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Developing a transfection method for Didymium iridis

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Developing a transfection method for *Didymium iridis*

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ABSTRACT

The plasmodial slime mold and member of Mycetozoa, *Didymium iridis,* has been studied in a variety of contexts such as RNA editing (Traphagen et al., 2010; Hendrickson and Silliker, 2010a; Hendrickson and Silliker, 2010b), mitochondrial inheritance (Silliker et al., 2002), biological speciation (Betterley and Collins, 1983; Clark et al., 1991) and mating competency (Shipley and Holt, 1982). Further studies are hindered by the lack of a transfection protocol, which would allow for gene manipulation in *D. iridis* (hereafter Didymium). Transfection methods developed in the related slime molds, *Physarum polycephalum* (Burland et al., 1993) and *Dictyostelium discoideum* (Fey et al., 1995; Pang et al., 1999), have only been successful when native regulatory sequences were used. This study tested whether Didymium could recognize vectors with regulatory elements (a promoter and terminator) from related slime molds, and whether vectors with regulatory elements native to Didymium would be recognized and expressed when introduced by standard transfection methods.

We constructed vectors using overlap extension PCR and the Gibson Assembly. These vectors were comprised of a cloning vector backbone, regulatory elements from close relatives of Didymium or Didymium, and a green fluorescent - reporter gene *gfp*. Four plasmids were introduced into Pan 2-16 amoebae; pDicty, pPhys, pDidy 1.0 and pDidy 2.0, by means of electroporation, lipofection, and XFECT transfection. Expression of the reporter gene, *gfp,* from these constructs was observed by fluorescence microscopy. Though *gfp* expression was observed with Didymium and Physarum based vectors, the number of transformants by any transfection method employed was extremely low. However, for each method of transfection, there was consistency in the parameters that worked, even with different constructs.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i
ABSTRACT iii
TABLE OF CONTENTS iv
INTRODUCTION 1
REVIEW OF LITERATURE 4
Organism of interest: *Didymium iridis* 4
Transfection: concept and technique 7
Transfection in closely related slime molds 8
Physarum 8
Dictyostelium 11
Development of native regulatory elements: Profilin A 15
Transfection methods 16
Experimental Design 17
METHODOLOGY 18
Pan 2-16 cultivation and growth curves 18
Polymerase chain reaction (PCR) of fragments 18
Gel electrophoresis, staining and UV imaging 23
Vectors and fragments 23
Overlap extension “stitching” PCR 25
TOPO Cloning 27
Gibson Assembly 27
Clone sequencing 28
Sequence Analysis: Sequencher and BLAST verification 28
Preparation of plasmid DNA for transfection 29
Transfection techniques overview 29
Electroporation: Parameter adjusted ranges, cell handling, and sampling 30
FUGENE: Parameter adjusted-ranges, cell handling, and sampling 32
XFECT: Parameters adjusted-ranges, cell handling and sampling 33
Imaging: Phase-Contrast and Fluorescence 34
RESULTS 36
Overview of design and construction of pDidy plasmids 36
Constructing pDidy 1.0 37

Stitched fragments 39
Construct map 40
*Hind*III restriction digest verification 42
Source and experimental sequence comparisons of pDidy 1.0 43
Constructing pDidy 2.0 44
Stitched fragments 45
Construct map 47
*Pst*I and *Hind*III double restriction digest of pDidy 2.0 48
Source and experimental sequence comparisons of pDidy 2.0 49
Overview of construction of pPhys 51
Construct map 54
*Eco*R1 restriction digest of pPhys 56
Source and experimental sequence comparisons of pPhys 57
Summary of plasmids and features 59
Baseline Pan 2-16 observations 60
Electroporation 62
Electroporation parameters testing 62
Electroporation: fluorescence observations 66
Lipofection with FuGENE HD 70
Lipofection parameter testing 70
Lipofection (FUGENE) fluorescence observations 71
XFECT Polymer Transfection 75
XFECT parameter testing 75
XFECT polymer fluorescence observations 76
Comparison of transfection methods 80
DISCUSSION 81
LITERATURE CITED 84
APPENDICES 89
Appendix A: pPhys sequence 89
Appendix B: pDidy 1.0 sequence 92
Appendix C: pDidy 2.0 sequence 94

INTRODUCTION

Didymium belongs to the phylum Mycetozoa within the class Myxomycetes (Olive, 1975). The Mycetozoa group is composed of three classes of slime molds called the Protostelids, Dictyostelids and the Myxogastrids (Myxomycetes). The phylum Mycetozoa houses two close relatives of Didymium called *Physarum polycephalum* (from the Myxomycetes) and *Dictyostelium discoideum* (from the Dictyostelid), and will be referred to as Physarum and Dictyostelium from here after, respectively. A shared characteristic between these slime molds is the state of being a free living amoeba in nature, with the only difference being that the myxomycetes can further develop into multinucleated plasmodia with thousands of mitochondria and nuclei (Collins and Betterley, 1982**)**. On the other hand, as a cellular slime mold, Dictyostelium lives a majority of its life as individual cells that are able to communicate with one another through cell signaling to cooperatively form an asexual fruiting body (Loomis, 2014).

Physarum and Dictyostelium are both model organisms in their respective classes used to study aspects of slime mold biology such as cell motility, cell signaling and the lifecycle progression. Additionally, the mitochondrial genomes of Physarum (Takano et al., 2001) and Dictyostelium have been sequenced **(**Echinger et al., 2005). Studies on Didymium have focused on ecological aspects such as mating competency (Shipley and Holt, 1982) and biological speciation (Collins and Betterley, 1983; Clark et al., 1991). Previous molecular research shows that Didymium exhibits unique mitochondrial inheritance patterns (Silliker et al., 2002) as well as complex RNA editing (Traphagen et al. 2010; Hendrickson and Silliker, 2010a; Hendrickson and Silliker, 2010b).

In order to further study Didymium, we need to be able to modify and introduce genes into Didymium. In various cell types, this is achieved by shuttling in exogenous DNA into the cell by manipulation of the cell membrane through various transfection methods. Transfection has not been previously studied in Didymium, however it has been successful in its close relatives; Physarum and Dictyostelium. Previous studies with Physarum and Dictyostelium indicate that native regulatory elements (e.g. promoters and terminators) are needed in order for the host cell to recognize the vector. Promoters are regions of DNA that initiate transcription, while terminators are regions of DNA that terminate transcription. This approach has been successful and routinely used in Dictyostelium and with lesser success in Physarum.

The purpose of this study was to create recognizable genetic constructs that could be introduced into Didymium cells using some conventional transfection methods as well as a newer method. In this study, individual vectors with a reporter gene (*gfp*) flanked by a promoter and terminator native to Didymium, Physarum and Dictyostelium were designed and constructed to be transfected into Didymium cells by electroporation, lipofection and XFECT polymer transfection. Transfected cells were then observed using fluorescence microscopy to detect the presence of GFP. It was predicted that based on how closely related Physarum and Dictyostelium are to Didymium, constructs containing regulatory elements from those close relatives would be recognized in Didymium amoebae. It was also predicted that the construct containing the Physarum regulatory elements constructed with *gfp* would be more readily recognized since it belongs to the same myxomycete class as Didymium does.

Using the polymerase chain reaction (PCR), regulatory elements (promoter and terminator) were amplified from Physarum and Didymium clones. In combination with the reporter gene *gfp*, regulatory elements were “stitched” together with *gfp* using overlap extension PCR. This long

[promoter-*gfp*-terminator] fragment of DNA was then cloned using a TOPO cloning vector and the Gibson Assembly. Constructs were then introduced into Didymium Pan 2-16 amoebae using electroporation, lipofection and XFECT polymer transfection methods. The basis of these experiments was to develop a method for transient gene expression in Didymium using successful techniques from close relatives as described in previous research.

REVIEW OF LITERATURE

Organism of interest: Didymium iridis

Didymium is classified in the kingdom Amoebozoa within the phylum of Mycetozoa. It is within the Mycetozoa phylum that the class of Myxomycetes, to which Didymium belongs, is housed. Myxomycetes are commonly referred to as plasmodial slime molds which describes the defining characteristic for this class. Plasmodia are wall-less, cytoplasmic streaming, multinucleate free living single celled organisms that can span anywhere from hundreds of microns to a number of centimeters across (Clark and Haskins, 2015). Plasmodia are able to feed on bacteria, fungi, and essentially any organism smaller than it. A unique attribute of the myxomycetes is a life cycle composed of a haploid amoebal stage and a multinucleated diploid stage as seen in figure 1 (Alexopoulos et al., 1996).

The life stages of Didymium are influenced by environmental conditions such as food availability and presence of water (Wang et al., 2017). Didymium spores give rise to amoebae which can be flagellated (myxoflagellate) or amoeboid in shape (myxoamoebae). As cell density increases, a population threshold is reached causing sexually compatible cells to become competent to mate and undergo syngamy (Collins and Betterley, 1982). During syngamy cytoplasm and nuclei fuse to form a diploid zygote. After rounds of nuclear and mitochondrial divisions, a one celled cytoplasmic mass forms with thousands of nuclei and mitochondria, this is called a plasmodium (Collins, 1976). The plasmodium is able to move freely engulfing food in the form of microorganisms, yeast and bacteria. Under unfavorable conditions such as dry conditions and food depletion, a plasmodia will undergo meiosis and sporulate.

Initially, Didymium was classified as a fungus based on its spore producing capabilities, however due to its unique plasmodial cell structure and ability to consume organisms smaller than itself through phagocytosis, it was reclassified (Collins and Betterley, 1982). As a member of the

Myxomycetes class, Didymium is more closely related to animals and fungi than to plants (Baldauf, 2003). Didymium can be found growing widely in temperate and tropical regions earning it the title of a cosmopolitan species (Collins and Betterley, 1982). Specific places where it can be found growing are on leaves, soil and decaying logs and bark (Olive, 1975).

The availability of a transfection method for Didymium could greatly extend genetic studies in this organism. Genetic research in Didymium has relied upon matings between sexually compatible cells. Early Didymium research involved studying biological speciation in isolates from different regions (Collins and Betterley, 1976; Clark et al., 1991). Worldwide surveys of Didymium strains reveal that morphologically identifiable Didymium isolates comprise a collection of mating strategies and cryptic species (Clark and Stephenson, 1990). This organism is referred to as a cosmopolitan species, which stems from Didymium being able to be found in virtually every habitat in the world (Shipley and Holt, 1982). Mitochondrial inheritance also has been studied in Didymium (Silliker and Collins, 1988). Inheritance is largely uniparental, but the specific patterns are complex and even environmentally determined (Silliker et al., 2002). Mitochondrial sequences in Didymium has revealed RNA nucleotide editing events that shows similarities and differences with Physarum (Traphagen et al. 2010; Hendrickson and Silliker, 2010a).

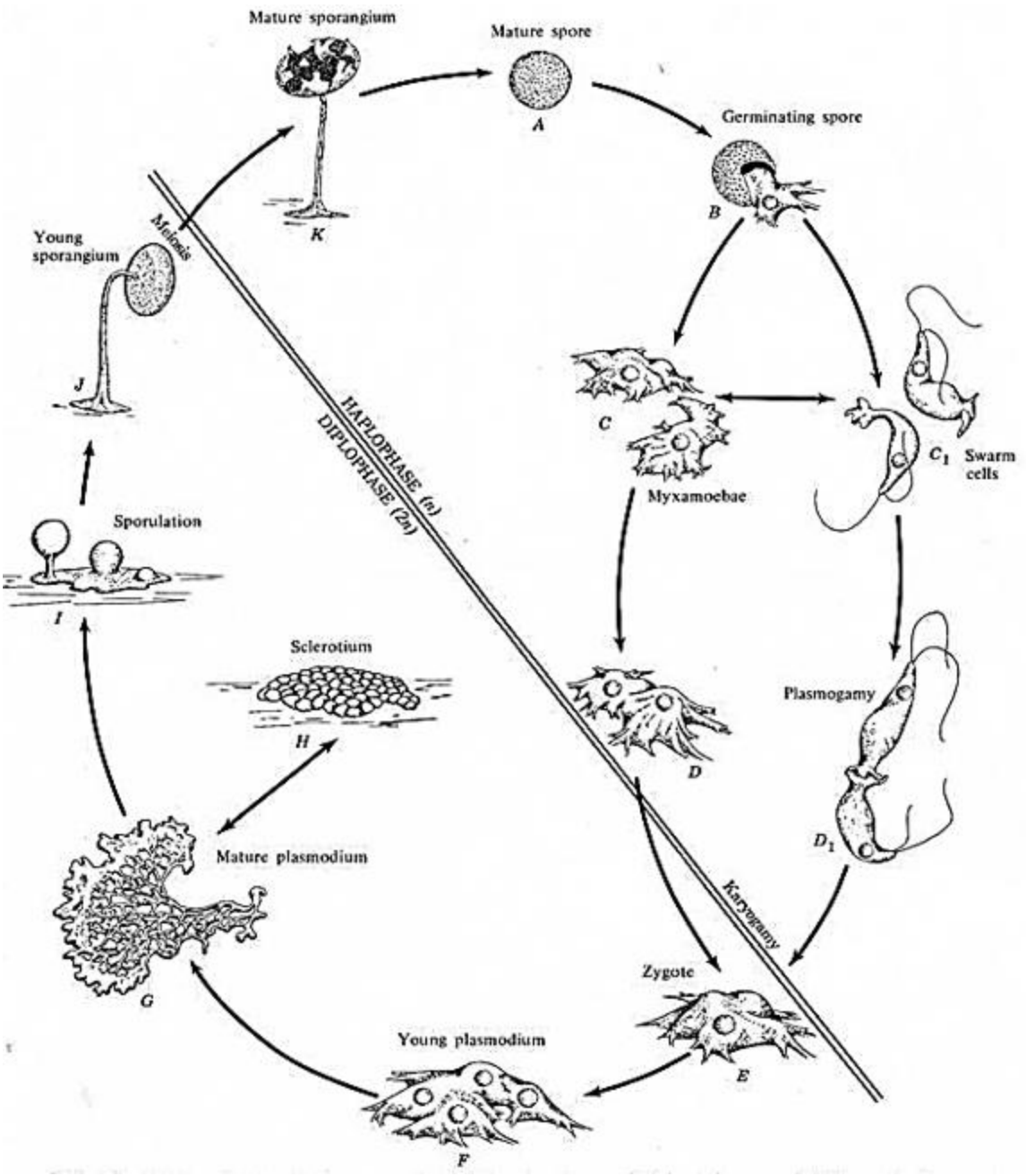


Figure 1. Myxomycete lifecycle. The lifecycle of a heterothallic myxomycete includes free-living haploid and diploid stages; the plasmodial stage is unique to this group of organisms. -From Introductory Mycology 1996 C.J. Alexopoulos, C. W. Mims, and M. Blackwell.

Transfection: concept and technique

Transfection is the deliberate introduction of genetic material into a eukaryotic cell through different techniques (Sambrook and Russell, 2001). Transfection techniques are powerful tools that allow for further studying of eukaryotic organisms at the molecular level. In most cases, DNA or RNA is introduced into a eukaryotic cell with the goal of specific protein production using the natural molecular processes of the cell. In most cases, a DNA vector containing a selectable marker, reporter gene, origin of replication, and recognizable regulatory elements are transfected into eukaryotic cells.

There are a variety of conventional transfection methods that are commonly used. Electroporation makes use of electrical fields to punch holes in a eukaryotic cell's membrane. Electroporation is efficient in getting genetic material into a cell relatively fast, but the shock can be harsh and may interfere with cell recovery. Unlike other transfection methods, electroporation requires optimization of multiple parameters for each cell type being treated. Lipofection, on the other hand, uses lipids to encase DNA vectors that are then introduced into the cell through lipid to lipid merging with the membrane. Using lipofection to introduce vectors into eukaryotic cells is fast, can lead to a fast recovery, but can be expensive. The high expense leads to a decrease in opportunities to optimize for each cell type. Calcium phosphate precipitation is another method that uses calcium chloride and a phosphate buffer in combination to coat DNA which then is attracted to the cell membrane and introduced into the eukaryotic cell through endocytosis. This method is relatively easy to use, but depending on the reagents being used it can be time consuming as well as being toxic at high concentrations. Since the method deals with phosphate precipitation, minor changes in pH can drastically alter the precipitation and transfection efficiency making it difficult to achieve consistent results.

Other transfection methods that are less conventional include: viral mediated transfection, nanoparticle and polymer transfection, cationic-mediated lipid transfection, gene gun, and direct injection. The best transfection methods are those that are relatively easy to perform consistently and efficiently. The phylum mycetozoa houses two model organisms: Physarum (plasmodial slime molds) and Dictyostelium (cellular slime molds) that have established transfection methods successfully used in furthering research for their respective classes.

Transfection in closely related slime molds

Physarum

Belonging to the same myxomycete class as Didymium, Physarum shares the same haploid to diploid life cycle as Didymium (Alexopoulos et al., 1996) (See Figure 1: Myxomycete life cycle). Like Didymium, the most conspicuous stage that characterizes Physarum is the one celled multinucleate mass of protoplasm, the plasmodium. When fully developed, plasmodia are able to internally move nutrients around in their endoplasm through a process called cytoplasmic streaming (Gotoh and Kuroda, 1982). As a result of this, it allows for the movement and crawling of the plasmodia. Apart from its overall biology, at the molecular level, the genome has been sequenced and transcriptomes have been characterized identifying loci, receptors and potential signaling mechanisms which have allowed for comparisons with other eukaryotic organisms (Schaap et al., 2015).

In addition to similar molecular mechanisms in the two myxomycetes, the life cycle and natural behavior are almost identical between the two slime molds. Given these similarities, Physarum biology serves as a starting point for understanding the biology of Didymium. The phylogenetic divergence of Physarum and Didymium has been reported to have occurred some 400 to 500 million years ago as supported by ribosomal large subunit (LSU) RNA (rRNA) and

telomeric region size comparisons against other multicellular eukaryotes (Johansen et al., 1992). Unlike Didymium, Physarum has developed transfection methodologies that have furthered the field of myxomycete research.

In Physarum transfection studies, constitutive gene promoters and terminators have been used to drive the expression of a gene of interest. The type of genes that are easiest to observe are those that are always turned on and are abundantly expressed in Physarum such as an actin gene family called *ardA, ardB, ardC* and *ardD.* This family of actin genes composes a majority of Physarum actin that is found in the amoebal and plasmodial stage (Hamelin et al. 1988). Plasmids containing the promoter regions of the actin gene *ardC* have been able to be recognized when transfected into yeast conferring hygromycin resistance (Burland et al., 1991). This promoter also referred to as *PardC*, has been considered a great candidate as a driver of gene expression due to its prevalence and role in actin gene functionality. The same *PardC* promoter when linked to the hygromycin (*hph*) resistance gene, was used to successfully transfect and be expressed in host Physarum amoebae (Burland et al., 1993). Promoters from highly expressed genes, such as the actin genes, have been found to be particularly effective. This established Physarum regulatory elements as being genetically recognizable outside the host type and had potential to be versatile elements in gene expression. Additionally, they were used in the development of a transfection protocol for Physarum. In these transfection studies, the most common methods used have been electroporation and calcium phosphate precipitation.

Another transfection approach to Physarum was with the use of putative promoter regions of long terminal repeats "*Hpa*II-repeat" element linked to a bacterial chloramphenicol acetyltransferase (CAT) gene (Burland et al., 1992). This showed that taking a putative promoter from Physarum, fusing it with a bacterial gene, and then re-introducing it into Physarum was not

only possible, but was also recognizable and expressed. Physarum promoters have been observed to be recognized in yeast, which is an organism classified in a different kingdom. Promoters from highly expressed genes, such as the actin genes, have been found to be particularly effective. This established Physarum regulatory elements as being genetically recognizable outside the host type and had potential to be versatile elements in gene expression. Additionally, they were used in the development of a transfection protocol for Physarum.

Transient and stable expression have both been shown to be possible in Physarum. Stable expression through integration was possible in Physarum by introducing linearized plasmids. For example, one Physarum study took mutated variants of an actin gene in the form of linearized plasmid and electroporated these constructs to induce homologous gene replacement (Burland and Pallotta**,** 1995). The plasmid transfected into the Physarum cells in this study contained mutant alleles for four isocoding genes which compose 83% of the actin present in both the amoeba and plasmodium phases of Physarum (Burland and Pallotta**,** 1995). From the few transfection methods used to study Physarum the most successful one has been electroporation.

