Start to Finish Detection of *Cryptosporidium* for in Field Use on Water Samples via Lauroylsarcosine sodium Salt (LSS) Extraction, Loop Mediated Isothermal Amplification (LAMP), and DNA Precipitation.

Honors Thesis
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by
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Abstract

Today, many diseases that are rare in developed nations are still prevalent in other countries due to limited technological access and resources. Among these diseases is Cryptosporidiosis, a common gastro-intestinal infection with currently no known treatments. Cryptosporidiosis is caused by the parasite *Cryptosporidium.* This parasite spreads through the environment as an oocyst, causing self-limiting diarrhea in healthy humans. These symptoms are often more dangerous in immuno-compromised individuals and children, whose immune systems are still developing. The rugged *Cryptosporidium* oocysts are able to survive treatment with most disinfectants. Thus, to prevent outbreaks, there must be early detection, followed by rapid treatment of the water supply and/or limited exposure to infected water supplies. Current methods of parasite testing for water samples are limited in their function by cost, access to materials, and ability to run equipment. Improvements in cost-effective, easy to use alternatives to traditional techniques, such as the use of lauroylsarcosine sodium salt (LSS) for DNA extraction and the loop mediated isothermal amplification (LAMP) method of amplification, will facilitate the detection of *Cryptosporidium* in the field and in impoverish nations.In this experiment, a five primer LAMP reaction was used in combination with the LSS reaction to eliminate otherwise essential electrical equipment in the detection process, expediting and easing in-field detection. The LSS was found to decrease the efficiency of the LAMP reaction, but its effects were mediated by changing the buffer used in the LAMP reaction. Amplicons were visualized using gel electrophoresis in this experiment. However, methods of detection were tested for incorporation into the test. These techniques will lead to the future of cost efficient detection of *Cryptosporidium* in water samples.

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Keywords: *Cryptosporidium*, Loop-Mediated Isothermal Amplification, parasite detection, cost-efficiency, optimization

Introduction

Field research in developing nations is challenging due to funding and equipment availability. However, when human health is concerned, a practical method of testing must be optimized to increase the likeliness of researchers performing this technique. On average, developing nations spend significantly less on public safety testing than developed nations do, despite organizations such as UNICEF identifying water quality monitoring as a growing priority (UNICEF, 2013). Unfortunately, this lack of funding for public health safety and scientific advancement leads to scarcity of safety testing for drinking water and other consumer resources, which negatively impacts the life expectancy of citizens. For the benefit of the citizens of developing nations, it is imperative that new methods of testing are developed which fit within the public safety testing budgets and resources of these nations.

Species of *Cryptosporidium* are among the parasites that pose a significant threat to human health, particularly in impoverished nations. Currently, ten percent of children worldwide die before the age of six from a diarrheal disease (Kotlof et al., 2013). *Cryptosporidium* is the second most common diarrheal disease for infants 0 to 11 months of age (Kotlof et al., 2013). Up to ninety-five percent of the population in developing countries tests positive for antibodies against this parasite. This proves that historically, *Cryptosporidium* has afflicted the citizens of underdeveloped or currently developing regions (Gutierrez, Y. 2000). With an infection rate reaching twenty percent in developing countries, there is a constant threat of *Cryptosporidium* infection for those who do not have access to proper methods of detection (Gutierrez, Y. 2000).

Cryptosporidium refers to a genus of organisms that are apicomplexian protozoans (Bakheit et al. 2008). A parasite of the human gastrointestinal tract, *Cryptosporidium* causes self-limiting or chronic diarrhea by infecting the epithelial cells of the microvilli (Fayer et al.

1990). While this infection is unpleasant, for those with healthy immune systems it is acute (Fayer et al. 1990). However, for those who are immuno-compromised, it can be a life threatening chronic condition (Fayer et al. 1990). The severity of the threat posed by *Cryptosporidium* in conjunction with its prevalence demonstrates a need for *Cryptosporidium* detection or treatment reform.

Cryptosporidium spreads through the environment as an oocyst, which is durable and resistant to external stressors including most household disinfectants (Gutierrez, Y. 2000). Since it is so difficult do destroy, a simple way to prevent a widespread outbreak is through early detection of *Cryptosporidium*, followed by containment until it can be properly treated. The oocyst is shed from an infected organism, which may be human or animal depending on the species of *Cryptosporidium*. While contact with an infected oocyst can occur via many routes, chance of contact is greatly increased in developing nations as they have fewer sanitation methods (Fayer and Xiao 2012; UNICEF, 2013). Unfortunately, methods of detecting *Cryptosporidium* on a frugal budget are limited. The purpose of this project was to develop an all-inclusive method of testing water samples, for the presence of *Cryptosporidium*, at a relatively low cost, while in the field. This was accomplished through the use of an LSS extraction followed by a five primer loop mediated isothermal amplification (LAMP) reaction (similar to PCR), which is then visualized by an isopropanol DNA precipitation reaction.

As *Cryptosporidium* oocysts are quite rugged, their detection has proven difficult, even with the most advanced diagnostic technology. Typically, oocysts are lysed using a freeze-thaw technique; however, this method relies heavily on the ability to rapidly change the temperature of a water sample. In a field research setting or in a developing nation, electricity may be scarce, thus alternating between freezing temperatures and warm temperatures may not be the best

method. In order to eliminate the need for extreme cold conditions, DNA extraction can be performed with the aid of lauroylsarcosine sodium salt (Sekikawa et al. 2011). This technique was selected because it is faster and operates at a uniform temperature. This process minimizes the need for electricity, and allows thermal regulation in a water bath heated with a flame, or a pre-prepared thermos at the correct temperature. Additionally, detection has been shown using this extraction method in conjunction with a six primer LAMP (Sekikawa et al. 2011). The only drawback to using the LSS extraction technique in the proposed overall method is that it has been shown to decrease the efficiency of a LAMP reaction (Sekikawa et al. 2011).

Once the DNA is extracted from oocysts, a five primer LAMP will be used to overcome the challenge of amplification. Typically polymerase chain reaction (PCR) is used for DNA amplification. However, PCR requires a thermocycler, which is an expensive piece of equipment and needs a constant power source for a minimum of three hours. The LAMP technique requires a set of four specially designed primers (FIP, BIP, F3, and B3) that adhere to the domains immediately flanking a target sequence and a specified type of DNA polymerase (bst DNA polymerase (Tomita et al. 2008)). *Bst* DNA Polymerase is from the *Bacillus stearothermophilus* DNA Polymerase protein that contains the 5′ → 3′ polymerase activity, but lacks 5′ →3′ exonuclease activity (New England BioLabs, Inc.). Unlike the gene amplification technique of PCR, LAMP takes place at a single temperature (63ºC (Momoda et. al, 2009)). The specialized bst DNA polymerase enzyme makes the cycling of temperatures unnecessary because it allows for the two strands of DNA to be weakly bound, so that extension can occur, but strands can still dissociate to allow for primer binding (Plutzer et. al, 2009). This process is sped up by the addition of the two optional primers (LB and LF primers) that were initially thought to be essential (Notomi et al. 2000; Gandelman et al. 2011). The LAMP technique has

been successful in amplifying a number of human parasites, including *Cryptosporidium* and *Giardia* (Momoda et al. 2009; Karanis et al, 2007; Plutzer et al. 2009). LAMP maintains its sensitivity in the presence of contaminants better than PCR (Skotarczak, 2010). Prior to the start of this project, *Cryptosporidium* detection via the LAMP method was conducted using the gp60 gene and 18S RNA gene in Japan, Germany and Austria (Karanis et al. 2007; Momodoa et al. 2010). In the past year, however, more articles have been published worldwide (Mohon et al. 2014; Nkbakht et al. 2014; Rigano et al. 2014; Wang et al. 2014). While my research was based largely on research from Japan, I sought to optimize the detection of *Cryptosporidium* in water samples using the 18S RNA gene and reagents that did not generate unnecessary financial burden, in the hopes that American researchers would adopt the method optimized here for use in the field.

