is the amount of time or length of DNA that polymerase can continue to synthesize DNA without detaching from the template (Voet and Voet 2000). *In vivo* DNA polymerase exhibits high levels of processivity, aided by accessory proteins such as PCNA (Voet and Voet 2000). It is possible that IPPase, by increasing the efficiency of the synthesis reaction, will allow for a longer fragment of DNA to be synthesized per polymerase binding event. Even if the amount of time the polymerase stays attached remains the same, the increased speed with IPPase would allow a longer

product to be produced. The direct effect of removing the inhibitory PP_i could also increase the processivity of polymerase. There has been evidence that an archaeal dUTPase increases PCR product lengths and possibly processivity (Hogrefe, Hansen et al. 2002). Achieving a longer PCR product with IPPase would be a step towards proving increased processivity. However, further assays would have to be performed to confirm a single binding event, rather than multiple polymerase bindings leading to longer PCR products.

Fidelity is another possible area where IPPase could have a positive effect. DNA polymerase is not 100% accurate, and occasionally adds an incorrect nucleotide to a growing DNA strand. There are assays being used to analyze the fidelity of different polymerases (Jozwiakowski and Connolly 2009). The same assays could be used to test IPPase's effect on the fidelity of DNA synthesis. IPPase drives the synthesis reaction forward thus making polymerase's activity less impeded, and possibly decreasing its error rate.

CHAPTER V

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