Comparing transfection studies in Physarum, the optimal voltage used in electroporation ranges from 0.8kV to 1.0 kV. Resistance parameters that have worked best were between 800 and 1000Ω, as well as having a consistent capacitance of 25μF (Burland et al., 1993; Burland and Bailey, 1995; Burland and Pallotta, 1995). Different electroporation volumes and cell densities have varied from study to study, however the recovery time and expression window in Physarum amoebae has remained consistent. Burland et al., 1993; Burland and Bailey, 1995; Burland and Pallotta, 1995, allowed Physarum cells to recover at 30℃ for 20 minutes post transfection. Following these protocols set for Physarum, antibiotic gene expression has generally been observed 5 to 7 days post transfection on agar plates. Expression of reporter genes, such as the

luciferase gene, has been noted to be present as early as 2 to 10 hours post transfection, with expression levels decreasing steadily over the next 20 hours (Bailey et al., 1994).

Didymium and Physarum share similarities in their life cycle, development, and genetics. Given these similarities, there was the potential for didymium to recognize Physarum regulatory elements if they managed to get into a cell. Additionally, since Physarum has been successfully transfected, a similar approach might also work in Didymium amoebae. Numerous studies using electroporation have been done on Physarum as it is the most accessible and simplest way to introduce exogenous DNA into cells. Lipofection as a transfection method has not been extensively used with Physarum or other myxomycetes. Conceptually speaking, myxomycetes cell membranes are composed of the same material as general eukaryotic cells, therefore, lipofection could be a good candidate to introduce DNA into Didymium cells. Although calcium phosphate precipitation has been used to transfect Physarum, results were inconsistent due to its sensitivity to pH. Since the calcium phosphate precipitation method is dependent on pH in order to create a precipitate to be placed onto cells, the variation in pH in the growth media may alter the precipitation step. Calcium phosphate in excess could also inhibit and promote cytotoxicity thereby killing off cells prematurely. Calcium has been observed to affect the growth and development of Physarum (Terry et al., 2009). For these reasons, we did not pursue this method in Didymium. The other Mycetozoan where there has been success in the development of a transfection protocol is Dictyostelium.

Dictyostelium

Dictyostelium discoideum is a cellular slime mold that belongs to the class of Dictyostelia. Dictyostelium can be found in forest soil, decaying wood and moribund plant structures (Olive, 1975). A unique characteristic of this slime mold is the ability to altruistically communicate with

other amoebae in order to aggregate into a slug like structure that functions as one unit. This "slug" is then able to undergo sorogenesis, the development of both a stalk and sorus, which then leads to reproduction by spore dispersal as described by Olive (1975). For cellular eukaryotes and cellular slime molds in particular, Dictyostelium has been considered the model organism. A database called DictyBase (www.DictyBase.org) has archival studies of Dictyostelium since 2003 (Basu et al., 2015). Aspects of Dictyostelium that have been extensively studied include genome, cell cycle, cell motility and cellular communication (Urushihara, 2009).

Genome mapping and sequencing efforts in Dictyostelium have led to the identification of signaling receptors, transporters, A-T rich sequence content and the identification of the number of chromosomes present (Eichinger et al., 2005). Restriction enzyme mediated integration (REMI) of linearized plasmids containing unique restriction sites have been used to regionally map six distinct chromosomes of Dictyostelium (Loomis et al., 1995). These findings and research were all possible as a result of conventional transfection methods being available for Dictyostelium (Lloyd et al., 1990; Fey et al., 1995; Gaudet et al., 2007).

The three most common methods of transfection used in Dictyostelium have been electroporation, calcium phosphate precipitation, and lipofection. Hygromycin was used as a selectable marker in plasmids that were introduced into the Ax4 strain of Dictyostelium using electroporation (Egelhoff et al., 1989). Some observations noted in transfection studies in Dictyostelium were the differences in expression of GFP due to the method transfection and antibiotic resistance cassette introduced into the cells. High copy number vectors and ones with different selectable markers have been shown to influence the expression of the reporter gene *gfp* (Pang et al., 1999). This was the first instance of genetic elements other than promoters affecting plasmid success and gene expression in cellular slime molds.

Like the Physarum studies, Dictyostelium gene expression studies make use of promoters that originally were associated with structural proteins such as actin. One promoter typically used in Dictyostelium transfection studies originates from the actin genes: *act*15 and *act*6. One of the earliest mentions of a developed transient expression system for Dictyostelium was reported using an *act*15 promoter coupled to a firefly luciferase gene within a vector that was electroporated or calcium phosphate precipitated into amoebae with successful results (Howard et al., 1988). In another transfection study using the same protocol design, the expression of *gfp* driven by the actin 15 promoter was successfully recognized not only in Dictyostelium, but also in another Dictyostelid called *Polysphondylium pallidum* when transfected by electroporation (Fey et al., 1995). This study in particular shows promoter recognition across different genera within the same Dictyosteliidae family of slime molds. Apart from vector recognition in Dictyostelium, the level of expression from a construct varies based on what is present on the vector and the method used to introduce the vector into a host cell.

The method of vector introduction into Dictyostelium cells has been noted to vary the level and type of expression of the selectable marker. Electroporation for instance has been observed to support single integration events into genomic DNA, while calcium phosphate precipitation has worked best for overexpression experiments due to the ability to introduce a high copy number of constructs (Gaudet et al., 2007). Electroporation and calcium phosphate precipitation have been the most effective transfection methods used to date. There have been studies reported that have used cationic lipid transfections called lipofection that have been successful in Dictyostelium, albeit rare (Lloyd et al., 1990). Comparing the methods of vector introduction, electroporation and calcium phosphate precipitation had low to no expression while lipofection (lipofectin reagent) had very low efficiency (Lloyd et al. 1990).

The transfection parameters that have generally been used for Dictyostelium, electroporation appears to be more varied depending on the study. In one example, voltages of 4.25 to 4.75 kV at 100µF as well as linearized and supercoiled vectors were used. They determined that increasing the amount of DNA used to electroporated with (e.g. above 20 µg per 0.8 mL) led to an increase in cell death (Howard et al., 1988). A decreased level of voltage of 1.2 kV at 3µF was successfully used by Egelhoff et al., 1989. Fey et al., 1995 had success at an even lower voltage of 0.8 kV at 3µF with a time constant of 0.8 to 1 ms with 10 to 20 µg of vector DNA. DictyBase.org states the optimal voltage set for electroporation of Dictyostelium is 0.85 kV at 25µF for two pulses (separated by a 5 second delay) with a 0.6 ms time constant (Gaudet et al., 2007). This appears to be the most commonly used method. Comparing all the electroporation methods for Dictyostelium, a majority of the methods include washing steps prior to electroporation. Washing amoebal cells prior to electroporation washes away growth media that amoebae might have on their membranes which may cause arcing within the cuvette causing massive cell death (Egelhoff et al., 1989). Apart from electroporation, lipofection has also been successfully performed in Dictyostelium. Lipofection in general has very straightforward approach since it is used for a range of eukaryotic cell types. Dictyostelium amoebae were grown, pelleted, isolated, and have had 10µg of vector DNA, water and lipofection reagent (lipofectin) introduced onto Dictyostelium cells dropwise while growing on plates with bacterial lawns (Lloyd et al., 1990). Following the successful parameters that have been used for both electroporation and lipofection in Dictyostelium, these transfection methods were tested in Didymium. Though Dictyostelium is not as closely related to Didymium as Physarum, the Dictyostelid class neighbors the Myxomycetes, allowing the potential for genes and regulatory elements to be recognized between Didymium and Dictyostelium.

Development of native regulatory elements: Profilin A

To develop an expression vector with regulatory elements native to Didymium, we focused on a gene previously cloned in our lab, profilin A. Profilins are actin binding proteins that aid in mobility and provide structural integrity to the cytoskeleton (Krishnan and Moens, 2009). Profilins are ubiquitous with different isoforms performing the same duties; they vary by small structural differences. For example, in the cellular slime mold Dictyostelium, profilin isoforms have been observed to compensate for one and another when the functionality of one is artificially decreased (Haugwitz et al., 1994). Playing a critical component in the internal structural integrity of a cell they are highly conserved and likely to be recognized across broad taxonomic classes.

There are two profilin types that have been identified in the myxomycetes; they share sequence similarities with other living organisms such as yeast, mice and humans (Binette et al. 1990). Versions of profilin A can be found in the amoebal and spore phases of both Physarum and Didymium, while versions of profilin P can be found in the plasmodial stage. The "A" in profilin A stands for Amoeba and the "P" in profilin P stands for plasmodia. Similar to the use of actin gene regulatory elements in Physarum transfections, we proposed to co-opt Didymium profilin A regulatory elements to drive reporter genes in Didymium.

In Physarum the promoter *PardC* of the *ardC* actin gene was also thought to contain an origin of replication, so the promoter acts both as a replicator and promoter in the transcription of genes (Pierron et al., 1999). The ardC gene has a terminator *TardC* which terminates the transcription of the gene. It seems plausible that the regulatory elements of this highly conserved Physarum gene could be recognized in Didymium. Both the *PardC* and *TardC* regulatory elements were cloned in a plasmid pTB41 (Burland et al., 1993). In our lab a 2.1 kb repeat sequence downstream of *ardC* was removed to create the plasmid pCN1 that was used as a source in this project.

Similarly, the promoter A15P from the Dictyostelium actin gene was fused with a luciferase gene to create an expression vector (Howard et al., 1988). Subsequently the actin 15 promoter was fused to *gfp* that was also used in this project, referred to as pDH-GFPABD120, or pDHygGFP (Pang et al., 1999). This construct contains an actin terminator (2H3-T). We tested whether this Dictyostelium construct would be recognized and expressed in Didymium.

Transfection methods

The transfection methods considered for this project are electroporation, lipofection (FuGENE HD) and polymer (XFECT) transfection. All transfection methods generally have the same end goal of introduction of exogenous DNA vectors into eukaryotic cells, with the only difference being the method by which it is achieved. The goal for this project was to see if constructs containing regulatory elements from closely related slime molds would be recognized when introduced into Didymium amoebae using three types of transfection methods. Two of these methods had been performed in other slime molds while none of the transfection methods had ever been tried in Didymium. Electroporation has been a simple, cheap and effective method of transfection in both Physarum and Dictyostelium. Electroporation was our initial focus in developing a transfection method in Didymium.

Although lipofection has mainly been used in Dictyostelium and there are only a few studies, it is worth testing in Didymium. The FuGENE lipofection reagent uses lipids to surround a genetic construct, which then allows for the lipid enclosed genetic construct to bind and merge into the cell membrane. Lastly, the XFECT polymer transfection method is a newer method not yet tried in myxomycetes. Like lipofection, the XFECT polymer transfection method uses the method of encasing plasmid DNA to be introduced into the cell. On the other hand, XFECT

polymer transfection uses polymers that complex and surround the DNA allowing for introduction into the cells by endocytosis.

Experimental Design

The goal of this research was to develop a vector and transfection method to facilitate gene manipulation in Didymium. This was accomplished by designing and constructing vectors that possessed a *gfp* reporter gene driven by promoters and terminators of close relatives of Didymium and Didymium that were then transfected into Didymium Pan 2-16 cells by electroporation, lipofection and XFECT polymer transfection. A Dictyostelium vector called pDH-GFPABD120 (Pang et al., 1999; DictyBase, http://dictybase.org/) that contained a *gfp* gene was tested in Didymium. Constructs with Didymium regulatory elements were made by amplifying promoter and terminator of the profilin A gene stitching it by overlap extension PCR to flank a *gfp* gene. This was repeated for another variant of *gfp* called *maxgfp* to make a second Didymium construct. The stitched fragment was then incorporated into a pUC19L vector through the Gibson Assembly. A similar overlap extension PCR approach was performed for the Physarum construct to stitch together *PardC* promoter and *TardC* terminator to *maxgfp*. The stitched fragment was cloned into a pCR2.1 TOPO vector. Transfection methods were tested in combination with the vectors after standardization of the transfection protocols. Post transfection observations were taken in 5 and 10 hour increments for 2-3 days to detect transient *gfp* expression by fluorescence microscopy.

METHODOLOGY

Pan 2-16 cultivation and growth curves

The Didymium Pan 2-16 strain is from the Central American Series tester clones isolated by Dr. O'Neal Ray Collins (Betterley and Collins, 1983). Pan refers to the source material (banana peel) being from Panama. Depending on the transfection method being performed, Pan 2-16 cells were grown in either 500 mL Erlenmeyer flasks in 125 mL of peptone-glucose-yeast medium (PGY) or culture tubes with 7 mL of PGY, supplemented with appropriately scaled volumes of heat-killed bacteria (HKB) as described by (Silliker et al., 1988**)**. *E.coli* was used in the preparation of HKB. Pan 2-16 cell counts were taken to adjust the inoculation volume to a starting concentration of $1.0x10^4$ cells/mL. Erlenmeyer flasks were placed onto a New Brunswick Scientific Shaker C10 platform shaker to shake for 3-4 days at 175 rpm and 23℃. Culture tubes were placed on a Lab Line Cell Gro rotator to grow at 23℃ between 3-4 days. After shaking for the allotted time, cell counts were taken to verify cell concentration and to adjust the concentration for the transfection method.

Growth curves were determined to gauge the timing duration of the exponential phase. The Pan 2-16 cells prepared for the lipofection and XFECT transfection methods were grown in 15 mL polypropylene conical tubes with 7 mL of PGY with 200 µL of HKB suspension. Growth curves were started at concentrations of $1.0 – 4.0x10^4$ cells/mL. Growth tubes were monitored every 24 hours for 7 days.

Polymerase chain reaction (PCR) of fragments

Primers were designed using Primer Dimer v.2.0 (Scientific & Educational Software, 1990). The lower annealing temperature of any primer pair was used as the annealing temperature for the PCR amplification profile. Primers used in PCR reactions were at a final concentration of

0.64 µM when mixed with dH_2O, 10 – 50 ng of DNA template and a MidSci Taq Plus Master Mix protocol recommended concentration in a final reaction volumes of 25 and 50 µL. A general PCR profile consisted of an initial denaturation step at 94℃ for 3:00 minutes followed by 30 cycles of [94℃ for 0:30 sec; T_{anneal} for 0:30 sec; 72℃ for 30 sec] followed by a final extension at 72℃ for 7 minutes and a cold hold at 4℃. A one minute extension time was used for every 1,000 base pairs amplified.

Tables I, II, and III list PCR fragments amplified by various primer pairs, their annealing temperatures, and sizes. These were used to construct: pDidy 1.0, pDidy 2.0 and pPhys. Profilin A fragments were amplified from previously cloned Didymium profilin A gene segments in clones ProA-R-2 (promoter) and ProA-F-1 (terminator). Physarum regulatory elements, promoter (*PardC)* and terminator (*TardC*) were amplified from pCN1C-1 derived from pTB41 (Burland et al., 1993). For fragments that were a bit more difficult to amplify due to nonspecific primer annealing, DNA fragments were gel purified using a Zymo Gel DNA Recovery Kit (Zymo Research, 2018b).

Table I: pDidy 1.0 PCR fragments, primer sequences, T_{anneal} (℃), and product sizes

DNA fragment	Primers	Sequence	T_{anneal} (℃)	Product size (bp)
ProAup	ProAup FW	5’-CTC GGT ACA AAT TGA CCC AAA GGT AAC TTT CA-3’	52.3	317
	ProAup RV	5’-CTA GCC ATC TAT AGT GAT TAA AGG ATG AGT-3’	52.2	
ProAup EXT	ProAup FW EXT	5’-AAT TCG AGC TCG GTA CAA ATT GAC-3’	55.6	325
	ProAup RV	5’-CTA GCC ATC TAT AGT GAT TAA AGG ATG AGT-3’	52.2	
ProAdown	ProAdown FW	5’-ACA AAT AAA TTA TTG TCT ATT TAG TAA TAA TTT CTG-3’	51.2	268
	ProAdown RV	5’-TGC ATG CCA AAA AGT CTT TTT TTA TTA TTA TTT ATG AT-3’	51.2	
ProAdown EXT	ProAdown FW	5’-ACA AAT AAA TTA TTG TCT ATT TAG TAA TAA TTT CTG-3’	51.2	282
	ProAdown RV EXT	5’-GAT TAC GCC AAG CTT GCA TGC CA-3’	60.8	
gfp	GFP FW	5’-CAC TAT AGA TGG CTA GCA AAG GAG-3’	50.1	736
	GFP RV	5’-ACA ATA ATT TAT TTG TAG AGC TCA TCC AT-3’	50.1	

Table II: pDidy 2.0 PCR fragments, primers sequences, T_{anneal} (°C), and product sizes

DNA fragment	Primers	Sequence	T_{anneal} (°C)	Product size (bp)
ProAup	ProAup FW	5'-CTC GGT ACA AAT TGA CCC AAA GGT AAC TTT CA-3'	52.3	323
	ProAup2.2 RV	5'-TTC ATG GCG GGC ATC TAT AGT GAT TAA AGG ATG AG-3'	62.0	
ProAup EXT	ProAup FW EXT	5'-AAT TCG AGC TCG GTA CAA ATT GAC-3'	55.6	331
	ProAup2.2 RV	5'-TTC ATG GCG GGC ATC TAT AGT GAT TAA AGG ATG AG-3'	62.0	
ProAdown	ProAdown2.1 FW	5'-GA G CTC GAT GAA TTA TTG TCT ATT TAG T-3'	53.2	270
	ProAdown RV	5'-TGC ATG CCA AAA AGT CTT TTT TTA TTA TTA TTT ATG AT-3'	51.2	
ProAdown EXT	ProAdown2.1 FW	5'-GA G CTC GAT GAA TTA TTG TCT ATT TAG T-3'	53.2	276
	ProAdown RV EXT	5'-GAT TAC GCC AAG CTT GCA TGC CA-3'	60.8	
maxgfp	PAGFP FW	5'-TTT AAT CAC TAT AGA TGC CCG CCA TGA AGA TCG AG-3'	61.9	719
	GFPTA RV	5'-ATT TAC TAA ATA GAC AAT AAT TCA TCG AGC-3'	52.3	

Table III: pPhys PCR fragments, primers sequences, T_{anneal} (°C), and product sizes

DNA fragment	Primers	Sequence	T_{anneal} (°C)	Product size (bp)
PardC	Pard C5 FW	5'-GAG CTC GGT ACG GAT CTC CAC ACT ATT-3'	61.1	1,122
	Pard C RV	5'-GGC ATA GCT TGA ACG TCT TCT CC-3'	57.8	
TardC	Tard C FW	5'-GAG CTC GAT GAA GTA GAT GCC GAC C-3'	60.8	994
	Tard C RV H	5'-GGT CTC GCG GTA GAC GTC GCA TGC TCC TCT AGA CT-3'	68.7	
TardC EXT	Tard C FW	5'-GAG CTC GAT GAA GTA GAT GCC GAC C-3'	60.8	1,007
	Tard C6 RV	5'-AAG CTT GCA TGC CGG GTC TC-3'	60.8	
maxgfp	pPhys GFP FW	5'-CGT TCA AGC TAT GCC CGC CAT GAA GAT CGA GTG CC-3'	68.1	709
	pPhys GFP2 RV	5'-GGT CGG CAT CTA CTT CAT CGA GC-3'	59.5	

Gel electrophoresis, staining and UV imaging

Amplified fragments of DNA were run on a 1% agarose gel made with Tris Acetate EDTA buffer (Sambrook and Russell, 2001). Gels were stained with ethidium bromide (EtBr) and imaged using a fluorchem HD2 UV imager.

Vectors and fragments

Constructs were designed to have both a promoter and terminator region from close relatives of Didymium and Didymium itself that would surround a reporter gene, *gfp*. The following is a summary of the vectors, fragments, and DNA sequences that were used to generate the constructs used in this study.

A Dictyostelium vector (pDH-GFP-ABD120, or pDHygGFP) (Pang et al., 1999) also referred to as pDicty in this study, was obtained from the DictyBase database (http://dictybase.org/). This plasmid has a hygromycin resistance cassette and *gfp* gene that are both flanked individually by an actin 15 gene promoter and terminator. The backbone cloning vector for pDicty was the pBluescript II KS. Figure 2 displays a map of pDicty with the genes and regulatory elements of Dictyostelium.