Ultimately, my purpose was to develop a system of reactions that allow for the detection of *Cryptosporidium* in water samples without the use of a thermocycler, or other sophisticated machinery. The implications of such a test extend far beyond the bounds of scientific inquiry, into the sphere of public health. The discovery of a cost-effective method of testing bodies of water for *Cryptosporidium* could increase the life expectancy of immuno-compromised individuals and those living in third world nations. Additionally, this test has the potential to raise the quality of living for many people living in at risk areas.

Materials and Methods

DNA Extraction with LSS (Lauroylsarcosine sodium salt)

In place of the traditional freeze-thaw method (Smith et al. 2009; Kim et al. 1991), the *Cryptosporidium* oocysts, initially suspended in water, were incubated at 90ºC with a 0.1% concentration of LSS for 15 min to lyse the rugged outer wall of the oocysts (Sekikawa *et al.* 2011). After DNA extraction, the samples were diluted 1:2 (LSS concentration 0.05% after this step) to obtain the target concentration of 0.01% LSS during the LAMP reaction (after sample is combined with LAMP reagents), reported in Sekikawa et al. (2011), as an optimal concentration for the LAMP reaction to function the sample must be diluted 1:10 by the start of the LAMP cycle.

Six Primer LAMP Protocol Optimization

Before the LAMP method could be optimized for a longer target sequence using five primers, LAMP had to be adapted for reagents available in the USA for a moderate cost. Since many enzymes sold in America come with buffers, the LAMP method was optimized to use some of these buffers, so that the reagents did not cause an additional financial burden. The LAMP procedure was modified from the Momoda et al. (2009), due to the availability of reagents within the United States of America.

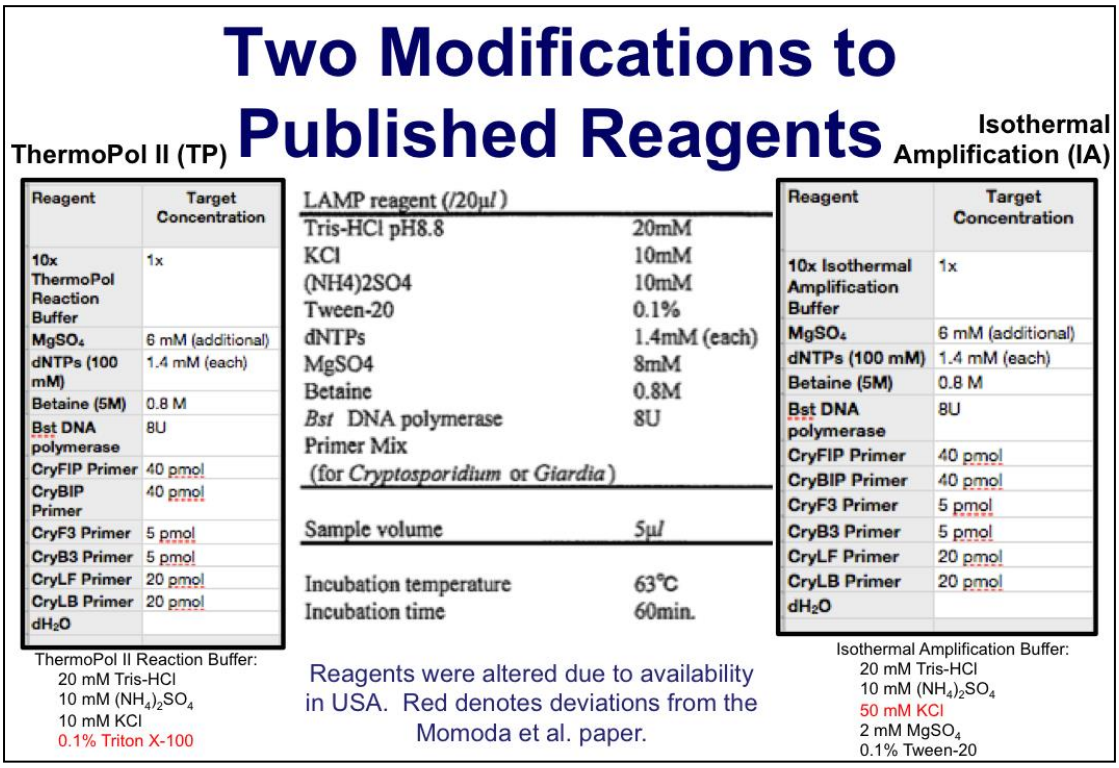
The primers used were the same as in Momoda et al. (2009) (Table 1). This set of primers target a fragment of *Cryptosporidium's* 18S RNA gene (Momoda et al. 2009). Two sets of reaction materials were compared for optimization (Fig. 1). The Isothermal Amplification Buffer based (IA) reaction was 25 µL total volume and included 1x Isothermal Amplification Buffer

(New England Biolabs), augmented with an additional 6mM $MgSO_4$, 1.4 mM of each dNTP, 40 pmol CryFIP primer, 40 pmol CryBIP primer, 5 pmol CryF3 primer, 5 pmol CryB3 primer, 20 pmol CryLF primer, 20 pmol CryLB primer, and 8U bstDNA polymerase with 5 µL template DNA (sample). These reactions contained 40 mM more KCl than those published in the Momoda et al. (2009) study. The ThermoPol Reaction Buffer based (TP) reaction was the same as the IA reaction with the exception that the 1x ThermoPol Reaction Buffer (New England Biolabs) replaced the 1X Isothermal Amplification Buffer. These reactions contained 0.1% TritonX-100, which was not included in the Momoda et al. (2009) study. All reaction volumes were supplemented with irradiated deionized water to reach the total reaction volume. Both reactions were incubated for sixty minutes at 63ºC (Momoda *et al.* 2009).

Table 1: Primers for 6 Primer Amplification (Sequences from Momoda et al. 2009)

Primer	Sequence
CryFIP	5’ TACTTAACTCATTCCAATTAGAAAACCCAGGGAGGTAGTGACAAG 3’
CryBIP	5’ ATAAACCCCTTTACAAGTATCAATTTATACGCTATTGGAGCTGG 3’
CryF3	5’ GCGCAAATTACCCAATCC 3’
CryB3	5’ ACTACGAGCTTTTTAACTGC 3’
CryLF	5’ CCAAAAAGTCCTGTATTG 3’
CryLB	5’ GAGGGOAAGTCTGGTG 3’

Figure 2: Reproduction of the reagents listed in Momoda et al. flanked by the two sets of reagents tested (2009).



LAMP Primer Design

LAMP primer design is constrained by many factors (Eiken Chemical). For example, the binding affinity between the domains immediately flanking either end of the target sequence is a limiting factor because these domains are combined, in multiple arrangements, to generate the basic sequence of the primers (Appendix 1). Any binding that occurs between domains F1, F2, F3, F1c, F2c, F3c, B1, B2, B3, B1c, B2c, or B3c other than between and domain and its complement (ex. F1 and F1c) hinders the appropriate binding of primers during the LAMP reaction and would be detrimental to the reaction propagation. PrimerExplorer V4 Software

generates a basic sequence for the LAMP primers (Eiken Chemical). This sequence is basic because restriction sites and other desired regions must be added manually to the sequence generated by PrimerExplorer V4.

The primers used in the five primer reactions were designed using PrimerExplorer V4 software, according to their manual (Eiken Chemical). PrimerExplorer V4 was manipulated to design the basic sequence of the primers that would target longest segment of *Cryptosporidium's* 18S RNA gene that was compatible with the LAMP primer constraints. The length of the target sequence was maximized at the expense of reaction speed because the LAMP portion of this project was intended to have a dual purpose. The overarching goal of this project was to determine a start to finish test for the detection of *Cryptosporidium* that exhibited ease of use within the field. However, the LAMP reaction was designed so that the amplified product could also be sequenced to determine the genotype of the *Cryptosporidium*. This is important because previous research has been detecting presence/absence of any species of *Cryptosporidium*, while the primers designed for this test were selected so that sequencing data (outside of a field work setting) could be obtained to determine the genotype of the *Cryptosporidium* detected. The data collected from this type of analysis could be used in epidemiological studies, since immuno-compromised individuals are sensitive to species of *Cryptosporidium* other than those that typically infect humans. The target sequence used (tcctgtttcgaaggaaatgggtaatcttttgaatatgcatcgtgatggggatagatcattgcaattattgatcttgaacgaggaattcctagtaag cgcaagtcatcagcttgcgctgattacgtccctgccctttgtacacaccgcccgtcgctcctaccgattgaatgatccggtgaattattcggac catactttgtagcaatacatgtaaggaaagtttcgtaaaccttatcatttagaggaaggagaagtcgtaacaaggttt) was 268 bp in length. While this gene is highly conserved between species of *Cryptosporidium*, there are distinct differences in this target region that would be detectable via sequencing (Altschul et al.