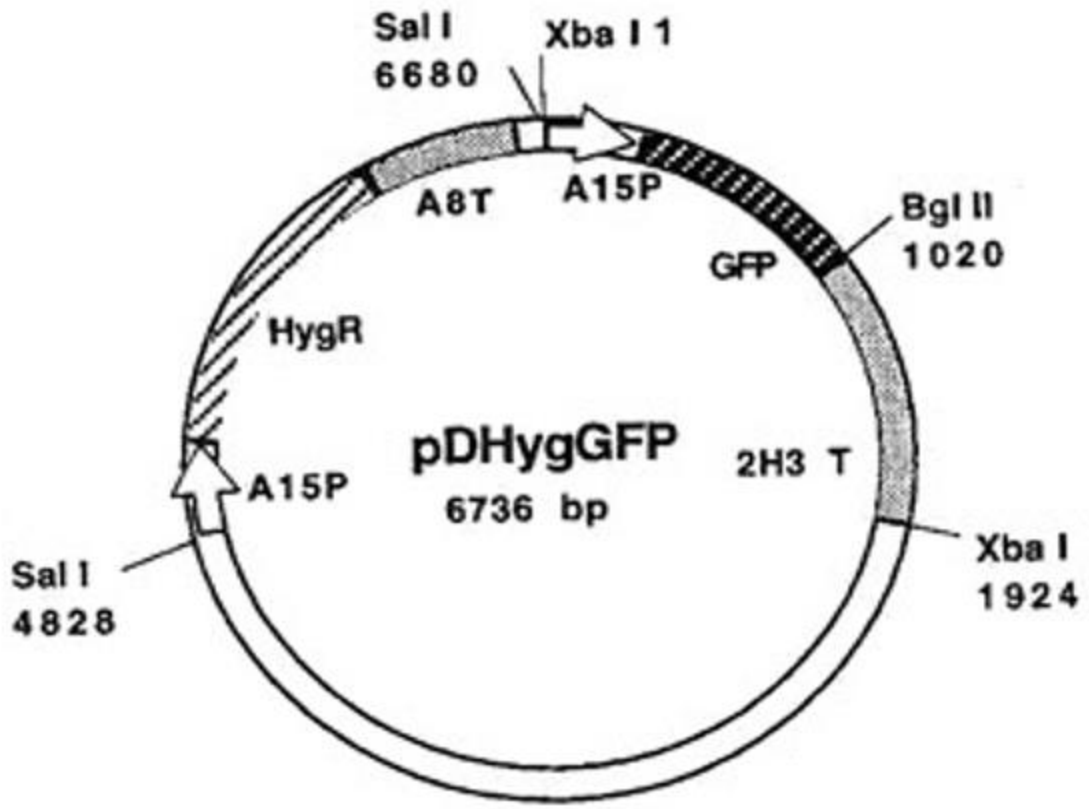


Figure 2. pDH-GFPABD120 (pDHygGFP). Referred to in this study as pDicty. The promoter A15P represents the actin 15 promoter from *D. discoideum*. The terminator used was the 2H3 terminator. Both regulatory elements originate from *D. discoideum* (Pang et al., 1999).

In order to generate a construct for Didymium with Didymium regulatory elements, we used pre-existing clones from our lab that had a profilin gene expressed in the amoebal stage of Didymium. Profilin is an actin binding protein that aids in mobility and provides structural integrity to the cytoskeleton. Profilin is an essential eukaryotic gene as well as being ubiquitous. Two profilin types occur in the myxomycete slime molds which share sequence similarities between other living organisms such as yeast, mice and humans (Binette et al., 1990). We selected regulatory elements from profilin A since it is a highly regulated gene in the amoebal

stage of Didymium and its regulatory elements were predicted to also be highly expressed. Two variants of *gfp* were used in Didymium constructs. In one construct, a standard *gfp* gene isolated from the plasmid pGLO (NCBI GenBank Accession #: U62637.1) was used, while in the second construct *maxgfp* was used from pMAXGFP (Amaxa Biosystems, 2018). A pUC19L (Gibson Assembly, Cat No. A13288) vector was used as the main cloning vector that would house the profilin A promoter, terminator and the variant of *gfp*.

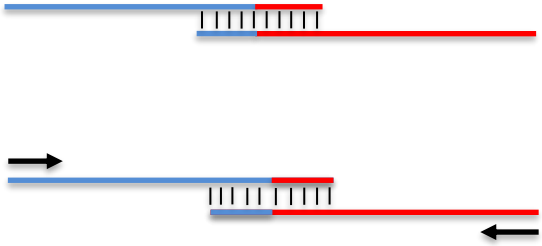
The Physarum construct was derived from the plasmid pTB41 (Burland et al., 1993) that contained a hygromycin resistance gene that was driven by the actin promoter and terminator. This pTB41 vector had a 2 kb section of a Physarum repeated sequence that was removed to create the pCN1 plasmid. From this pCN1 clone, we amplified both the promoter and terminator regions. The promoter, *PardC* of the *ardC* actin gene of Physarum, also functions as an origin of replication. Therefore, the promoter acts both as a replicator and promoter (Pierron et al., 1999).

Overlap extension "stitching" PCR

Overlap Extension PCR, or PCR "stitching", was used to join DNA fragments. This method uses the outer-most forward and reverse primers between both fragments to amplify the compound fragment joined internally by an overlapping sequence in common (Shevchuk et al., 2004 and Horton et al., 2013; see figure 3).

1. **PCR amplify individual fragments**

2. **Outer primers are used in PCR Stitching**



3. **Stitched DNA product**

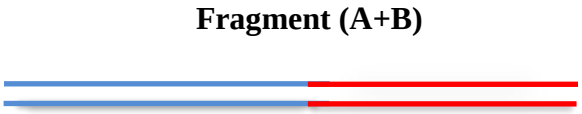


Figure 3. Overlap extension PCR (stitching). Primers containing compatible end regions to neighboring DNA sequence are used in PCR to add extensions into a neighboring DNA fragment or sequence. Two fragments with compatible ends can be stitched together using outside primers to prime and amplify inwards. The results is two separate DNA fragments becoming one continuous fragment.

TOPO Cloning

Stitched fragments were TOPO cloned into either a pCR2.1 or pCR4 cloning vector. Standard TOPO cloning (Invitrogen, 2018b and Invitrogen, 2018c) kit protocols were followed. TOPO cloning involves taking gel purified stitched products and mixing them with a salt solution and [1/5] of the recommended concentration of TOPO vector. The reaction was incubated at room temperature for 30 minutes. The newly formed vector was then mixed with competent TOP10 *E. coli* on ice for 30 minutes, and then heat shocked at 42℃ for 30 seconds. Transformed *E.coli* cells were allowed to recover with 250 µL of SOC media in an Amerex Instruments orbital incubator shaker for 1 hour. Cells were then plated onto lysogeny broth (Lennox), or LB, plates made with 100 µg/mL of ampicillin and allowed to grow for 18-24 hours for colony isolations.

Gibson Assembly

Following the GeneArt Seamless Plus Cloning and Assembly kit manual (Invitrogen, 2013), stitched fragments [ProAup-gfp-ProAdown] and [ProAup-*maxgfp*-ProAdown] were both mixed according to the kit protocol, with the provided PUC19L vector to generate a final construct. The Gibson Assembly was used to combine fragments with overlapping compatible ends into a pUC19L vector (Gibson et al., 2009). An insert to vector ratio of 2:1 was calculated when combining the pUC19L vector and fragments of interest. Once constructed, the plasmid was cloned into TOP10 competent Invitrogen *E.coli* cells using the kit transformation procedure (Invitrogen, 2013). Cells were then plated on LB plates with 100 µg/mL of ampicillin antibiotic and grown at 37℃ for colony isolations.

Overlap extension PCR was utilized for the creation of pDidy 1.0, pDidy 2.0 and pPhys. Promoters were stitched to the variant of the reporter gene, *gfp*, and then stitched as a larger fragment to the terminating sequence. The pDidy constructs were constructed in parallel and were

stitched and gel purified continuously until the large [ProAup-gfp-ProAdown] was generated and then cloned into a pUC19L vector. Unlike the pDidy constructs, the pPhys construct was generated by stitching together the *PardC* and *maxgfp* fragments and unexpectedly cloning this larger [*PardC- maxgfp*] fragment into a pUC19L vector. The *TardC* fragment was amplified and cloned into a pCR2.1 vector. These fragments, [*PardC-maxgfp*] and *TardC* were then stitched together using their respective outer primers; Pard C.5 FW and Tard C.6 RV. This generated the larger [*PardC-maxgfp-TardC*] (2796 bp) which was cloned into pCR2.1 as well.

Clone sequencing

Clones containing our genetic constructs were grown and isolated using a standard miniprep procedure (Ausubel et al., 1989). Isolated plasmid DNA was concentrated using a Zymo Clean kit (Zymo Research, 2018a**)** and resuspended in 20 µL of dH_2O. A standard sequencing reaction was performed using 150 ng of plasmid DNA template, 0.5 µL of a FW or RV primer (0.16 µM), 1.5 µL 5X sequencing buffer, 1.0 µL of BigDye Terminator Mix, and DH_2O up to a final volume of 10 µL (AppliedBiosystems, 2002). Samples were precipitated and suspended in 15 µL of Hi-Dye Formamide reagent prior to sequencing. Samples were run on the ABI Prism 310 DNA sequencer to obtain DNA sequences.

Sequence Analysis: Sequencher and BLAST verification

Sequence data was analyzed using Sequencher v.4.0 (Gene Codes Corporation, 1999). Sequences were screened to identify any base changes compared to the source material. The identity of the constructed plasmid was verified by sequencing the entire integrated fragment, which contained regulatory elements and the reporter gene *gfp* or *maxgfp*. The sequence data was uploaded to the National Center for Biotechnology Information (NCBI)

(http://www.ncbi.nlm.nih.gov/) nucleotide sequence database to compare with other sequences in the database.

Preparation of plasmid DNA for transfection

The plasmids pDicty, pPhys and pDidy were isolated and purified using either a Qiagen plasmid isolation midi kit (Qiagen, 2001) or an Invitrogen Pure Link Maxiprep kit (Invitrogen, 2018). Once isolated, the concentration of the samples were measured with a ND-1000 spectrophotometer nanodrop and stored at 4C.

Transfection techniques overview

Plasmid constructs were prepared and introduced into Didymium Pan 2-16 amoebae. Figure 4 illustrates the three techniques employed in this project in order to deliver the plasmid. Electroporation makes use of electrical fields to open up the cell membrane of the amoebae in order for the vector to enter the cell. Lipofection makes use of lipids to surround and house a plasmid in order to deliver it to the cell by lipid to lipid interaction and merging. Polymer transfection (XFECT) has affinity for genetic material, allowing multiple XFECR polymers to bind and surround a plasmid which is then introduced into the amoebal cell by endocytosis.

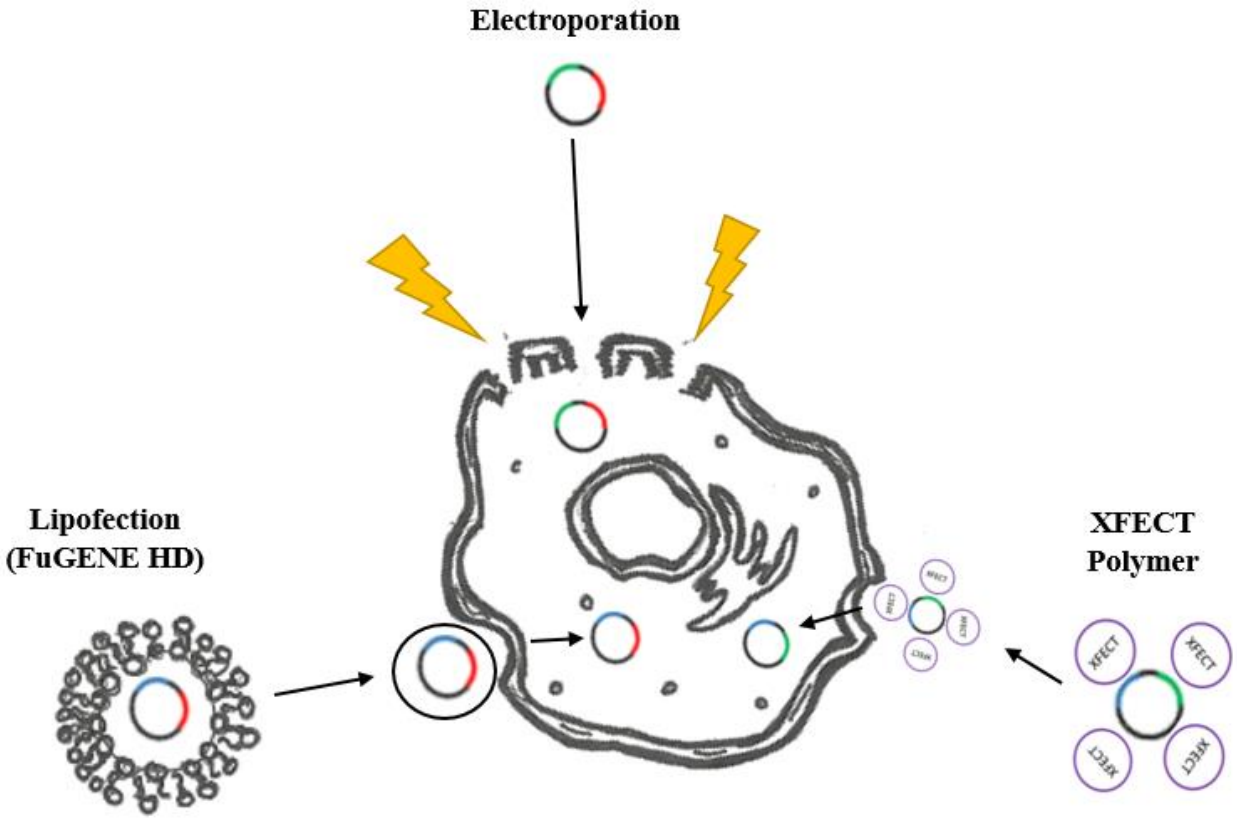


Figure 4. Overview of transfection methods. Constructed vectors were introduced into Didymium Pan 2-16 amoebae by electroporation, lipofection and XFECT polymer transfection.

Electroporation: Parameter adjusted ranges, cell handling, and sampling

Didymium cells were first prepared by inoculating the equivalent of $1.0x10^4$ cells/mL in volume into 500 mL Erlenmeyer flasks and shaken at 175 rpm on a New Brunswick Scientific c10 platform shaker at 23C for 3 to 4 days. After shaking for 3-4 days, cell counts using a hemocytometer were performed in order to determine the cell concentration at the end of the incubation period. Then 125 mL of Pan 2-16 cells were transferred and split into 3 conical tubes and pelleted in a Beckman Allegra 21R Centrifuge at 700 x g for 5 minutes. The supernatant was removed and cells were washed twice with 25 mL of HBS buffer (40 mM sucrose; 10mM HEPES;

pH 8.2). During the second wash steps, the pellets from the 125 mL were combined into one tube. The loose Pan 2-16 cell pellet was then suspended in a volume of HBS buffer to a concentration of $1.25x10^7$ cells/mL as recommended by Fey et al., 1995, for the majority of electroporation experiments. The cells were then incubated on ice for 30 minutes prior to electroporation.

Before attempting to transfect Pan 2-16 cells it was necessary to establish boundaries of voltage and resistance to maximize cell survivability. As a starting point, the voltage and resistances of successful transfections with Physarum and Dictyostelium were considered. According to Burland et al., 1993, the highest voltage to transfect Physarum at is 0.9 kV. For Dictyostelium (Gaudet et al., 2007; Pang et al., 1999) 0.85 kV was the maximum. The resistances used in these protocols were set at 800 ohms. In previous Physarum transfection studies, the highest resistance was set at 1000 ohm (Burland and Pallotta, 1995).

The parameters from here were extended up into a maximum of 1 kV and a minimum of 0.2 kV. This range of voltages allowed for obtaining preliminary data on cell survivorship and cell morphology. Cells were prepared as mentioned in the cell preparation section and the plasmid pGLO was used as a negative control plasmid during the parameters determination since it should not affect the Pan 2-16 cells. A volume of 500 µL of the resuspended Pan 2-16 cells at 1.25×10^7 cells/mL were electroporated using an exponential pulse at 25 µF in a 4 mm cuvette within the range of established voltages and resistances in increments of 0.100 kV and 100 Ω. Cell counts were performed immediately after electroporation to determine cell survivability and the condition of cell's morphology post electroporation. The parameters that provided the highest level of cell survivorship were considered for future electroporation experiments.

After initial cell observations, electroporation experimentation began with the experimental plasmids. Using optimal parameters, DNA quantity was tested ranging from 500 ng to 15 µg in

500 ng increments and 1µg increments. During these trials, resistance and voltages were held constant at 300 Ω and 0.4 kV. Observing fluorescence in some samples at specific DNA quantities led to varying voltages and resistances while maintaining DNA quantity constant. Samples were allowed to recover in a 30℃ incubator in their respective cuvettes immediately post transfection for 10 minutes. Cell counts were taken immediately post transfection as well. After incubation, electroporated samples were transferred into sterile culture tubes with 7 mL of PGY and about 200 µL of HKB and placed on the rotator to further recover and grow at 23℃.

FUGENE: Parameter adjusted-ranges, cell handling, and sampling

The lipofection reagent used was called FuGENE HD and the protocol was followed as described in the manual (Promega, 2018). Pan 2-16 cells were grown to 50 to 80% percent of their max density in culture tubes in 7 mL of PGY and 250 µL of HKB on a rotator at 23℃. The lipofection complex was prepared by adding 8.8 µg of plasmid DNA into dH_2O for a total volume of 414 µL. This created a 0.020 µg/µL concentration of plasmid dilution. The plasmid dilution was mixed by finger flicking and then 26 µL of the FuGENE HD reagent were added into the plasmid dilution. The lipo-plasmid mixture was mixed carefully by pipetting up and down in the centrifuge tube 15 times. The complex was left to incubate at 23℃ for 10 minutes. From this tube, 400 µL of mixed complex was transferred into a Nunc EasYFlask 25cm^2 culture flask, also referred to as a Nunc flask from here on, with the 7.25 mL of Pan 2-16, PGY and HKB culture. The Pan 2-16 cells and complex were gently mixed by swirling and the cells were left to incubate at room temperature (23℃) overnight without agitation.

This was the general protocol used for transfecting Pan 2-16 cells. The first 2 attempts in transfecting Pan 2-16 cells with each plasmid were directly performed as instructed by the reagent protocol. Additional parameters were altered to gauge if DNA concentrations had an effect on the

expression of GFP. This included doubling and halving the amount of DNA used in the complex solution makeup. Two methods of cell culture was tested, recovery in rotating cultures tubes and in stationary Nunc flasks, both at 23℃. Recovery temperatures were varied between 23℃ and 30℃ for the first trials. After observing fluorescence results, the recovery temperature for the remaining lipofection experiments was held constant at 23 ℃.

XFECT: Parameters adjusted-ranges, cell handling and sampling

Pan 2-16 cells were grown on a rotator to a concentration of 1.0 x 10^6 cells/mL. In a 50 mL conical tube, 1 mL of these cells were transferred over to be transfected using the XFECT transfection polymer. The protocol for the XFECT method was modified from the manual XFECT Transfection Reagent Protocol-At-A-Glance (PT5003-2) (Takara, 2018)**.** The protocol called from 5 µg of plasmid DNA in a total volume of 100 µL with XFECT reaction buffer (e.g. 18.4 µL of plasmid (271.74 ng/µL) into 81.6 µL of XFECT reaction buffer). After the contents were mixed, 1.5 µL of XFECT transfection polymer were added into the 100 µL total volume, vortexed and a quick spin brought the contents down. The mixture was incubated at 23℃ in a microcentrifuge tube for 10 minutes and then transferred into the 50 mL Falcon conical tube with the 2 mL of Pan 2-16 cells at 1.0 x 10^6 cells/mL and swirled.

After swirling and mixing gently in the 50 mL conical tube, the tube was placed in an isolated location away from light. The tubes were placed on their sides to allow the 2 mL to spread out ensuring good aeration during transfection and recovery (see Figure 5).

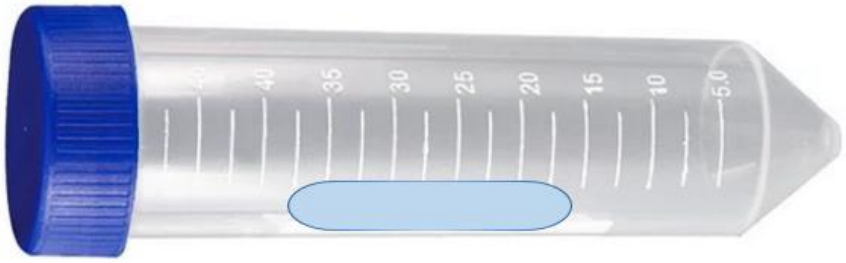


Figure 5. XFECT conical tube setup. A conical tube with 2 mL of Pan 2-16 amoebae mixed with XFECT polymer. The conical tube was placed on its side to provide good aeration for the cells.

Two incubation times were tested: 4 hours and overnight (typically 18 hours) XFECT exposure. Samples were then quick spun, aspirated and suspended in 2 mL of fresh PGY and 200µL of HKB. Recovery and growth of the cells occurred in the 50 mL conical tubes. For fluorescence observations, 100 µL of sample were placed into a microcentrifuge tube and quick spun to concentrate cells into a loose pellet. The loose pellet was transferred onto a microscope slide for preparation for fluorescence microscopy observations.

Imaging: Phase-Contrast and Fluorescence

Pan 2-16 cells were observed immediately after each transfection method using a phase contrast microscope. Cell counts were performed immediately after every transfection method to observe morphology and general health of the cell. Depending on the transfection type, cells were transferred over to fresh 7 mL PGY tubes with anywhere from 200 to 400 µL of HKB and allowed to recover for 2 to 4 days. Aliquots of 250 µL of Pan 2-16 cells were transferred into microcentrifuge tubes and cells were loosely pelleted by quick spinning for 7 seconds using a table top minifuge. From the loosely pelleted cells, 15 µL of pellet were pipetted onto a microscope slide. This was done to observe transfection by electroporation and lipofection. For XFECT

polymer transfection, 100µL of cell sample was transferred onto a microscope slide. During the recovery days, observations were made in approximately 5 and 10 hour increments.

A Nikon Eclipse 80i microscope coupled with a mercury lamp was used for phase-contrast and fluorescence observations of all samples. The microscopes fluorescein isothiocyanate (FITC) excitation and emission filters block and direct specific wavelengths of light that have interacted with a sample. According to the Nikon Eclipse 80i fluorescence microscope manual, the FITC filter is used for wavelengths of 490 – 520 nm. GFP has an excitation wavelength of 490 nm and an emission wavelength of 510 nm. When using the fluorescence microscope, cells were first observed at 100X total magnification using phase contrast, then the microscope was switched to the fluorescence settings. Magnification was increased for imaging. Cells were screened in left to right and right to left direction covering the entire coverslip. When a fluorescent cell was found, a 400X and 1000X total magnification setting was used to take an image of the fluorescing cell using a DinoScope camera and a Samsung J7 smartphone.

RESULTS

Overview of design and construction of pDidy plasmids

The pDidy plasmid was constructed in three phases (see figure 6). In the first phase PCR was used to amplify fragments from clones that contained regulatory elements from Didymium and fragments of *gfp* and *maxgfp* (see Table I and II). Gel analysis verified that the fragments were the correct size (Table I and II). Some fragments needed to be gel purified. In phase two, extension primers added nucleotides to the Didymium promoter ProAup and terminator ProAdown to create overlapping ends. In phase three, purified fragments were combined together using overlap extension PCR before integration into a vector using the Gibson Assembly.

Constructing pDidy 1.0

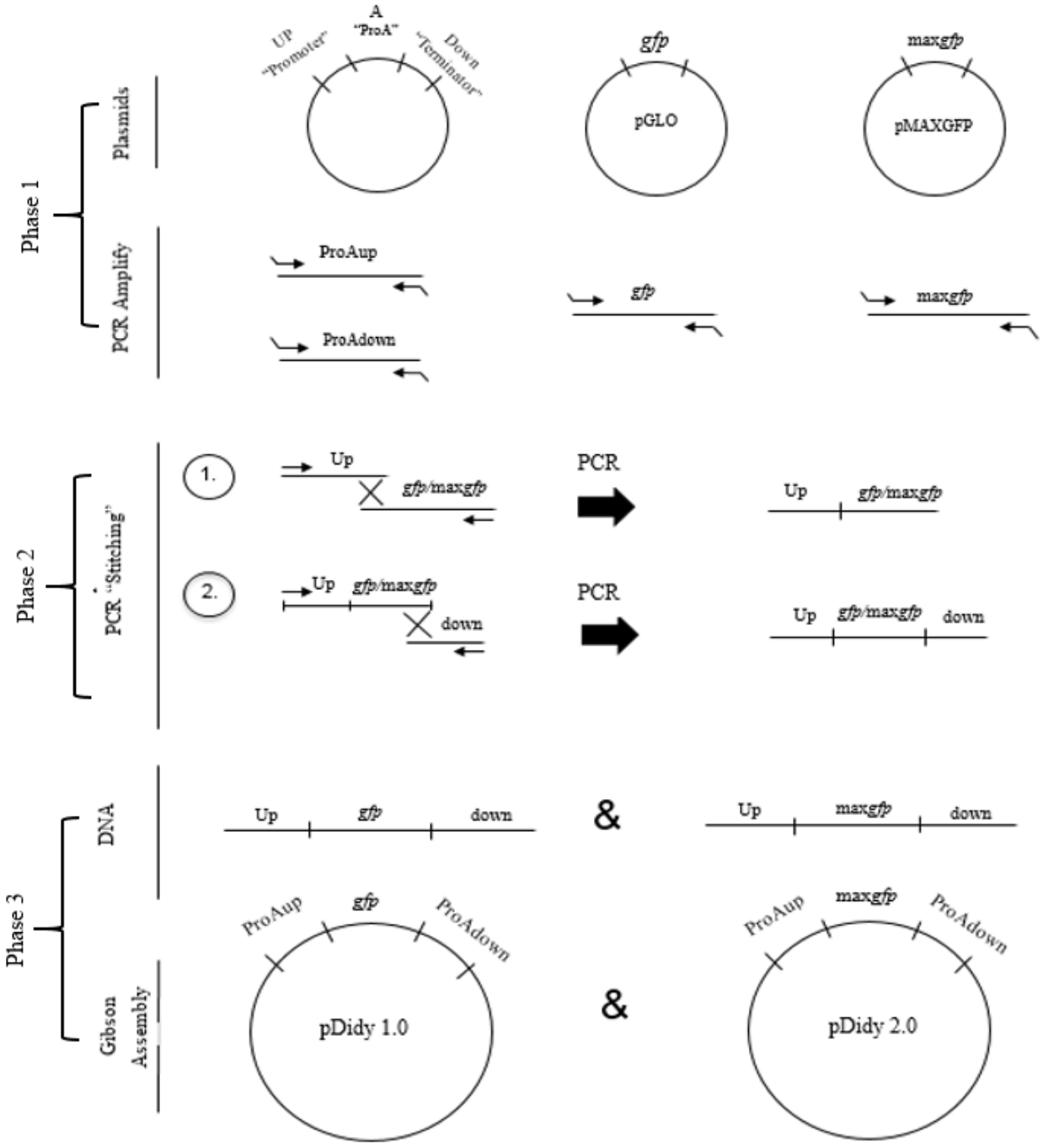
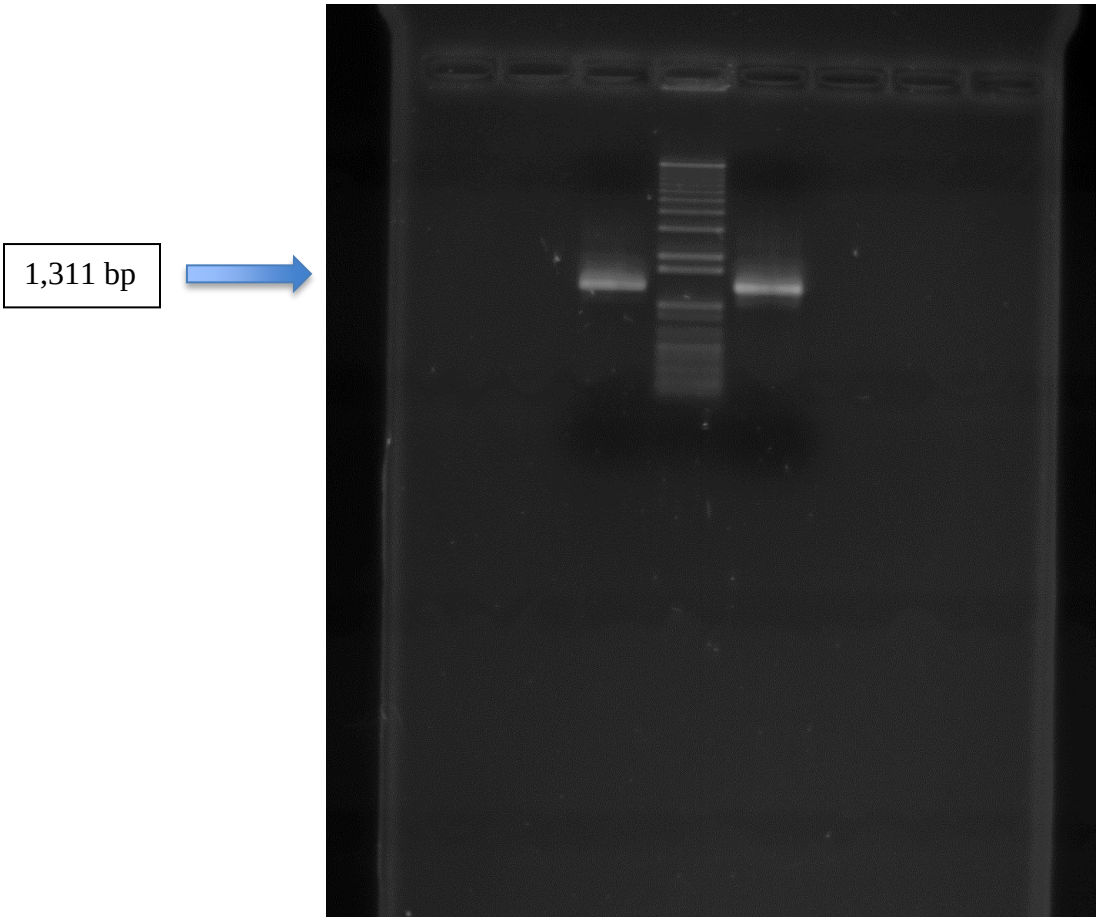


Figure 6. Workflow of pDidy 1.0 and 2.0 construction. Individual DNA fragments consisting of the Didymium regulatory elements (Promoter and terminator) and either *gfp* or *maxgfp,* were amplified using PCR. Individual fragments were stitched together using outer primers to generate one larger continuous fragment. The larger fragment was cloned into a pUC19L vector using the Gibson Assembly.

Since the promoter ProAup is a smaller DNA fragment compared to *gfp* and ProAdown, it was first stitched to *gfp*, to make [ProAup-*gfp*] (1,045 bp), then the joined fragments were stitched to ProAdown to generate [ProAup-*gfp*-ProAdown] (1,311 bp). The annealing temperature used between outer primers was the lower temperature to ensure proper annealing for both. The final purified stitched product size was verified by gel analysis (Figure 7). This fragment was cloned into the pUC19L vector (2,659 bp) using the Gibson Assembly. The total size of the pDidy 1.0 plasmid is 3,953 bp. The sum of the fragment sizes is greater than the final plasmid size due to the overlapping base pairs. A map of the plasmid highlighting the regulatory elements surrounding the *gfp* gene, as well as vector sequences is presented in figure 8.



DNA (Fragment or Plasmid)	Primers used		T_{anneal} (°C)	Product length (bp)
	Forward Primer	Reverse Primer		
ProAup-*gfp*	ProAup FW EXT	GFP RV	50.1	1,045
[ProAup-*gfp*-ProAdown]	ProAup FW EXT	ProAdown RV EXT	55.6	1,311

Figure 7. Gel purified pDidy 1.0 stitched fragments. Using PCR stitching, Didymium regulatory elements were stitched to a *gfp* gene and run on a 1% agarose gel. Lane 3 and 5 shows the gel purified product of [ProAup-*gfp*-ProAdown] at a size of 1,311 bp. Lane 4 is a standard 1 kb^+ ladder. Not pictured is the intermediate product of [ProAup-*gfp*] at 1,045 bp.

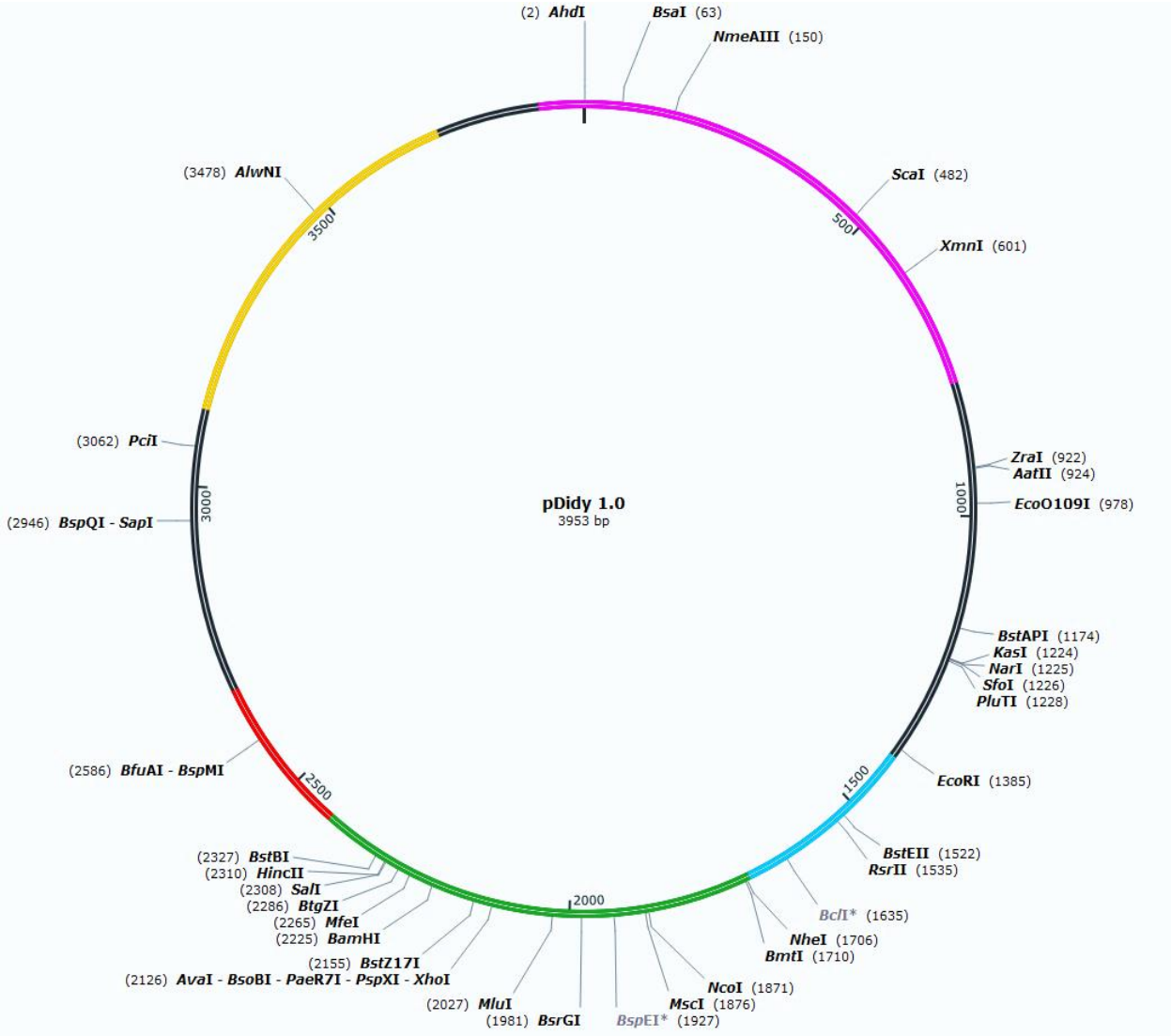


Figure 8. pDidy 1.0 plasmid map. The size of the plasmid is 3,953 bp with a pUC19 vector backbone. The plasmid has color coded regions: Ori C (yellow), ampicillin resistance gene (purple), ProAup promoter (blue), *maxgfp* (green) and ProAdown terminator (red).

When the pDidy 1.0 construct was digested with *Hind*III the predicted products were 3,083 bp and 855 bp fragments. The results are in agreement with predictions (see figure 9). The pDidy 1.0 plasmid was sequenced to further analyze the constructed plasmid. Several clones were sequenced with the same PCR primers used to amplify individual fragments. The entire insert was sequenced in three overlapping segments of 500 – 700 bp. Comparison of the construct sequence to the source material revealed that no mutations were introduced by PCR or other manipulations (figure 10).

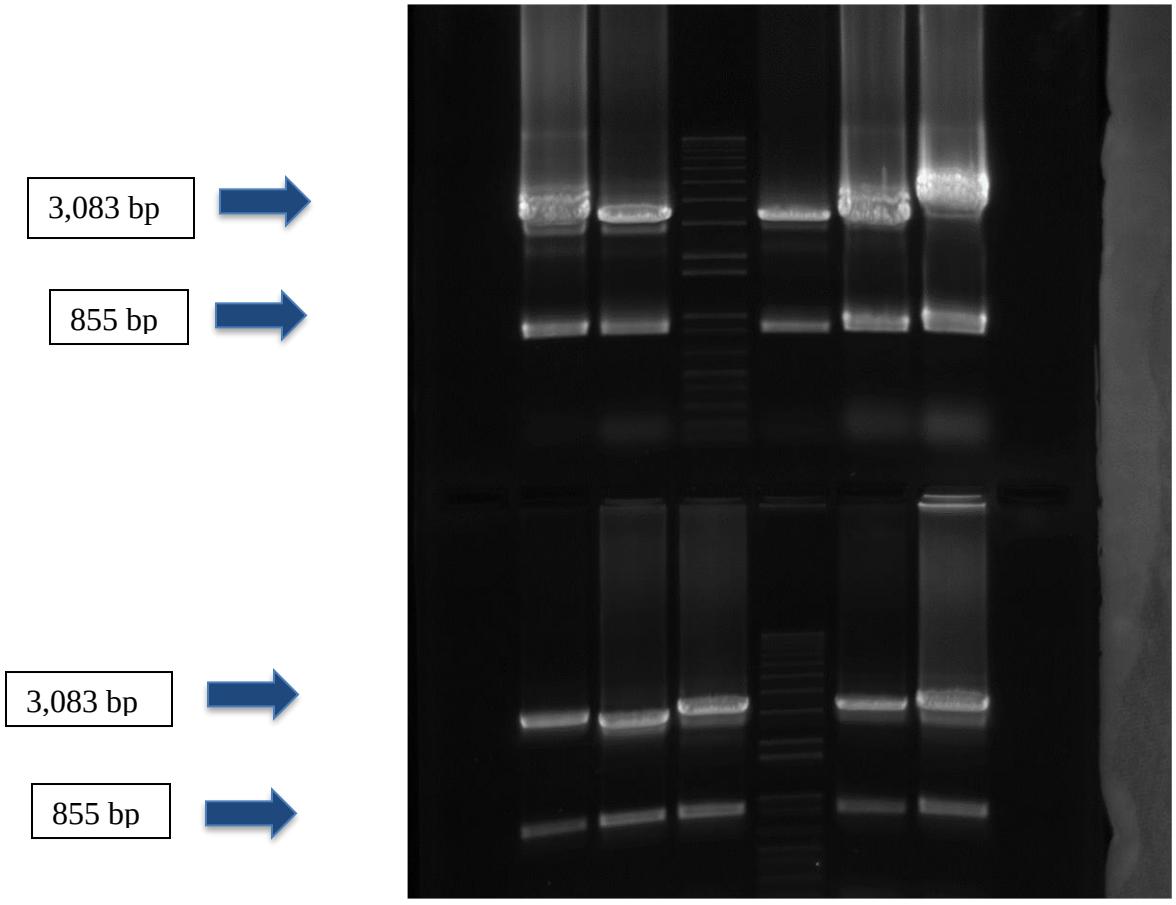


Figure 9. *Hind*III restriction digest of pDidy 1.0. Top lanes 1-6 show pDidy 1.0 clones #1-5 with a 1 Kb^{+} marker in lane 3. Bottom lanes 7-12 show pDidy 1.0 clones #6-10 with a 1 Kb^{+} ladder in lane 10. All digested clones display two positive bands at 3,083 bp and 855 bp.