1990). Only five primers were identified for this target sequence. There was no LB primer identified.

NEBcutter was used to identify restriction enzymes that would not cut within the target sequence (New England BioLabs, Inc.). The enzyme chosen was Dra I (cut site TTT/AAA) (New England BioLabs, Inc.).

Restriction enzyme sites were manually designed into the FIP and BIP primers before the primers were ordered to allow for sequencing. The cut site was inserted between the F1c and F2 domains in the FIP primer and between the B1c and B2 domains in the BIP. Since the LAMP reaction amplifies the target sequence in a chain like manner (Appendix 2), these restriction sites are used to turn a LAMP product into the same form as a PCR product (one copy of target sequence per DNA segment). The five primers are listed in Table 2:

Table 2: Primers for 5 Primer Amplification

Primer	Sequence
Cry1113FIP	5'CCTCGTTCAAGATCAATAATTGCAATTTAAATTTTCCTGTTTCG AAGGAAATGG 3'
Cry1113BIP	5'TTCGGACCATACTTTGTAGCAATACTTTAAATTTAAACCTTGT TACGACTTCTCC 3'
Cry1113F3	5' ACTGATGCATCCATCAAGT 3'
Cry1113B3	5' TCTGCAGGTTCACCTACG 3'
Cry1113LF	5' CCCCATCACGATGCATATTCAAAAG 3'

Five Primer LAMP Protocol Optimization

When the LAMP primers were designed to target a longer segment of the 18S RNA gene, only five were identified. Consequently, the protocol for the LAMP reaction was optimized to adjust for this. The LAMP procedure was repeated with varying concentrations of $MgSO_4$ to determine if the ion concentration needed to be altered to adapt to the decreased number of primers. The concentrations tested were 2 mM, 4 mM, 6 mM, 8 mM (control), 10 mM, 12 mM, and 14 mM. Next, the reaction time was optimized, incase the decrease in primer number impacted the speed of the reaction's progress. The times tested were 0.5 h, 1 h, 1.5 h, and 2 h.

Isopropanol precipitation of DNA

A DNA precipitation reaction was performed using a QiAgen's Isopropanol precipitation of DNA protocol (Qiagen). Steps 2-6 were followed. In brief, the samples were combined with 0.6 volumes of isopropanol and centrifuged for 15-30 min at 10,000-15,000g, Then the supernatant was discarded, the pellet was washed with 70% ethanol and the samples were centrifuged again for half as long. Tubes were analyzed for the presence of a pellet, after precipitation with 80% isopropanol.

Restriction Fragment Polymorphism

To confirm the correct amplicon sequence, a restriction fragment polymorphism reaction was performed. Four µL of Dra I, 4 µL of its corresponding buffer, 22µL of irradiated deionized water, and 5 µL of LAMP product were combined. The reactions were incubated at 37ºC for 12 h to ensure that the DNA was cut completely. The results were then visualized using agarose gel electrophoresis.

Results

DNA Extraction Via LSS (Lauroylsarcosine sodium salt) vs. Freeze-Thaw

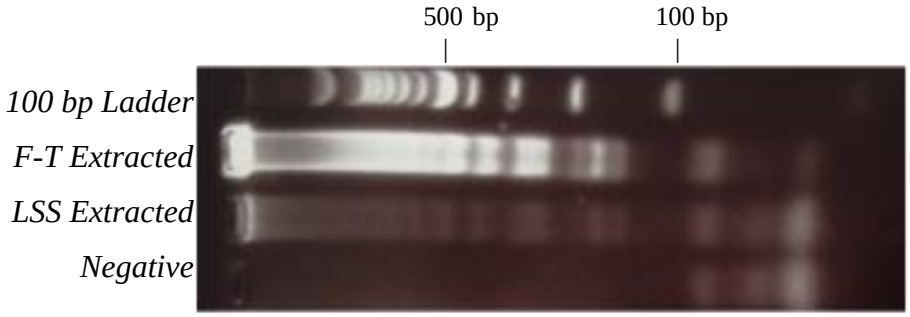


Figure 3: Gel Electrophoresis showing the same sample extracted using both F-T and LSS methods. Note the difference in florescence between the two samples, which shows the LSS decreases the efficiency of the LAMP reaction. These samples were both amplified using the same LAMP procedure.

The LSS extraction showed a lower DNA concentration of amplicon than the Freeze – Thaw (F-T) method of extraction (Fig. 3). Both lanes containing sample show a characteristic LAMP banding pattern, comprising of a vast number of bands of varying length corresponding to chains of varying numbers of copies of the target sequence, which ultimately present as a smear due to the high DNA concentration and proximity of the different bands (Momoda et al, 2009). However, the banding patterns vary in florescence.. Since the LSS banding patter is fainter, there is a decrease in the efficiency of the LAMP reaction in conjunction with LSS. However, this decrease is not sufficient enough to outweigh the in field benefit that LSS provides, as a positive can still be observed using LSS extraction and LAMP amplification.

Six Primer LAMP Protocol Optimization

The results of the six primer LAMP optimization reaction were viewed using gel electrophoresis (Fig. 4). No amplicon observed by electrophoresis in ¾ of the TP Buffer-prepared samples (TP1 and TP-2, samples; TP-4, negative control). No amplicon observed by

electrophoresis in ¼ of the IA buffered (1A-4, negative control). The TP buffer did not amplify all three samples, while the IA buffer did.

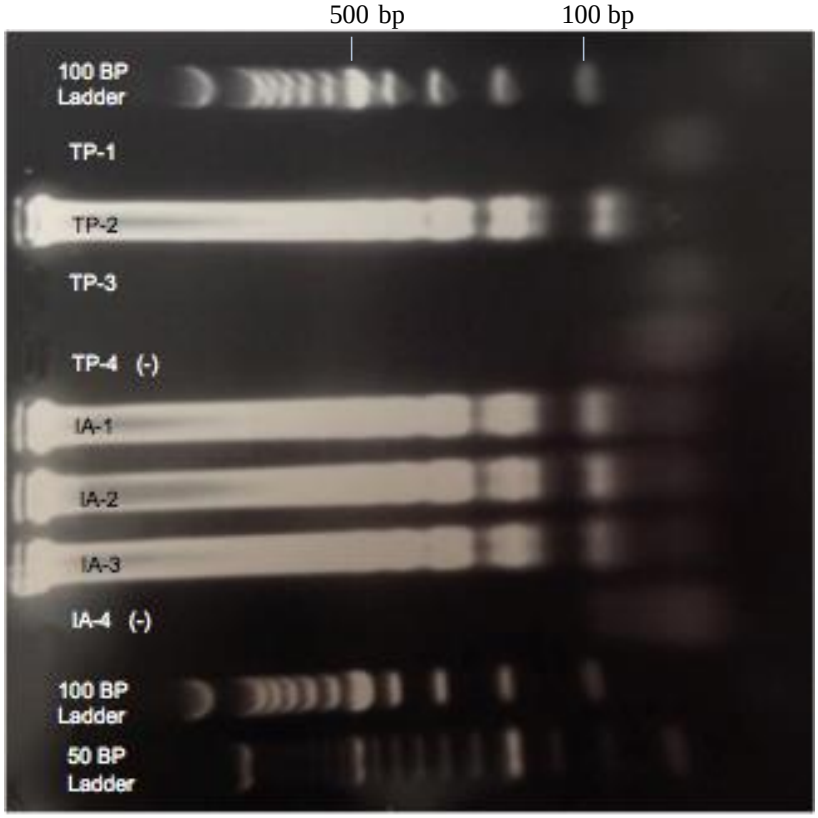


Figure 4: Gel Electrophoresis showing three different samples and a negative control, for each of the two sets of reagents. All three wells corresponding to the IA buffer have characteristic LAMP banding, while two of the TP lanes shows no banding when the samples were known to be positives. This suggests the TP reaction conditions are inferior to the IA reaction conditions.