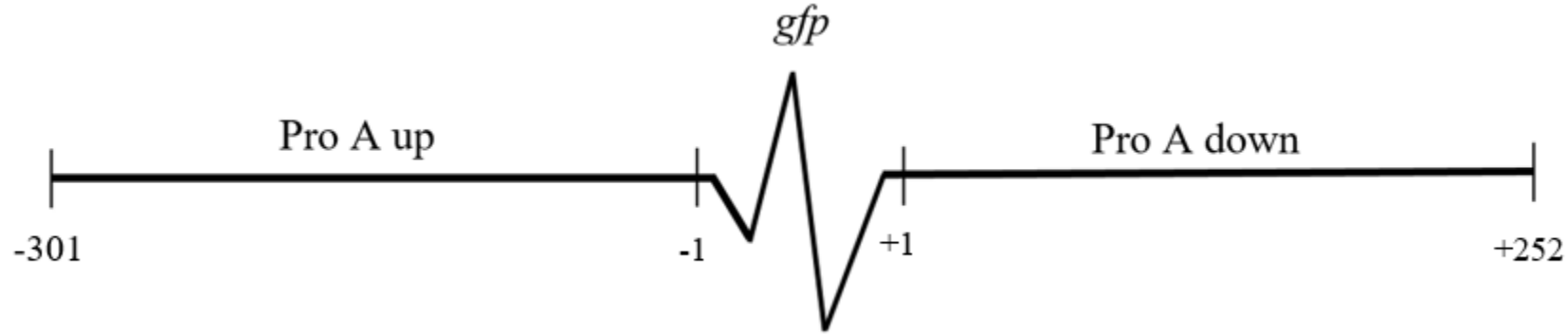
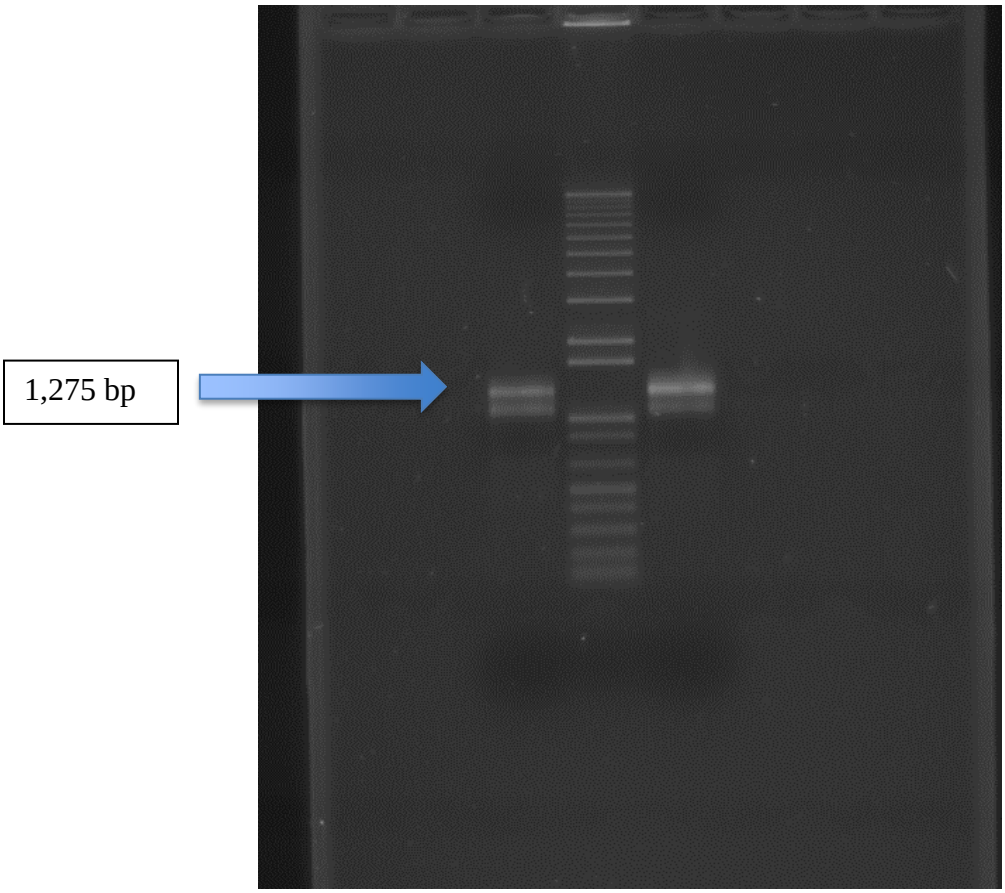


Figure 10. pDidy 1.0 clone sequence compared to the source material. No mutations or disagreements were found in the sequenced pDidy 1.0 construct.

Constructing pDidy 2.0

The primers used to amplify pDidy 2.0 are listed in Table II, which shows the resulting sizes. The overall construction of pDidy 2.0 is diagrammed in figure 6, and is similar to the process of constructing pDidy 1.0. Purified pDidy 2.0 amplification products were stitched together. The ProAup promoter was first joined to the *maxgfp* gene to form [ProAup-*maxgfp*] (1,022 bp), then joined to ProAdown to generate [ProAup-*maxgfp*-ProAdown] (1,275 bp). Stitched products were then verified and purified (see figure 11).



DNA (Fragment or Plasmid)	Primers used		T_{anneal} (°C)	Product length (bp)
	Forward Primer	Reverse Primer		
[ProAup-max*gfp*]	ProAup FW EXT	GFPTA RV	52.3	1,022
[ProAup-max*gfp*-ProAdown]	ProAup FW EXT	ProAdown RV EXT	55.6	1,275

Figure 11. Gel purified pDidy 2.0 stitched fragments. PCR stitching was used to generate one large continuous fragment consisting of a Didymium profilin A promoter and terminator attached to a *maxgfp* gene. Lanes 3 and 5 of this 1% agarose gel show the stitched product [ProAup-max*gfp*-ProAdown] at 1,275 bp. The upper band in each lane is the correct sized stitched fragment. Not pictured is the intermediate product of [ProAup-*maxgfp*] at a size of 1,022 bp.

Like the pDidy 1.0 construct, the [ProAup-*maxgfp*-ProAdown] fragment was cloned into the 2,659 bp linearized cloning vector (pUC19L) using the Gibson Assembly to create a 3,895 bp plasmid. Figure 12 shows the predicted map of pDidy 2.0. A double restriction digest with *Pst*I and *Hind*III was performed to confirm the presence of 3,509 and 386 bp bands predicted by the map (see figure 13).

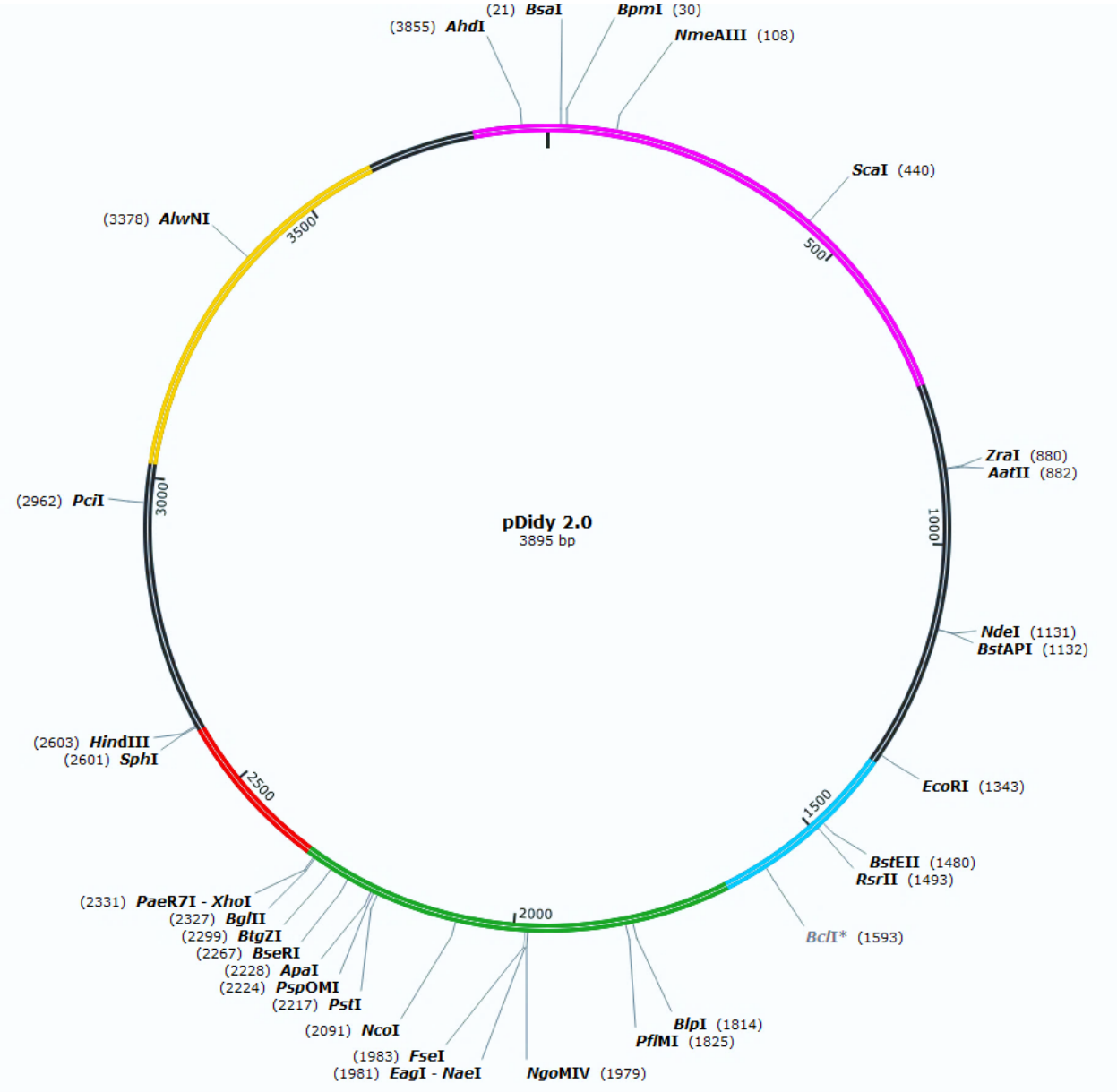


Figure 12. pDidy 2.0 plasmid map. The size of the plasmid is 3,895 bp with a pUC19 vector backbone. The plasmid has color coded regions: Ori C (yellow), ampicillin resistance gene (purple), ProAup promoter (blue), *maxgfp* (green) and ProAdown terminator (red).

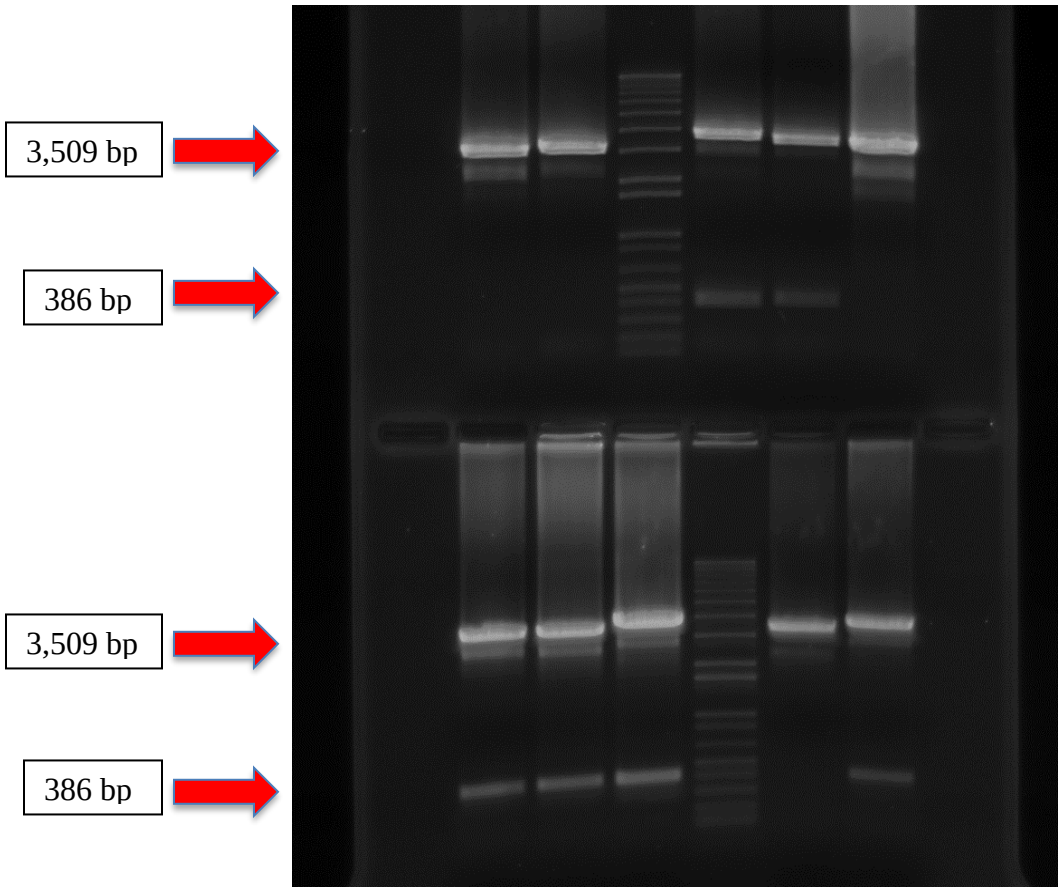


Figure 13. *Pst*I and *Hind*III double restriction digest of pDidy 2.0. Top lanes 1-6 show pDidy 2.0 clones #1-5 (left to right) with a1Kb+ ladder in lane 3. Bottom lanes 7-12 show clones #6-10 (left to right), with a $1Kb^{+}$ ladder in lane 10. Clones number 3, 4, 5-7 and 10 show correct size bands at 3509 bp and 386 bp.

Source and experimental sequence comparisons of pDidy 2.0

The pDidy 2.0 plasmid was sequenced to further analyze the constructed plasmid. As previously described for pDidy 1.0 sequencing, several clones were sequenced with the same PCR primers used in the amplification process. The insert was sequenced in three overlapping segments ranging from 500 – 700 bp. Contigs were generated with the source material and revealed three mutations in the insert (see figure 14). Two mutations were found in the ProAup promoter; both were G substitutions for A. In the ProAdown terminator, an A addition was found. The *maxgfp* gene sequence was unchanged when compared to the original sequence. Relative to the location of the *maxgfp,* the mutations in ProAup are -218 and -176 upstream of the transcription start site. The ProAdown terminator mutation is +218 downstream of the end of the *maxgfp* (see figure 14).

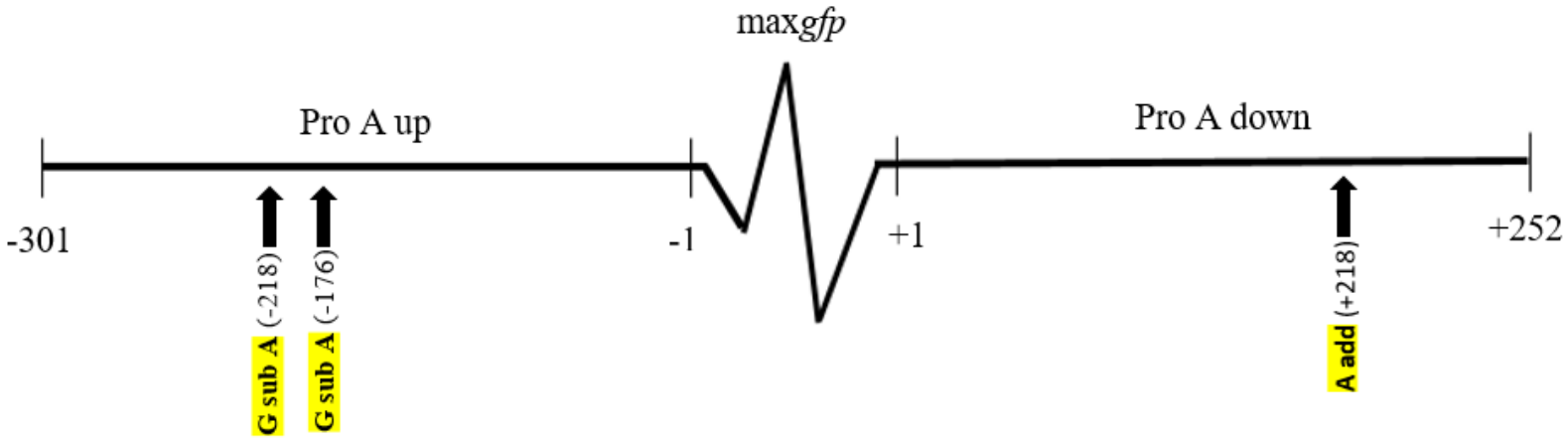


Figure 14. Mutational analysis of pDidy 2.0. Sequences were screened and compared to source material to identify mutations. Mutations were only present in non-coding regions of the sequence.

Overview of construction of pPhys

The pPhys construct was made in three main phases (see figure 15). The first phase included amplifying the regulatory elements: *PardC* (promoter) and *TardC* (terminator) and the reporter gene *maxgfp* from source plasmids (see Table III). In the second phase, the fragments were stitched together using overlap extension PCR (stitching). Intermediate stitched products were cloned to obtain stable stitched fragments. In phase three, the final stitched product was cloned into a pCR2.1 TOPO vector. Once constructed the pPhys plasmid was analyzed by restriction digestion and sequencing. A variety of mutations were identified; the least modified representative of pPhys was selected for transfection experiments into Pan 2-16 cells.

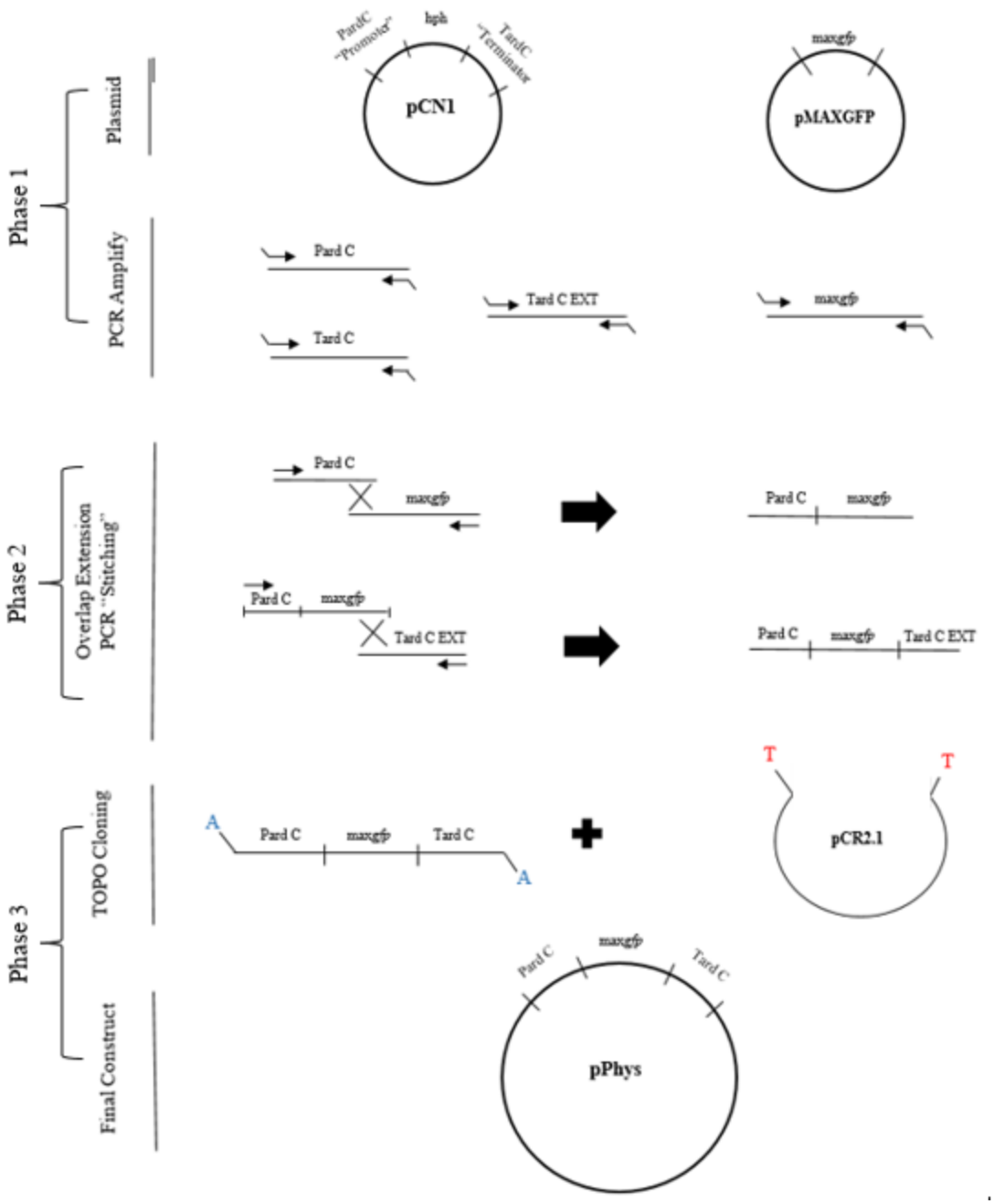
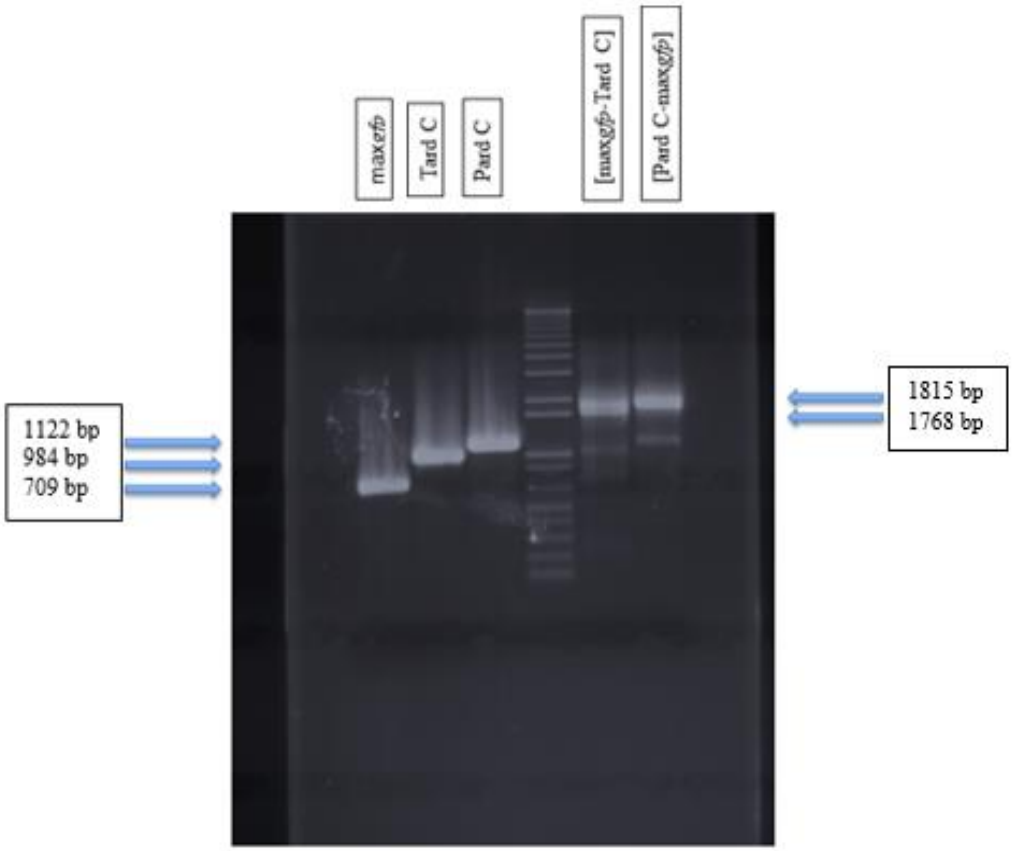


Figure 15. Workflow of pPhys construction. A summary of the construction of pPhys. At the TOPO cloning step, A and T overhangs were used to clone the stitched insert.

In detail, the purified pPhys amplification products were stitched together. *PardC* was joined to *maxgfp* to form [*PardC-maxgfp*] (1,814 bp). The stitched intermediate was then stitched to *TardC* to generate [*PardC-maxgfp-TardC*] (2,796 bp). The final stitched product was then verified and purified (see figure 16). The [*PardC-maxgfp-TardC*] fragment was then cloned into a TOPO pCR2.1 to create the final pPhys construct at a size of 6,725 bp (see figure 17). A restriction digest was performed with *Eco*R1 to verify the size of pPhys (see figure 18).



DNA (Fragment or Plasmid)	Primers used		T_{anneal} (°C)	Product length (bp)
	Forward Primer	Reverse Primer		
[*PardC-maxgfp*]	Pard C5 FW	pPhysGFP2 RV	59.5	1,815
[*PardC-maxgfp-TardC*]	Pard C5 FW	Tard C6 RV	60.8	2,796

Figure 16. pPhys fragments and intermediate stitched products. pPhys 1.0 fragments were amplified by PCR and analyzed on a 1% agarose gel. Lane 2 contains the *maxgfp* fragment (709 bp), lane 3 *TardC* fragment (984 bp), lane 4 *PardC* fragment (1122 bp) and lane 5 a 1 Kb^{+} ladder. Lane 6 shows a stitched product [*maxgfp - TardC*] (1768 bp) and lane 7 has the stitched product [*PardC – maxgfp*] (1815 bp).

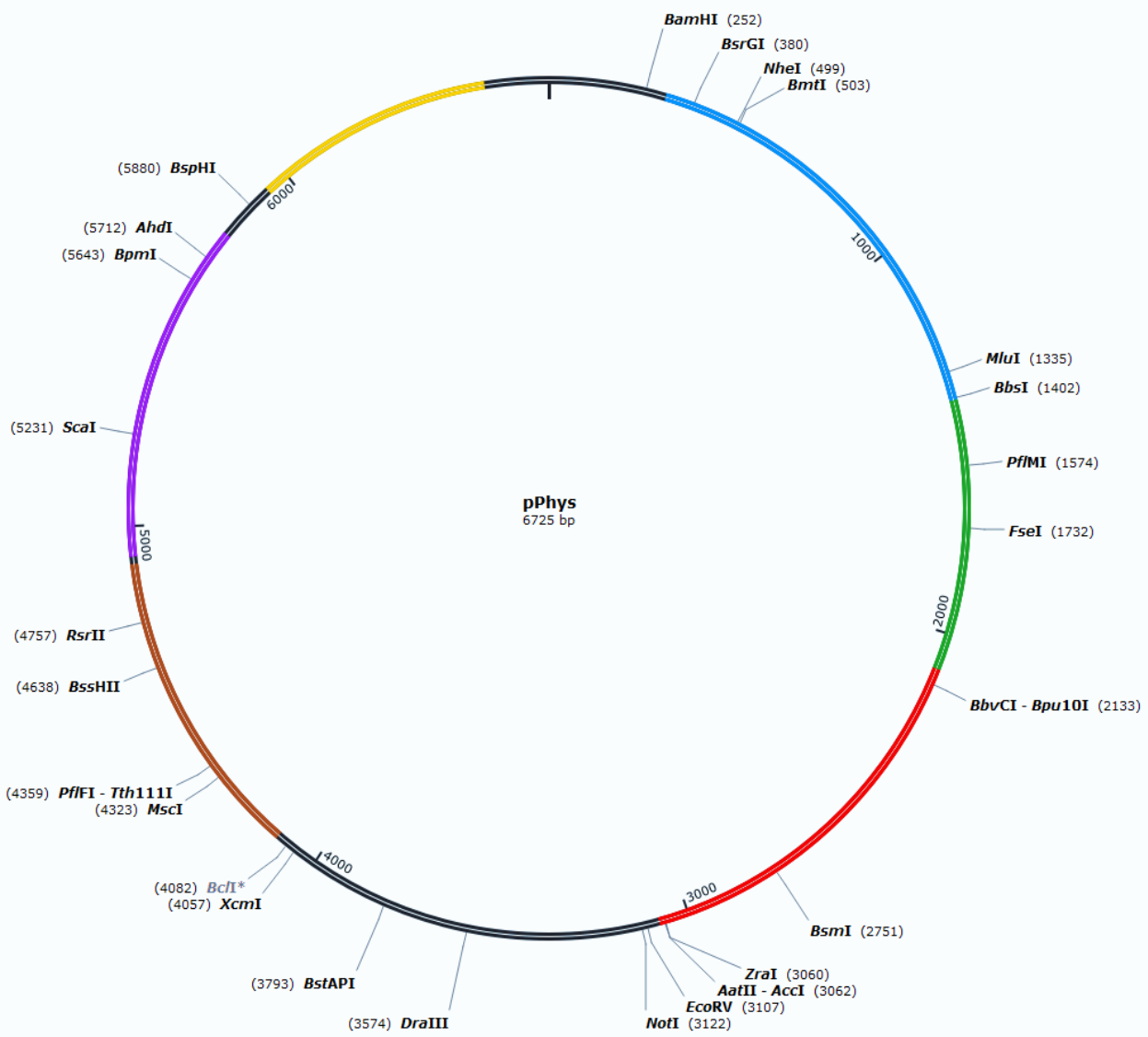


Figure 17. Constructed pPhys plasmid map. The size of the plasmid is 6,725 bp with a pCR2.1 vector backbone. The plasmid has color coated regions: Ori C (yellow), ampicillin resistance gene (purple), kanamycin resistance gene (brown), *PardC* promoter (blue), *maxgfp* (green) and *TardC* terminator (red).

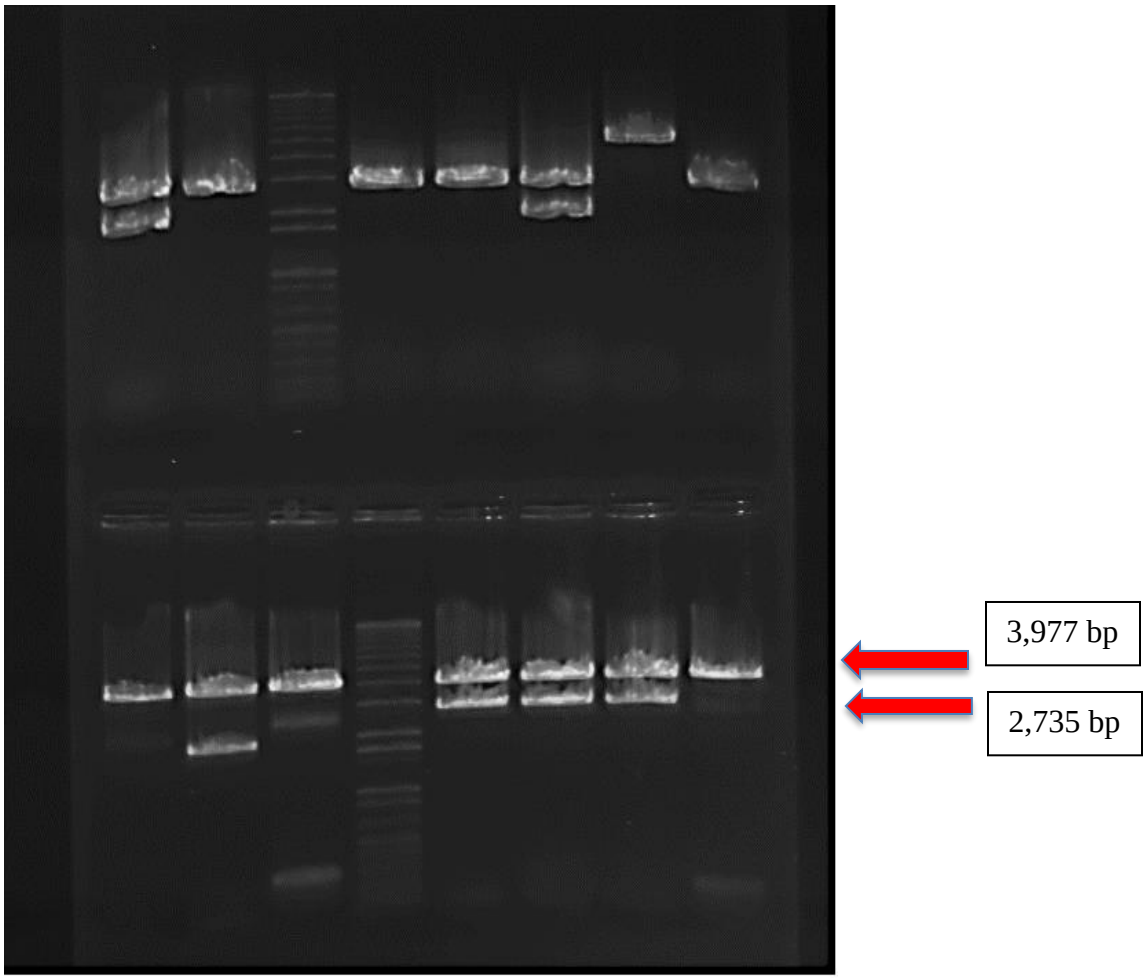


Figure 18. *Eco*R1 restriction digest of pPhys. Top lanes 1, 2 and 4-8 show digested products of attempted ligated pPhys constructs #9-15. Lanes 9-11 and 13-16 show positive digested clones of TOPO pCR2.1 [*PardC-maxgfp-TardC*] with a larger fragment at 3,977 bp and a secondary band at 2,735 bp. Lanes 3 and 12 contain a standard 1 kb^{+} ladder.

Source and experimental sequence comparisons of pPhys

The pPhys plasmid was sequenced to further analyze the constructed plasmid. Several clones were sequenced using the PCR primers used in earlier amplifications. The pPhys insert was sequenced in five overlapping segments ranging from 500 – 700 bp. When plasmid sequences were compared with the source material, six mutations were revealed in the *ParC* promoter, none in the *maxgfp* gene, and two in the *TardC* terminator (figure 19). The nature of the mutations and the location of the mutations relative to *maxgfp* are indicated.

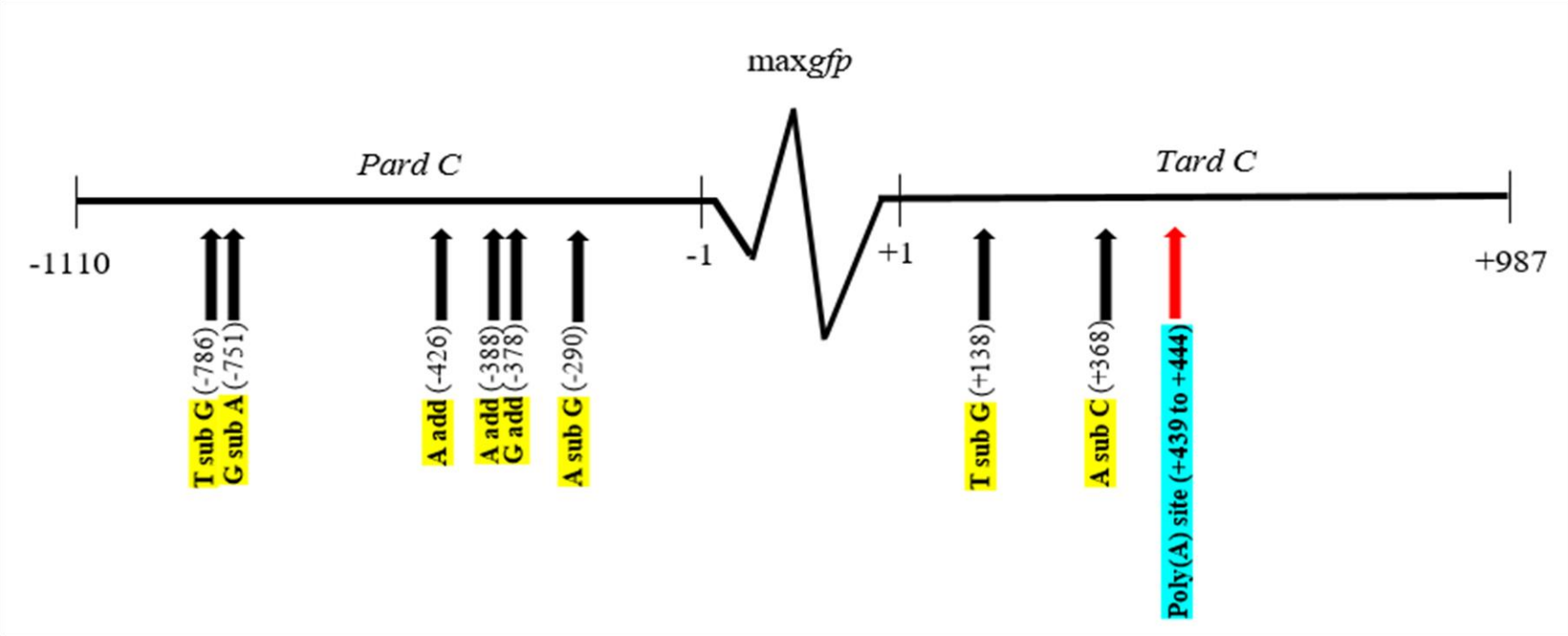


Figure 19. Plasmid sequence comparison with the source material. Experimental sequences of pPhys were compared to source sequences in order to identify mutations or misalignments. Negative and positive numbers in parentheses display the locations of mutations relative to *maxgfp* transcription start site.

Summary of plasmids and features

Four constructs were prepared for transfection into Didymium amoebae (Table IV). The plasmid pDicty (6,736 bp) consists of *gfp* being regulated by a Dictyostelium actin promoter and terminator: A15P and 2H3T, respectively. The two Didymium plasmids constructed were similar but differed in the *gfp* used. The pDidy 1.0 (3,953 bp) construct contained a *gfp* gene that was flanked by a profilin A promoter and terminator. Similarly, pDidy 2.0 (3,895 bp) had the profilin A promoter and terminator, but it had a *maxgfp* gene instead. Lastly, pPhys (6,705 bp) was regulated by the actin associated promoter and terminator *PardC* and *TardC*. These regulatory elements flanked *maxgfp*.

Table IV. Plasmids used in transfection experiments

Plasmids	Source	Vector size (bp)
pDH-GFP-ABD120 (pDicty)	DictyBase	6,736
pDidy 1.0	Constructed by overlap Extension PCR & Gibson Assembly	3,953
pDidy 2.0	Constructed by overlap Extension PCR & Gibson Assembly	3,895
pPhys	Constructed by overlap Extension PCR & TOPO Cloning	6,725

Baseline Pan 2-16 observations

Untransfected Pan 2-16 amoebae were observed with phase-contrast microscopy to establish a baseline morphology for healthy untransfected Pan 2-16 amoebae (see figure 20). Untransfected amoebae appear irregular to spherical in shape. The nucleus is a prominent feature in the cells, it is clear with a dark central nucleolus.

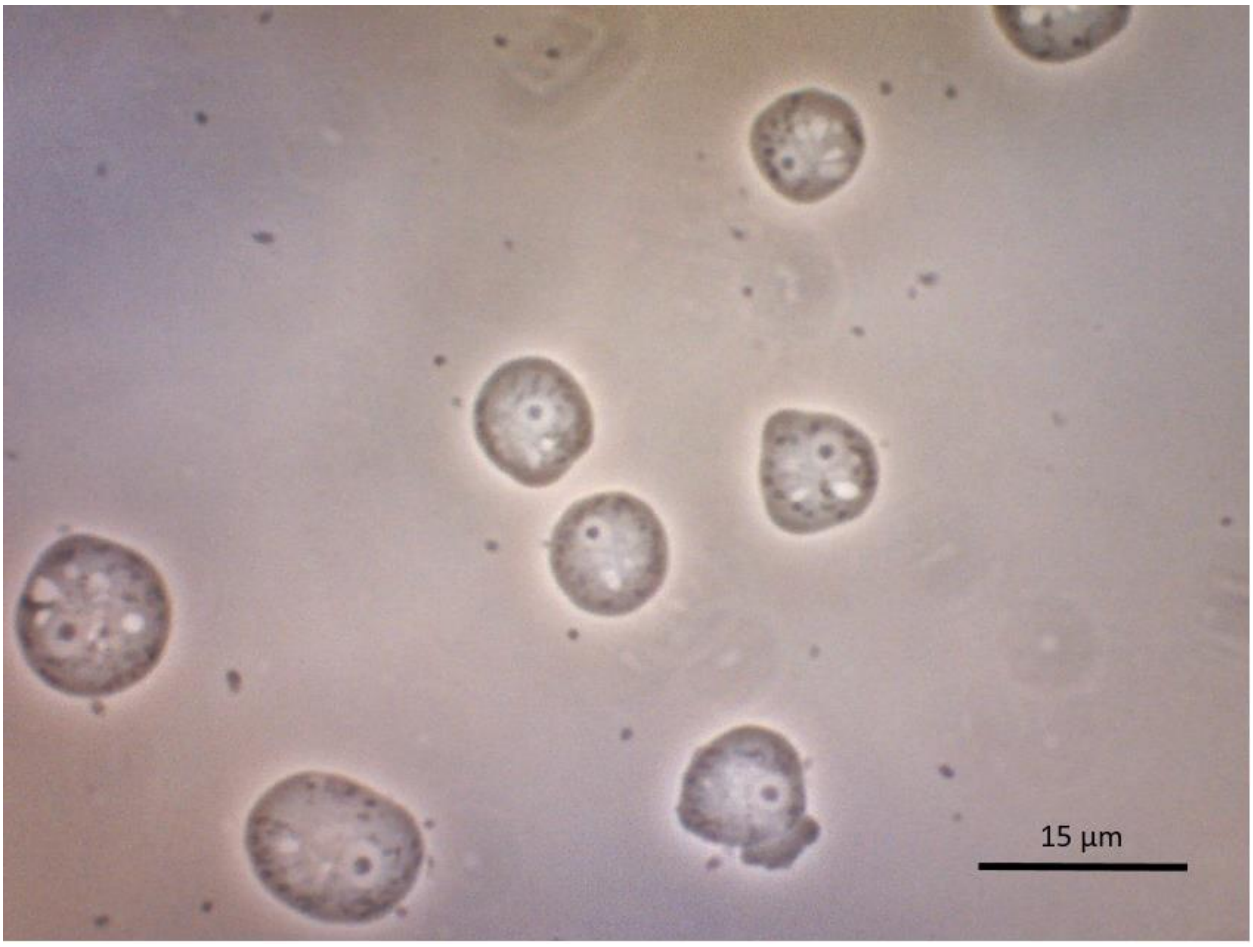


Figure 20. Pan 2-16 scale. Phase-contrast microscopy was used to observe untransfected Didymium Pan 2-16 to determine a baseline of healthy cells.