Five Primer LAMP Protocol Optimization

The five primer LAMP optimization reactions were performed to control for ion concentration and time variations that arose from the loss of one optional primer. From the gel electrophoresis from the optimization experiment testing the effect of ion concentration on five primer LAMP reactions, one can see that all concentrations above 2 mM $MgSO_4$ showed

significant banding in the characteristic LAMP banding pattern and were, therefore, successful amplifications (Fig. 5). The 12 mM $MgSO_4$ concentration showed a decrease in the luminescence of the bands. When repeated, this discrepancy between 12 mM and the other concentrations >2mM was still observed (Appendix 3).

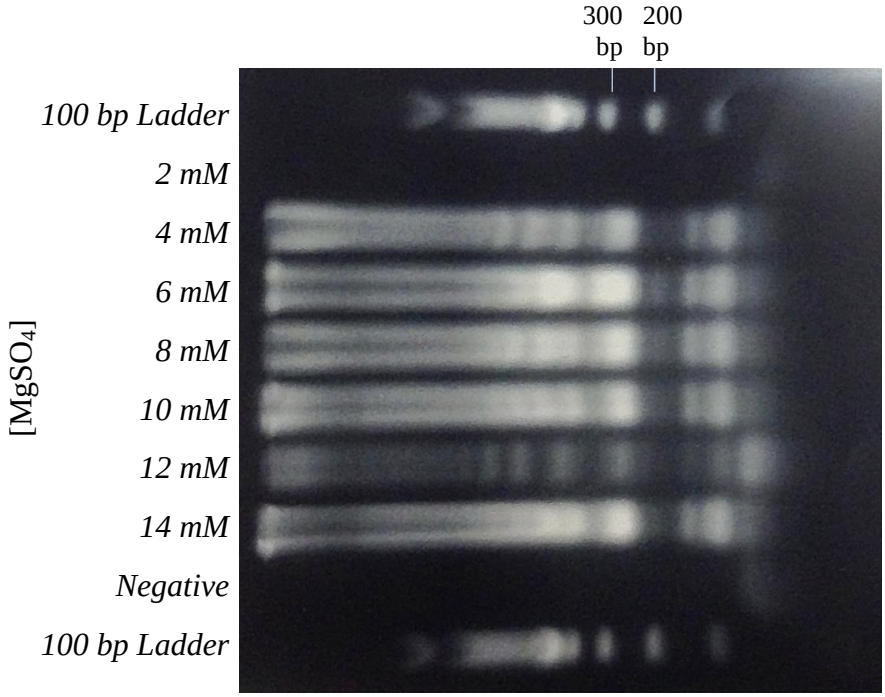


Figure 5: Gel Electrophoresis showing the LAMP reaction with varied ion concentration

Since the LB primer is an efficiency primer, it was anticipated that the LAMP reaction would need more time to progress to completion. However, this was not the case. The gel electrophoresis from the optimization of the reaction time with only five primers showed an even amount of florescence in all samples that were run for one hour or more (Fig. 6). Samples run for only thirty minutes had an insufficient amount of DNA to be detected in the gel.

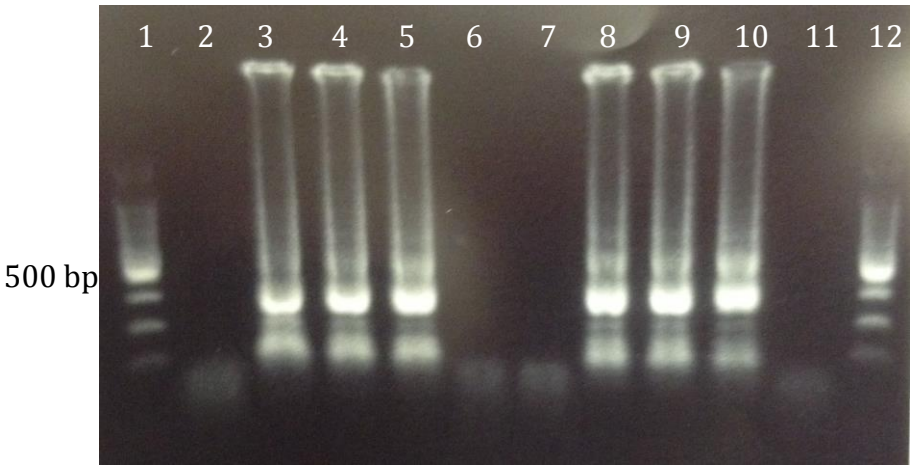


Figure 6: Gel Electrophoresis showing the LAMP reaction run at varying amounts of time. Lanes 2-11 are freeze-thaw purified Cryptosporidium. The samples in lanes 7-11 have LSS added to the reaction at the concentration that would exist if the oocysts had been LSS extracted (final concentration of 0.01% LSS). The samples from lanes 2 and 7 were incubated at 63ºC for 30 min. The samples from lanes 3 and 8 were incubated at 63ºC for an hour. The samples from lanes 4 and 9 were incubated at 63ºC for an hour and a half. The samples from lanes 5 and 10 were incubated at 63ºC for two hours. Lanes 6 and 11 are negative controls that were incubated at 63ºC for one hour (as the initial protocol called for). Lanes 1 and 12 are a 100 bp ladder.

Isopropanol precipitation of DNA

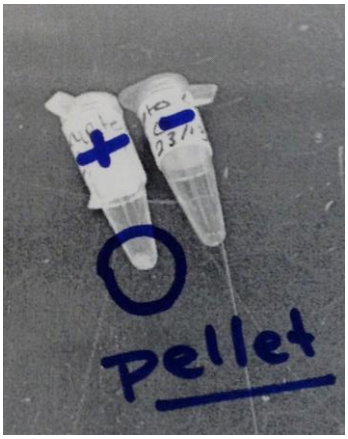


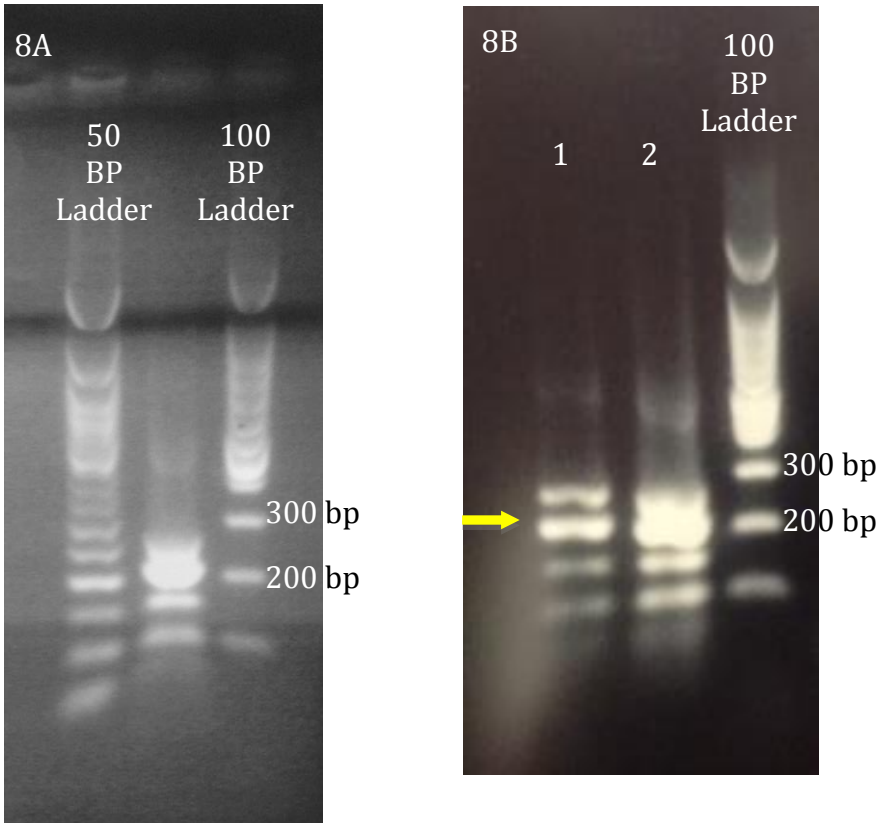
Figure 7: Pellet observed in the positive control during the DNA precipitation. No pellet was observed in the negative control.

The results from the isopropanol precipitation of DNA showed a visible pellet in the positive control and no pellet in the negative control (Fig. 7). This experiment was done as a practical option for result visualization in the field.

Restriction Fragment Polymorphism

The restriction fragment results showed a band at the expected length (between two hundred and three hundred base pairs) and two other bands that were shorter (approximately one hundred to one hundred seventy) than the target sequence (Fig. 8). These results confirm that the target sequence was amplified. The smaller bands were likely partial copies of the target sequence that were interrupted by another primer and cycle of replication.

Figure 8: Gel Electrophoresis showing the LAMP reaction products digested with DraI. The sample well in 8A and 8B lane 2 were loaded with 2 µL sample. 8B lane 1 was loaded with 1µL of sample. Note the one major band between 200 and 300 bp in the center lane in 8A and in lane 2 in 8B. This is likely the target sequence. Lane 1 in 8B also shows one band larger than the others that is likely the target sequence based on size.



Discussion

The purpose of this project was to design a start to finish test that incorporated known methods and new ones to easily and economically test water samples entirely in the field. Ultimately, the project was a success. The components of this reaction can be put together to generate a complete test for *Cryptosporidium* in water samples. The test is less expensive and more field friendly than the current test. Combining LSS extraction with LAMP extension and isopropanol precipitation of DNA will yield a start to finish test for *Cryptosporidium*.

While the LSS extraction showed diminished function of LAMP reagents or lower levels of extracted DNA compared to the Freeze – Thaw method of extraction, it still demonstrated a positive result. These results are consistent with those published by Sekikawa et al. (2011). Additionally, since LSS is a known PCR inhibitor, it is not surprising that it interacts with LAMP in a negative manner.

In pursuit of a start to finish test, the LAMP reaction was optimized to help minimize the negative effects of the LSS. The IA buffer amplifies a wider range of species of *Cryptosporidium*, including *C. parvum* (which was known to be the only species of *Cryptosporidium* in sample #1 in Fig. 4). This is consistent with the findings of Sekikawa et al. (2011) that claim that Tween-20 (found in IA buffer) is better at restoring the bst DNA polymerase sensitivity than Triton-X for a wide range of (found in the TP buffer). The banding patterns observed during the six primer LAMP reaction were consistent with previously reported banding patterns (Momoda et al. 2009; Tomita et al. 2008). From these experiments, it appears that the TP buffer requires more specific binding as it does not positively identify a range of species of *Cryptosporidium*. More trials are needed to understand the relationship between LSS inhibition of DNA polymerase and the necessary specificity of the target sequence. From these

experiments, it appears that the IA buffer is better suited for a detection test, for it will pick up a wider variety of species of *Cryptosporidium* and some not dangerous ones, while the TP buffer will allow some dangerous strains to go undetected. As is the policy of the US government when screening potential blood donors, testing done to maintain human, and more specifically public health, must err on the side of false positives rather than have false negatives, to minimize risk to the society (Linden, J. 1997).

The target sequence for the LAMP reaction was optimized for specificity. The tradeoff of a longer target with fewer primers enables more specific amplification. This specificity allows the LAMP reaction that was designed to have multiple uses. The longer target sequence is beneficial for the start to finish detection test because it only requires five primers, which decreases the overall cost of the assay. However, it also allows for sequencing of the amplified sequence. Sequencing the amplified target, while not a field friendly method, could be used at a later time for epidemiological studies. The longer sequence allows for differentiation of *Cryptosporidium* genotypes. Since certain genotypes of *Cryptosporidium* are infective only for immuno-compromised individuals, while others are infective to all humans, studying which genotypes are present in a given body of water is an area of current interest. Similar studies have been conducted with PCR products (Xiao et al. 2001; Peng et al. 2003). Optimizing the five-primer system yielded results similar to the six-primer system. The concentration of $MgSO_4$ does not significantly impact the results of a LAMP reaction, unless the concentration is below a threshold value, which was between 2 mM and 4mM in this study. This shows that the five primer reaction conditions are similar to the six-primer reaction system, for they have the same ion concentrations as initially used in the six primer experiments (Momoda et al. 2009). However, these results are in contrast to some published LAMP methods, which cite

concentrations of $MgSO_4$ that are significantly higher, such as the 8 mM used by Tomita et al. (2008), are detrimental to the reaction. The 12 mM $MgSO_4$ concentration lane shows an anomaly (Fig. 5). This was thought to be from human error loading the wells. However, the gel was repeated with the same exact results (Appendix 3). Since the results were the same, the only possible explanation is an error in the execution of the LAMP procedure, which could not be repeated due to a shortage of two primers.

Since the primers were designed to accommodate a longer than average target, only five primers could be identified. Lacking one speed primer (the LB primer), it is likely that the reaction progresses slower than otherwise, so it was postulated that the reaction may have needed to be run for longer. The minimum amount of time for a LAMP reaction with ample extracted DNA is between half an hour and an hour (Fig. 7). These results show that when there is an excess of extracted DNA present, the LAMP reaction can run with only five primers at a reaction rate comparable to a six-primer reaction (Tomita et al. 2008). More research trials need to be done to determine optimal reaction times based on expected concentration of *Cryptosporidium* oocysts. While these results did not support the hypothesis that increased time would increase final concentration of DNA enough to be perceived by florescence, perhaps if there were less initial sample, the amount of time may become a more pressing issue. In this case with an excess of DNA, it is likely that the reagents were being exhausted within the first hour, otherwise an upward trend would be noticed in the concentration of the DNA as the samples were incubated longer. These results were confirmed with a restriction fragment polymorphism reaction. The LAMP products were cut with DraI, the enzyme who's cut site was added to the primers. The products were clearly delineated into multiple bands (Fig. 8B, lane 1). The majority of the DNA was found in the band that was the target length; however, there was some DNA that was

shorter. These bands are likely composed of terminal copies of the chain of target sequences. Since LAMP chains have multiple copies of the target sequence together, linked by the FIP and BIP primers (for a diagram of LAMP, see Appendix 2), the enzymes that are copying the end copies of the gene may not have been fully finished sequencing when the digest occurred. Since there is so much DNA, perhaps these partials were copied as well.

Having successfully amplified a large target sequence, the next and final step in method to test water samples is to incorporate the best method of visualization. The best method of visualization, accounting for ease of field use and cost, was DNA precipitation (Fig. 7). This method of visualization is simple to execute in the field with cleaned samples. There are some concerns that water samples that have not been purified will yield a false positive, as the particles that cloud the water will form a pellet despite a lack of *Cryptosporidium*, as well as, false positives from non-specific priming. The degree to which the sample can be cleaned is important for this method of detection. This test is not suited for samples that cannot be cleaned of debris. However, it is unlikely that the precipitate is a false positive from non specific priming because bst DNA polymerase, used in LAMP, has been shown to minimize non-specific priming when used at 63ºC (Cai et al. 2010). Additionally, since restriction sites were added to the primers, the results can later be verified through more rigorous testing if necessary. For this study, this method worked well because the sample had a DNA concentration of 3920 µg/mL when analyzed with a spectrophotometer (ten times the typical concentration received from a PCR) and the sample was free of debris. However, this method likely has a higher threshold of detection than some other options, such as a field spectrophotometer SYBR Safe. These methods were both rejected for economic reasons. The field spectrophotometer is more accurate and would be recommended as an alternate method of visualization because it gives a measure of

DNA concentration. However, the field spectrophotometer is more difficult to maintain than the isopropanol precipitation reaction. Additionally, it is a costly investment unless it is going to be used frequently. Similarly, the imager for the SYBR Safe gel stain is over $1000. While the blue light transilluminator can be replaced with another blue light source, the orange gel through which the results are visualized must be the corresponding wavelength to cause the appropriate amount of interference. Future research pursuits include incorporating a molecular beacon into the primers so that the amplified DNA will fluoresce. However, this method as well would be a significantly increased expense. Ultimately, the isopropanol DNA precipitation reaction was incorporated into this assay because it demonstrated ease of use in the field at a relatively low cost.