At every observation time, cells were observed by phase-contrast before switching to fluorescence microscopy. Untransfected cells were observed under fluorescence to determine if

the cell naturally emit fluorescence (see figure 21). The Pan 2-16 cells do not give off any fluorescent signals by themselves and this served as our negative control.

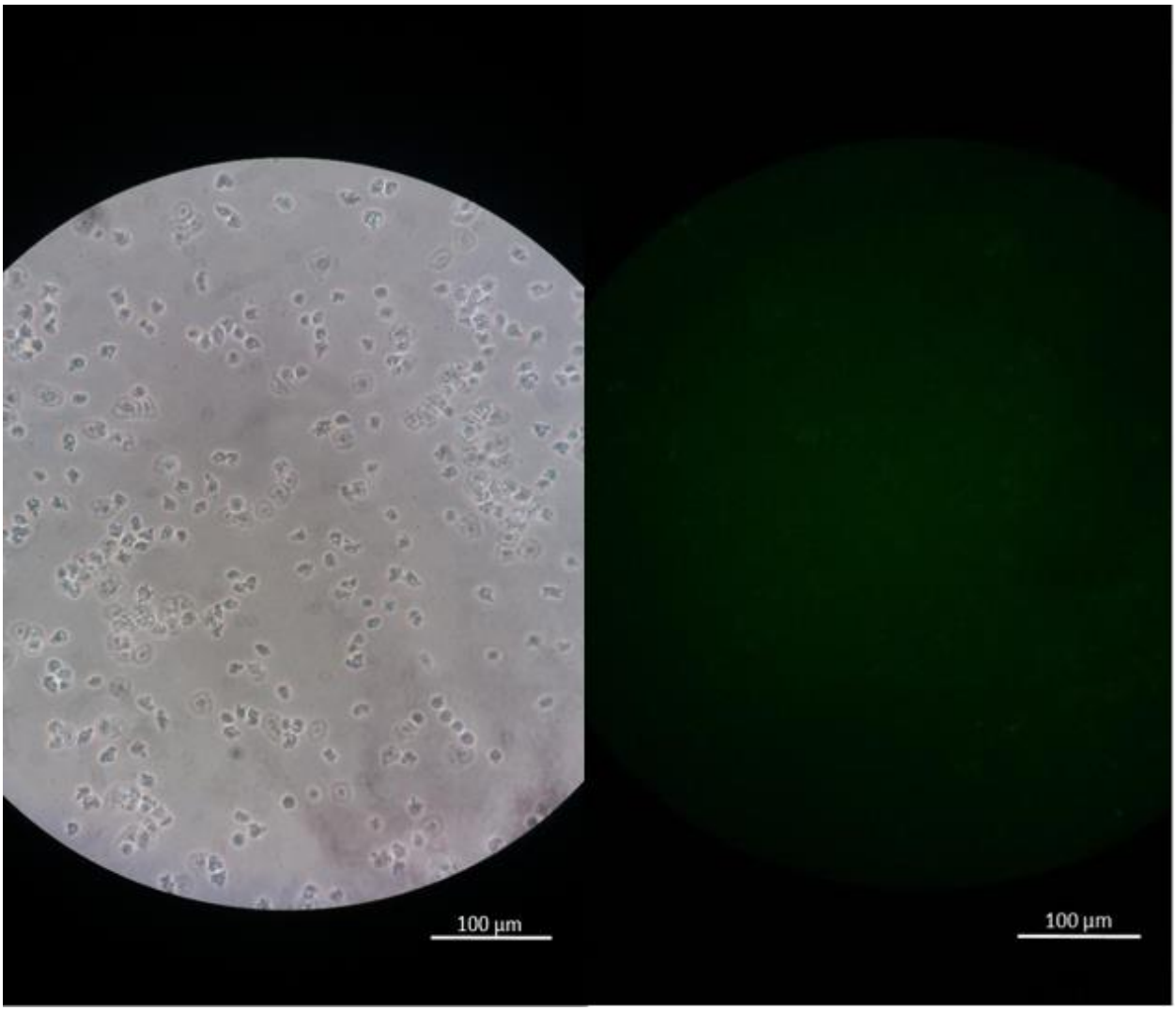


Figure 21. Pan 2-16 negative control. Untransfected Pan 2-16 cells were observed to determine any baseline fluorescence. None was observed.

Electroporation

Electroporation parameters testing

Prior to introducing constructs into Didymium Pan 2-16 amoebae, cell survivorship under various electroporation parameters was tested in order to gauge a range that would allow for amoebae to survive the electroporation shock and remain healthy (Table V). Based on previous Physarum and Dictyostelium studies mentioned in the introduction, a range of voltages (kV) and resistances (Ω) were selected and tested on Didymium cells. Each treatment started with approximately $1.0x10^8$ cells/mL and performed in triplicate. Cell survivorship immediately after treatment was calculated. For voltages, an upper limit of 1 kV and a lower limit of 0.2 kV with varying resistances in increments of 100 Ω from 100 -1000 Ω were also tested. A linear trend at a constant voltage of 0.40 kV with resistances ranging from 100 – 1000 Ω was determined (see figure 22). These voltage and resistance combinations tested are displayed in Table V.

Table V. Cell counts and optimization of electroporation parameters

Voltage (kV)	Resistance (Ω)	Cell concentrations (cells/mL)
0.85	1000	6.50×10^4
0.50	∞*	7.00×10^5
0.50	1000	2.00×10^5
0.50	800	2.45×10^5
0.45	1000	9.70×10^5
0.45	800	1.60×10^5
0.40	1000	1.03×10^6
0.40	900	1.80×10^6
0.40	800	2.78×10^6
0.40	700	6.73×10^6
0.40	600	8.98×10^6
0.40	500	9.83×10^6
0.40	400	1.66×10^7
0.40	300	3.31×10^7
0.40	200	3.65×10^7
0.40	100	8.00×10^7
n/a**	n/a**	1.28×10^8 ***

*The infinity symbol (∞) indicates a default setting of above 1000 Ω for the BioRad GenePulser.

** Cells that were not electroporated.

*** The starting concentration for parameter testing was a calculated 1.28×10^8 cells/mL.

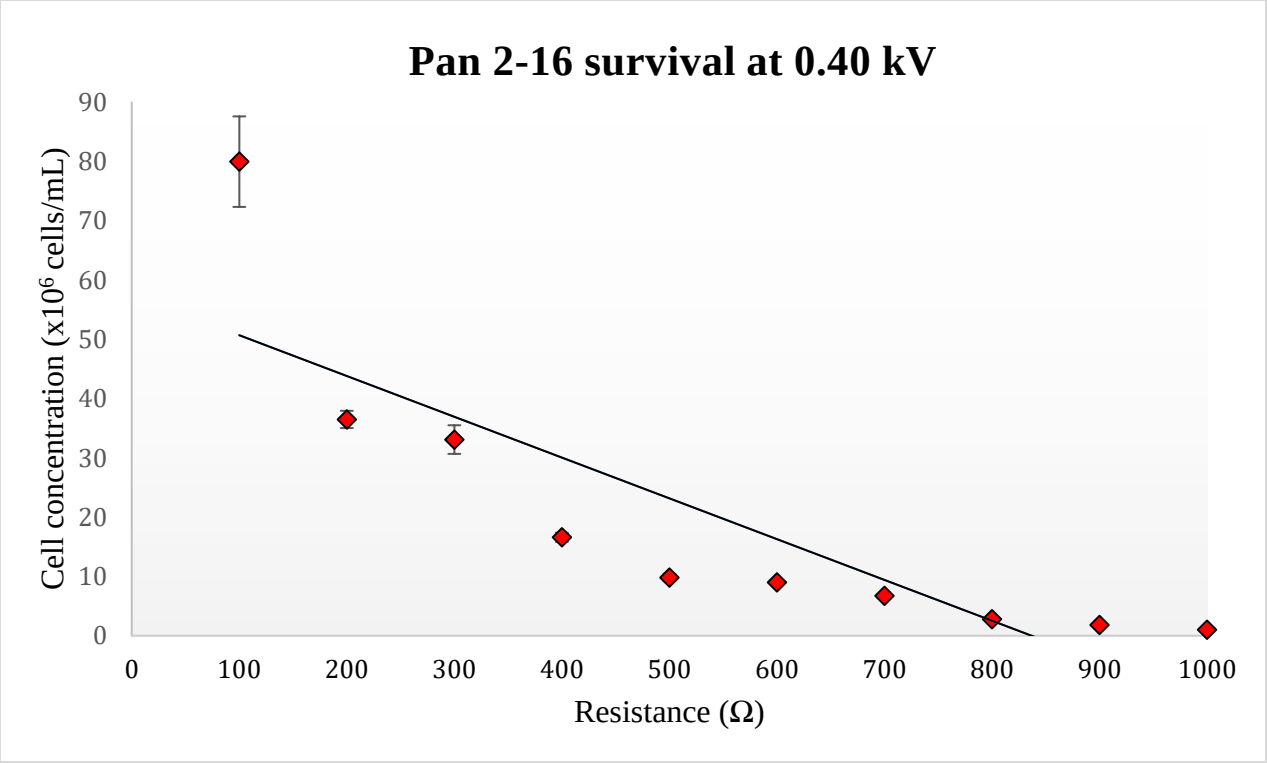


Figure 22. Electroporation resistance testing at a constant 0.40 kV. Cell survivorship was determined at resistances ranging from 100 to 1000 Ω. The initial cell concentration for these tests was 1.28 x10^6 cells/mL.

The general trend for the survivorship Pan 2-16 cells using electroporation can be noted in Table V and figure 22. Higher voltages near 1 kV regardless of resistances, obliterated a majority of the amoebae, so voltages past 0.85 kV were abandoned. Whereas, voltages below 0.40 kV allowed for a higher cell survival. The highest survivorship was seen with a low voltage of 0.40 kV and low resistances of 100 - 400 Ω. These trends are summarized in Table VI.

Table VI. Electroporation patterns

Voltage (kV)	Resistance (Ω)	Survivorship
High	High	0-15%
Low	High	15-50%
Low	Low	>50%

Immediately after electroporation, cells were allowed to recover in a 30°C incubator for 10 minutes and then were placed onto a rotator at room temperature. Electroporated Pan 2-16 cells generally appeared spiky. Cells that were exposed to the highest voltages developed a dark brown and orange rusted coloration within five to ten minutes of rotating. After recovery, some cells maintained their general shape, while many ghost membranes and cells vacant of contents were evidence of considerable cell death. Although the electrical field administered during the electroporation was brief and exponential, cells did recover when voltages were lower, even at high resistances.

The range of electroporation parameters tested for each construct is listed in Table VII. During preliminary experimentation, a range of cell concentrations was compared to gauge the effect of different cell concentrations. Each condition was performed in triplicate. The quantity of DNA tested in a constant volume of 500 µL was: 250, 500, 1000, 1500, 2000, 3000, 5000, and 10,000 ng. The best results were obtained with a DNA quantity in the range of 3000 – 5000 ng, a voltage of 400 V, and a resistance of 300 Ω; these conditions yielded fluorescence results. Like earlier studies with Physarum (Burland and Bailey, 1995), an exponential pulse and a 30°C recovery was successful in Didymium.

Table VII. Electroporation parameters and conditions tested

Parameter/conditions	Conditions and Ranges
Cell Concentration (cells/mL)	$1.00x10^8$, $1.25x10^7$, $1.0x10^7$
DNA Concentration (ng)	250, 500, 1000, 1500, 2000, 3000, 5000, 10,000
Voltage (V)	300, 400, 700, 800, 1000
Resistance (Ω)	200, 300, 700, 1000
Pulse Type	Two-pulses, Exponential, Square wave
Recovery Temperature (℃)	23, 30
Construct 30min Incubation (℃)	0, 22

*Highlighted values indicate parameters/conditions that yielded fluorescence.

Electroporation: fluorescence observations

Post electroporation Pan 2-16 amoebae were observed immediately under fluorescence every five hours and repeated with observation times every ten hours. Cells were focused by first using phase-contrast microscopy before switching to fluorescence. After at least five hours of recovery, cells became spherical and appeared healthier compared to immediately after electroporation. No fluorescence was ever detected for any of the electroporation experiments performed with the pDicty construct. When pDidy 1.0 was tested, fluorescence was observed in a few Pan 2-16 amoebae at a time. The parameters highlighted in table VII indicate the conditions that yielded fluorescence results in Pan 2-16 using the pDidy 1.0 and pPhys plasmids. The fluorescence signal with pDidy 1.0 varied from faint to strong. Figure 23 shows a strong fluorescent signal. However, when pDidy 2.0 was electroporated using the best parameter set

found with pDidy 1.0 no fluorescence was observed. Figure 24 shows fluorescing cells that have been electroporated with the pPhys construct using the same parameters previously mentioned.

Fluorescence was observed using pDidy 1.0 and pPhys at 15 to 25 hours post electroporation. The earliest time at which the pDidy 1.0 construct showed fluorescence was around 15 hours with the signal fading 10 hours after at the 25 hour mark. Fluorescence using the pPhys plasmid was observed at 20 hours while the latest signal was observed fluorescing around 25 hours. While searching for fluorescent cells, cellular and non-cellular debris was also observed. Debris can sometimes give off fluorescent signals due to the interaction of the exciting blue light on the debris. It was relatively easy to determine intact fluorescent cells compared to fluorescing debris based on shape and movement. Figure 23 shows the best image obtained of a fluorescent cell in the same frame as a non-fluorescent cell.

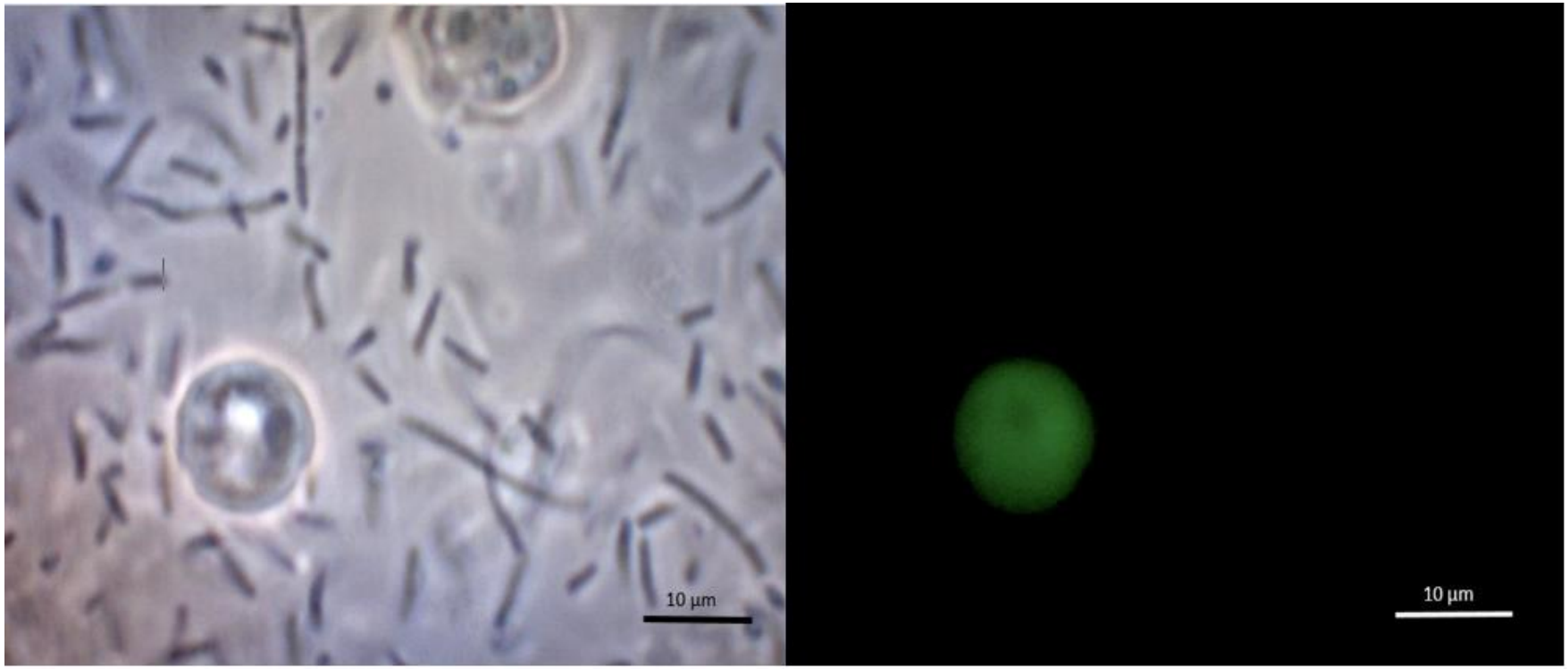


Figure 23. Electroporated Pan 2-16 amoebae with pDidy 1.0 fluorescence observation. A Pan 2-16 amoeba fluorescing 15 hours post electroporation.

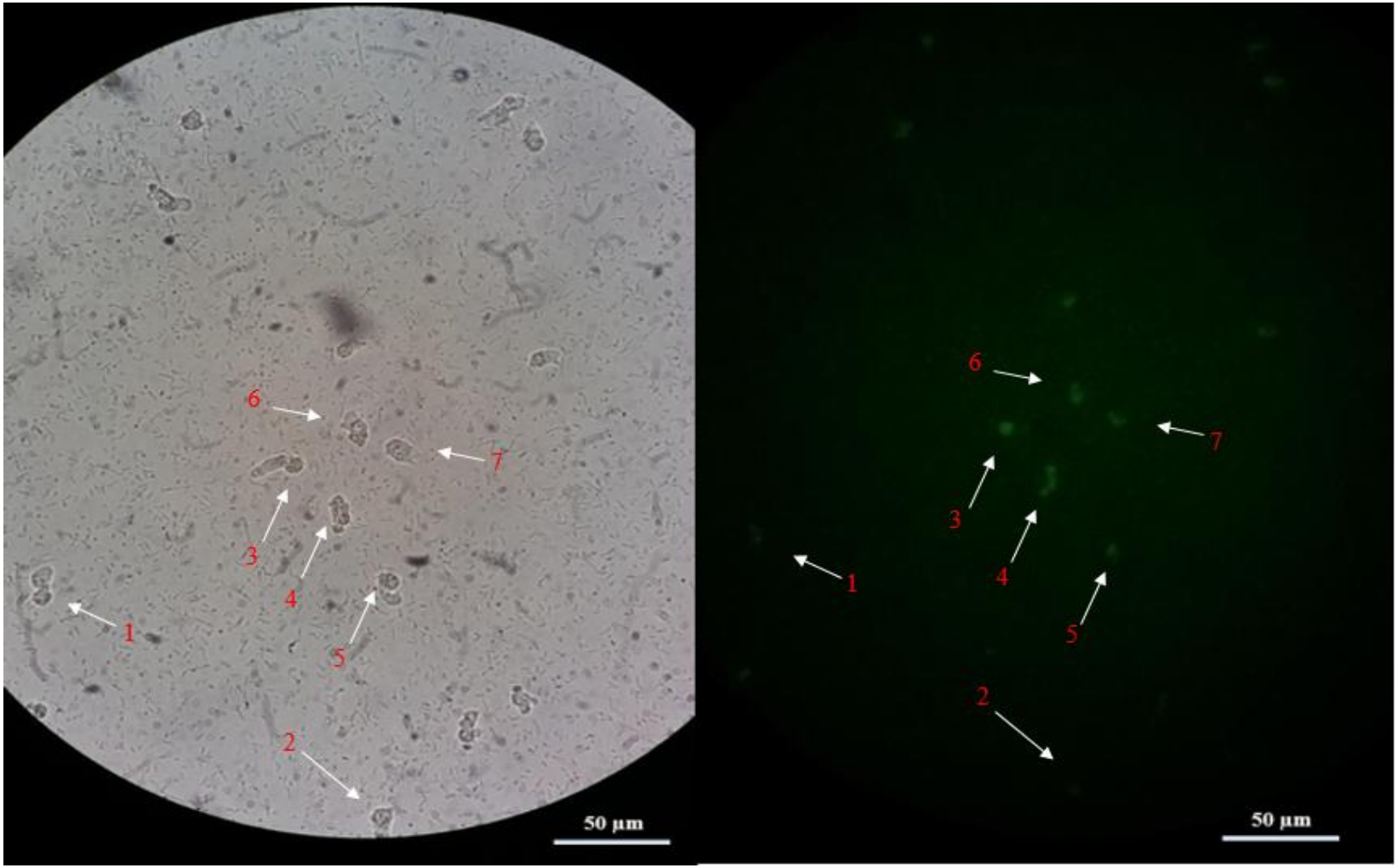


Figure 24. Electroporated Pan 2-16 amoebae with pPhys fluorescence observation. Pan 2-16 amoebae fluorescing 20 hours post electroporation. Arrows 1 and 2 point to non-fluorescing cells, while arrows 3 – 7 point to fluorescing cells.