The LSS extraction in conjunction with the LAMP reaction can be used to test water samples with a variety of primer numbers. When maximizing the target sequence length, sometimes other aspects of a reaction must be sacrificed. In order to have the longest sequence possible, five primers were used instead of six. While this was cost efficient, it was postulated to delay the reaction; however, no reduction in reaction speed was observed. It is possible that when testing samples that have very low concentrations of oocysts, some reaction rate delay may be observed, thus it may be beneficial to increase the reaction time. Ion concentration, however, does not have any impact on the reaction, as long as a minimum threshold value is reached. In the future, trials to determine the detection limits of this start to finish test for *Cryptosporidium* in water samples should be conducted.

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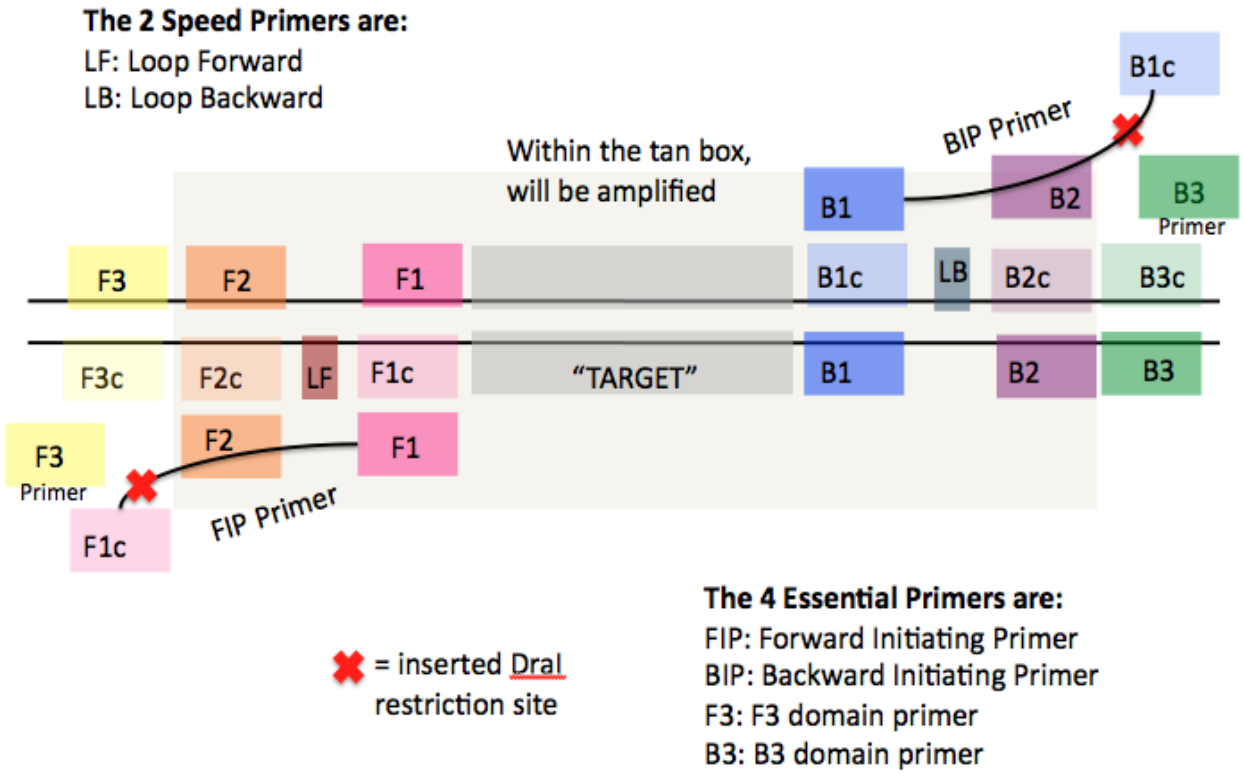
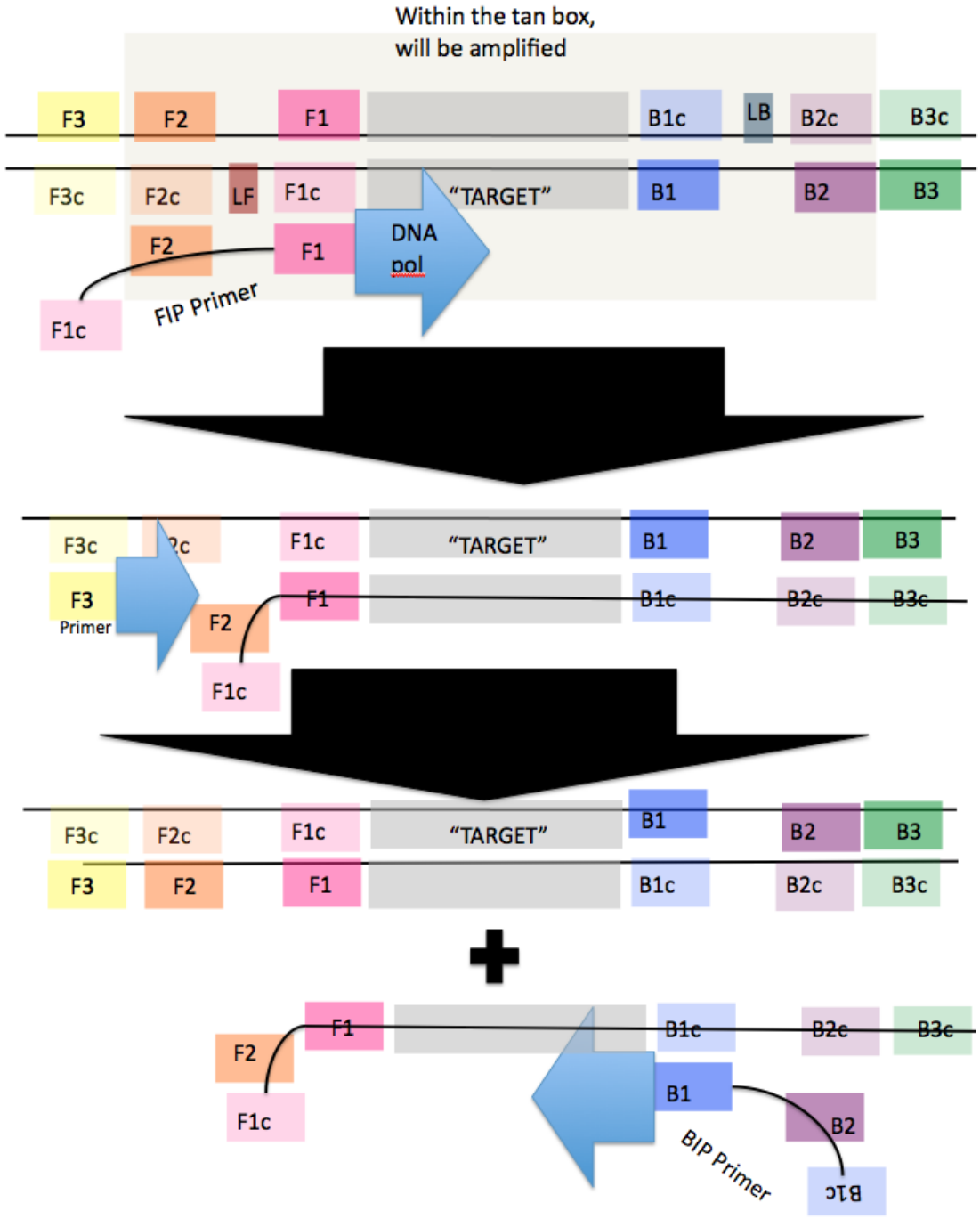
Appendix

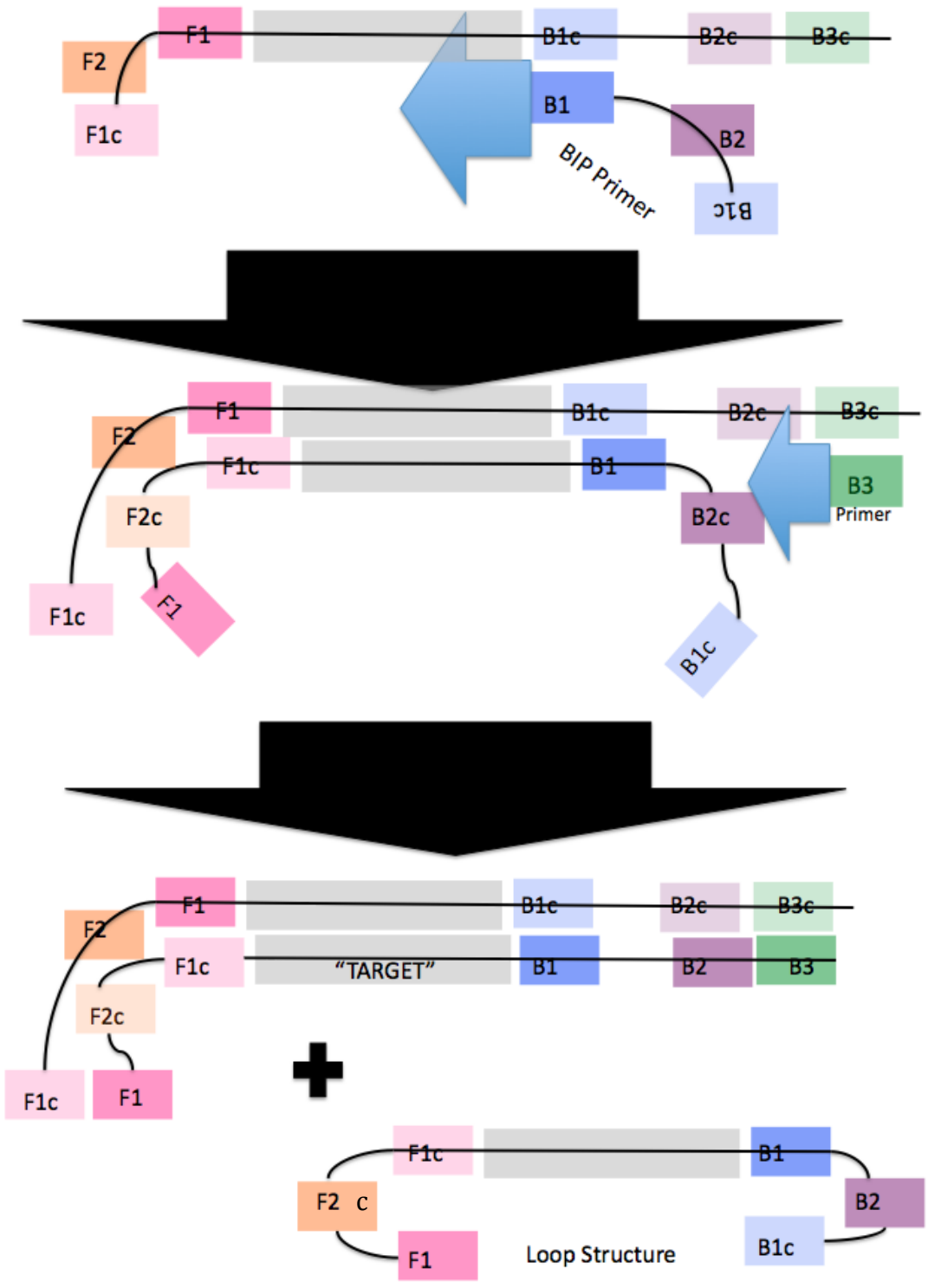
Appendix 1: Diagram of target sequence and primers



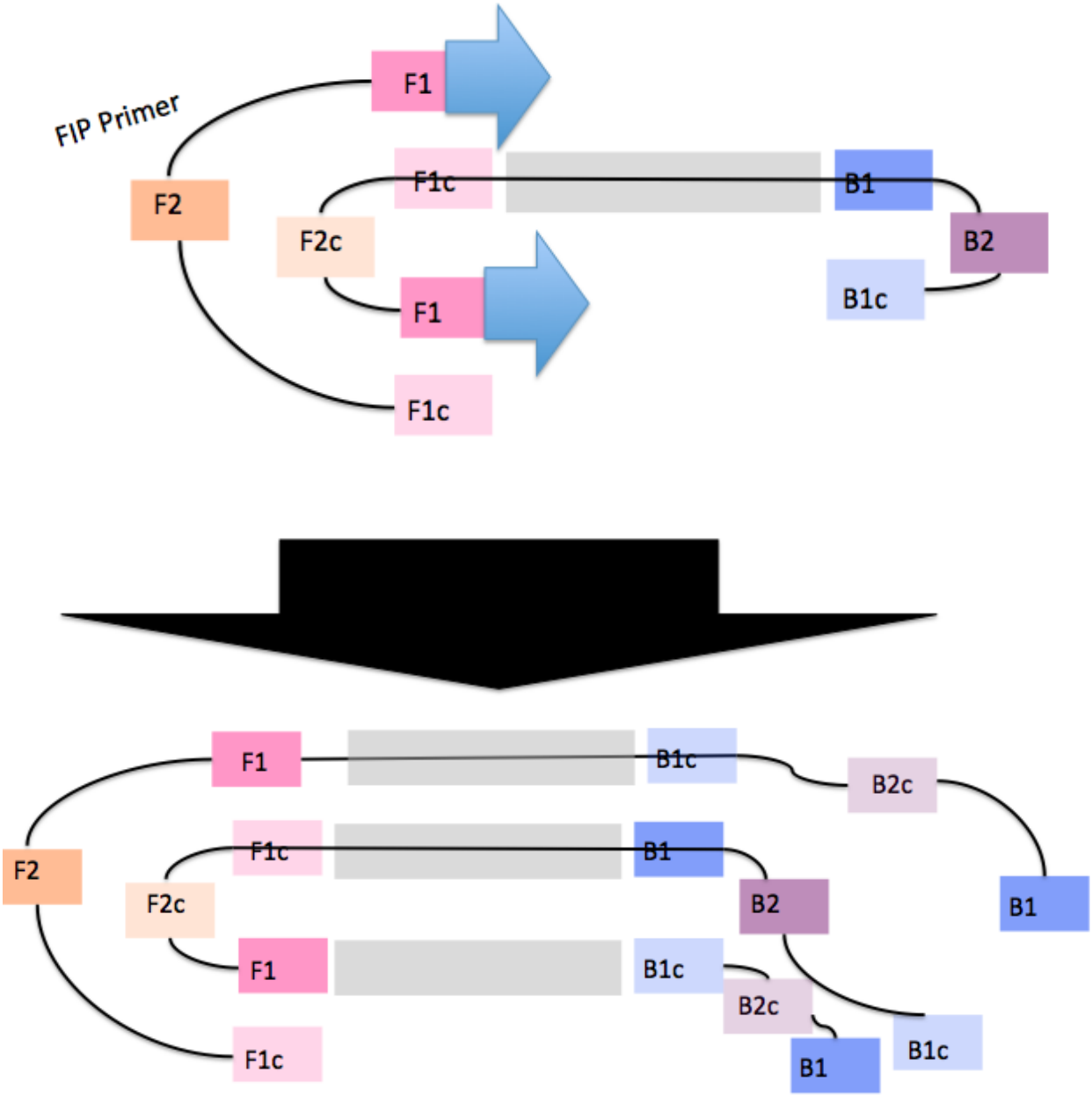
The colored boxes are domains within the sequence of the gene. The primers are constructed to use the same sequences. The two most complex sequences are the FIP and the BIP. The FIP is the F1c + F2 + F1. The BIP is the B1c + B2 + B1. Multiple primers bind to the same location so that the binding and extension of one primer forces another to detach.

Appendix 2: Diagram of LAMP Reaction (4 essential primers)



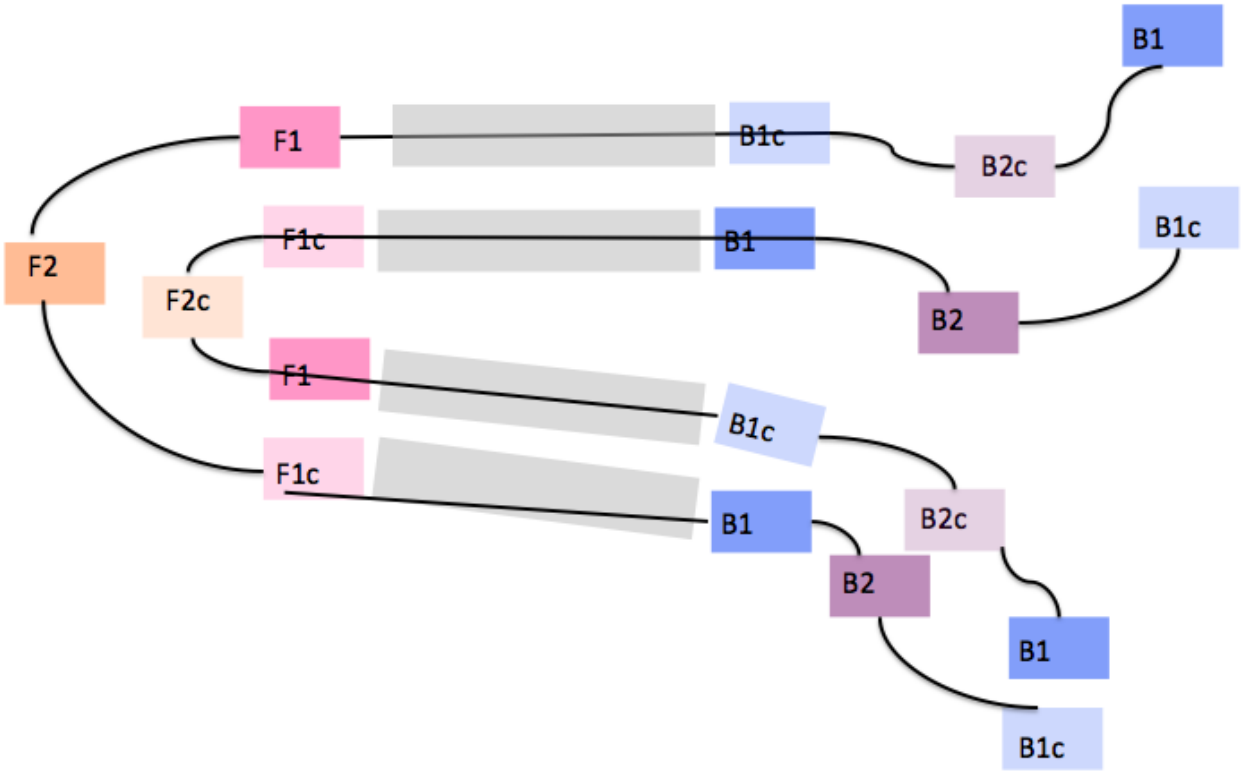

F1
B1c
B2c
B3c
F2
B1
F1c
B2
BIP Primer
B1c
F1
B1c
B2c
B3c
F2
F1c
B1
B3
Primer
F2c
B2c
F1c
F1
B1c
F1
B1c
B2c
B3c
F2
F1c
"TARGET"
B1
B2
B3
F2c
F1c
F1
F1c
B1
B2
F2 c
F1
Loop Structure
B1c

From the Loop Structure amplification can occur from both sides via FIP or BIP. This is an example of amplification from two sides using FIP, the BIP would simply bind on the right instead of the left.



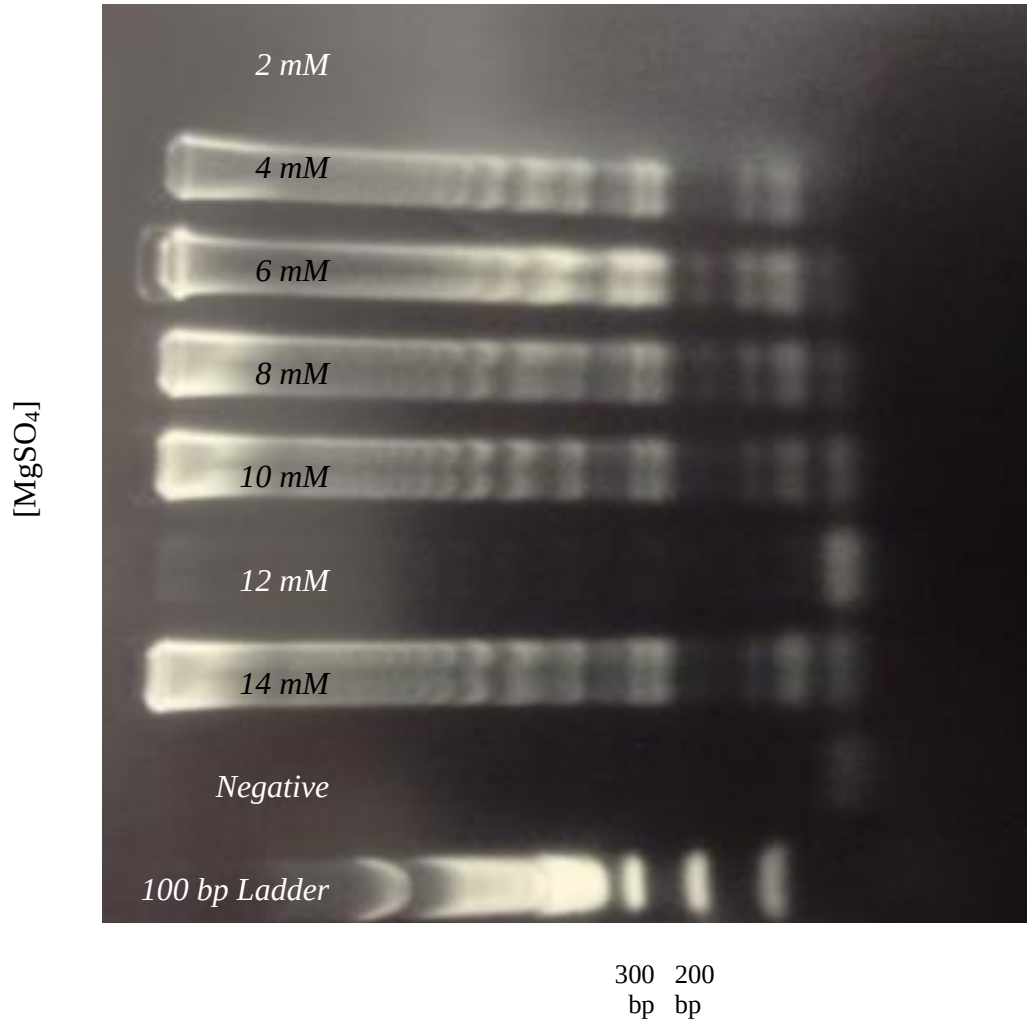
At this point, the upper strand dissociates because it is a loop structure and another FIP comes in and binds the same place it would have in the upper picture on this page. Amplifying on both sides from that FIP binding yields a structure that has two copies of the target double stranded.

It looks like:



And from here you can see how these strands dissociated and the same process continues ultimately creating long chains of target linked together by F1 F2 F1c or B1 B2 B1c.

Appendix 3: New version of Fig. 5



A new gel electrophoresis of (Fig 5). This show the exact same conclusions as the gel presented earlier in the paper. On this gel, the lane marked 6mM appears to be florescing more than the concentrations flanking it. However, this is not a reliable measure, so the only definitive statement that can be made is that as long as the concentration of $MgSO_4$ is above the threshold value (between 2 mM and 4 mM) the reaction can occur.