Lipofection with FuGENE HD

Lipofection parameter testing

The lipofection transfection method involves encasing a plasmid in lipids to form a liposome that can fuse with a cell membrane. The lipofection reagent FuGENE HD was used in this study. Cultures initiated with a starting concentration of 1 - $5x10^5$ cells/mL plateaued at about $4.5x10^6$ cells/mL. For our experiments, cultures were started out at $4.0x10^5$ cells/mL and treated at a cell density of $3.5x10^6$ cells/mL estimated to be 80% of the maximum (stationary phase). The FuGENE HD kit recommends administering liposomes at a range of 50 - 80% of the max cell density (Promega, 2018).

Cells and the lipo-plasmid mixture were incubated overnight at room temperature. Two types of vessels were tested: a rotating 15 mL conical tube and a stationary 25 cm^2 Nunc EasYFlask. The rotating conical tube treatments were unsuccessful, suggesting that the lipofection efficiency was decreased by the constant motion. Pan 2-16 cells tested in a stationary mini-Nunc EasYFlask had sufficient aeration without agitation. Incubation times of 12 and 24 hour exposure were tested. The lipofection incubation time was terminated by removal of FuGENE HD and a changing of the media. Cell observations were made in 5 hour increments.

The various parameters tested are summarized in Table VIII. After preliminary tests, a cell density of 80% was kept constant in all subsequent lipofection experiments. We tested the effect of varying the DNA amount. The 8.8 µg of plasmid DNA worked best with our cell type. Recovery temperatures of 22 and 30℃ were tested. Stationary recovery at 30℃ led to cells clumping together more, while the 22℃ recovery temperature allowed cells to be less clumped and to fluoresce. The incubation time was the last parameter tested with an overnight incubation (12

hours) or 24 hour exposure of the FuGENE HD and plasmid mix. The overnight exposure led to the fluorescence observed.

Table VIII: Lipofection (FuGENE HD) parameters and conditions tested

Parameter	Conditions and Ranges
Percent of max cell density (%) transfected*	50, 65, 80
DNA amount (µg)	4.4, 8.8 and 17.6
Recovery Temperature (°C)	22 and 30
Incubation Vessel	15 mL conical tube (rotating) and Nunc EasYFlask (stationary)
(FuGENE + Pan 2-16) Incubation Time (hrs)	12 and 24

*Max density of Pan 2-16 averages $4.33x10^6$ cells/mL.

Lipofection (FUGENE) fluorescence observations

When observing cells under phase-contrast and fluorescence microscopy, cells appeared in grape like clusters. This allowed for easy cell imaging. For all lipofection experiments, there was a high level of debris scattered throughout in the culture. This debris appeared in the form of globular masses. Unlike electroporation debris, this lipofection debris might have been a result of the FuGENE reagent not being fully spread onto the cells. The mixture was very viscous. This might also explain the cell clumping. These globular masses at times appeared to fluoresce but were easily dismissed as cells because they lacked nuclei and other internal cellular features. The

FuGENE HD reagent possibly makes the cell membrane more fluid which would allow for cell clumping to occur (see figure 25).

Lipofected Pan 2-16 cells were able to fluoresce when pDidy 1.0 and 2.0 plasmids were used. The pDidy 1.0 transfected cells fluoresced brighter compared to the pDidy 2.0 transfected cells. Figure 25 shows a cluster where the right most cell shows high levels of fluorescence. This cell has a nucleus and was mobile while being observed. Fluorescent Pan 2-16 cells lipofected with pDidy 2.0 can be seen in figure 26. A lower magnification was used to obtain a wider field of view to capture non-fluorescing Pan 2-16 cells as well. Both the pDicty and pPhys plasmids did not result in any degree of fluorescence with the FuGENE HD reagent. The timing of fluorescence as a result of the lipofection method with plasmids pDidy 1.0 and 2.0, was on average between 20 and 25 hours, though only few fluorescent cells were found. In both cases, the last observation of fluorescence occurred at hour 25. The level of fluorescence decreased steadily over the five hour period.

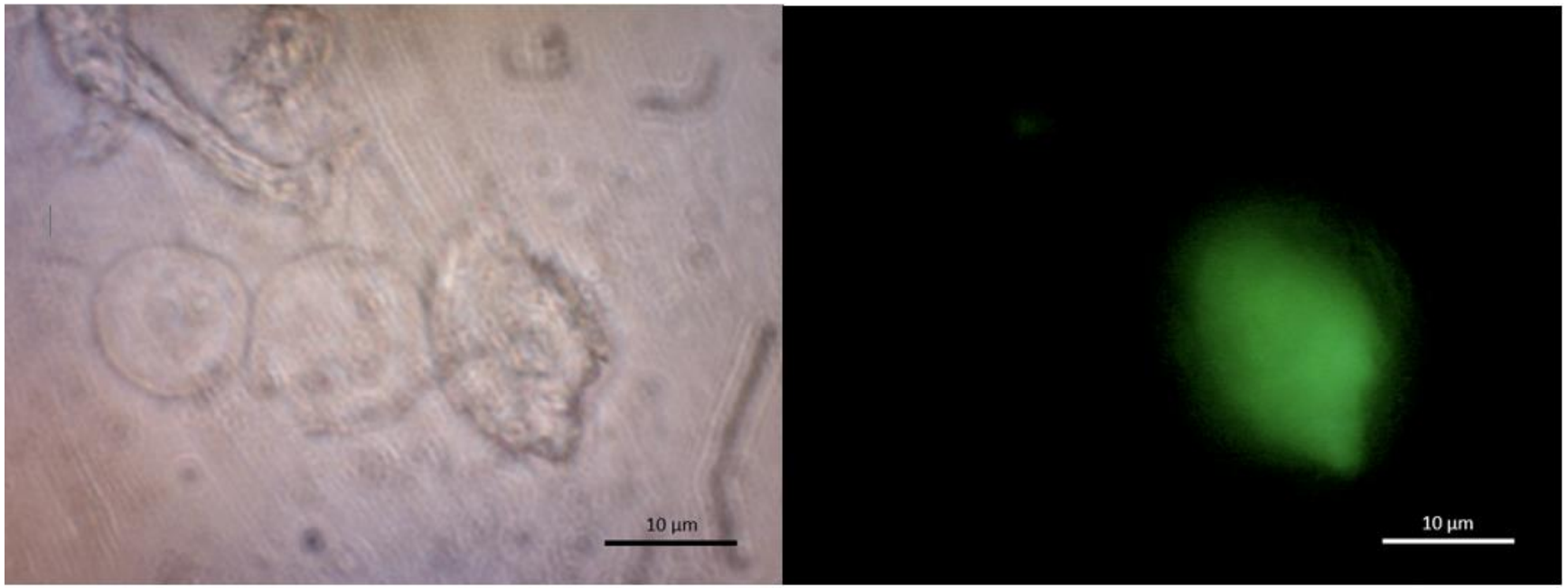


Figure 25. Lipofected Pan 2-16 amoebae with pDidy 1.0 fluorescence observation. Pan 2-16 cells were lipofected using the FuGENE HD reagent with pDidy 1.0. Nuclei can be observed in the three cells along with debris. Fluorescence was observed in the far right cell at 19 hours post exposure to pDidy 1.0.

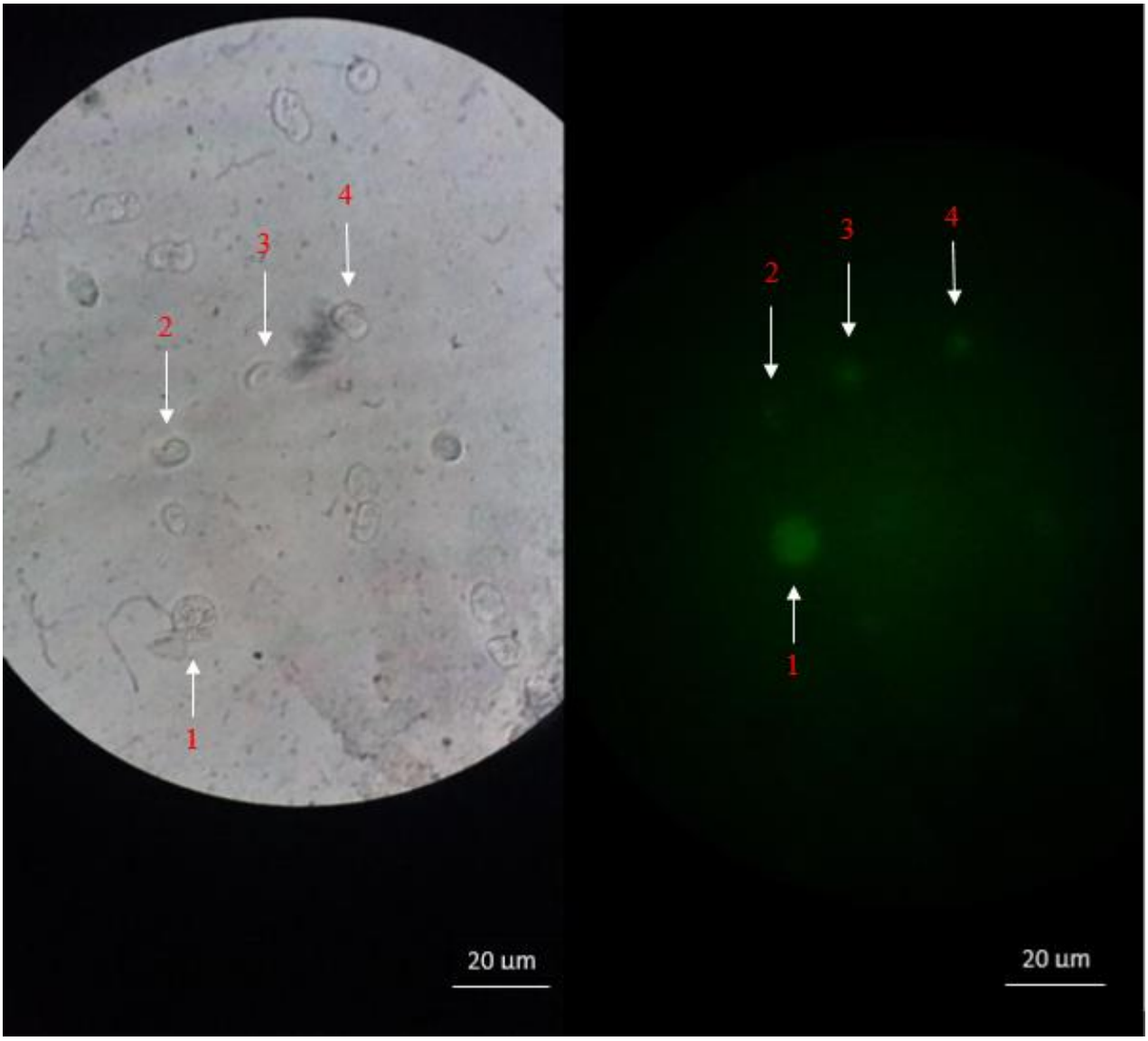


Figure 26. Lipofected pan 2-16 with pDidy 2.0 fluorescence observation. The pDidy 2.0 plasmid was used to lipofect Pan 2-16 amoebae. This image was taken 24 hours post lipofection. Arrows 1 - 4 all point to fluorescing cells.

XFECT Polymer Transfection

XFECT parameter testing

The XFECT polymer transfection method is relatively new and has not been previously tried on Dictyostelium, Physarum or Didymium. Like the lipofection method, the XFECT polymer transfection method uses polymers to complex with plasmids to encase the DNA which is then introduced into target cells by endocytosis. The XFECT kit protocol provided general guidelines for eukaryotic cells; these were modified to better suit working with Didymium. Table IX shows the parameters tested using the XFECT method.

Pan 2-16 cells were grown in rotating culture tubes at room temperature to reach desired cell concentrations. Two cell concentrations were tested with this method: $1.0x10^6$ cells/mL and $2.0x10^6$ cells/mL. Three different incubation vessels were tested in order to determine the best container to provide proper aeration to growing Pan 2-16 cells. Based on the XFECT protocol, 1 mL of cells at $1.0x10^6$ cells/mL were used. The most suitable incubation container was a 50 mL conical tube. A key factor in determining a proper incubation vessel was to spread out the cells for aeration while maintaining a certain density. When rested on its side, the curve of the 50 mL tube kept the 1 mL of cells in a flattened droplet (figure 5). The DNA amounts tested were according to the kit recommendation 5 µg, but 10 µg was also tested. No fluorescence was found with 10µg. The 5 µg amount was successful in yielding fluorescence in a few cells. Incubation time of the [XFECT polymer + DNA] complex and the 1 mL of Pan 2-16 cells was also tested. Three incubation times were tested: 4, 8 and 12 hours. Cells appeared to respond better to the 4 hour incubation time yielding positive fluorescent cells. Since the cells were still actively growing, the 4 hour incubation might have provided the best nutrient availability during plasmid exposure. The

8 and 12 hour incubations did not provide any fluorescent signals. Incubation times were terminated by changing of the media to remove the presence of the XFECT reagent.

Table IX. XFECT parameters and conditions tested

Parameter	Conditions and Ranges
Cell Concentration (cells/mL)	$1.0x10^6$, $2.0x10^6$
DNA amount (µg)	5 and 10
Incubation Vessel	15 and 50 mL conical tube, Nunc EasYFlask
Incubation Time	4, 8 and 12 hours

XFECT polymer fluorescence observations

After exposure to the XFECT treatment, Pan 2-16 cells had a healthy rounded shape. Like the FuGENE reagent, the XFECT reagent had the effect of causing cells to clump together in groups of four to eight cells. A likely similar effect might have had taken place, where the cell membrane became more fluid, thereby allowing cells to stick to one another. About 100 µL of transfected cells were taken and transferred into a microcentrifuge tube, which was then quickly spun to form a loose pellet. From this loose pellet, 15 µL of the loose pellet was placed onto a microscope slide to observe by phase-contrast and fluorescence microscopy. Like with the lipofection results, the only constructs resulting in fluorescence were pDidy 1.0 and 2.0.

The highlighted parameters shown in table IX are those that yielded fluorescent cells with both the pDidy plasmids. Observations were made between four and five hours post exposure due to the smaller incubation time for the XFECT method. The constructs pDicty and pPhys did not

result in fluorescent cells. In all cases, if a fluorescent signal was observed, it was faint. Figure 27 shows two cells with a moderate degree of fluorescence. Figure 28 shows cells transfected with pDidy 2.0, though faint, multiple cells fluoresced. The average time range for GFP expression using XFECT polymer transfection was 24 to 28 hours for pDidy 1.0. On the other hand, the pDidy 2.0 plasmid had an expression window of 22 to 30 hours. In both cases, the window of time when cells were fluorescing was nearly identical. Between the two plasmids, qualitatively pDidy 2.0 gave rise to slightly more transformants compared to pDidy 1.0 but the signals were very faint for both constructs.

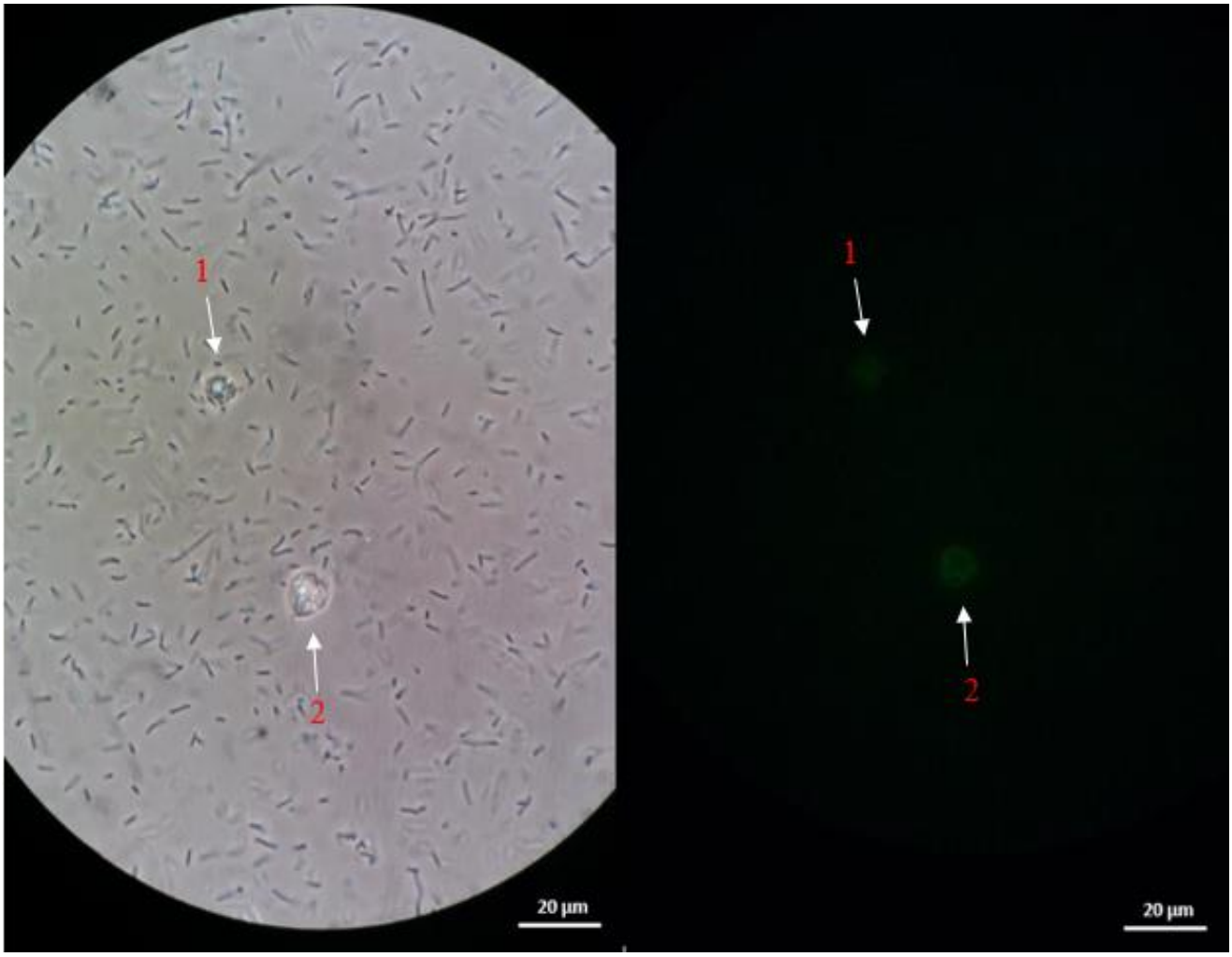


Figure 27. Fluorescence observation of XFECT transfected pDidy 1.0. Two amoebae fluorescing 24 hours post transfection. Arrows 1 and 2 point to fluorescing cells.

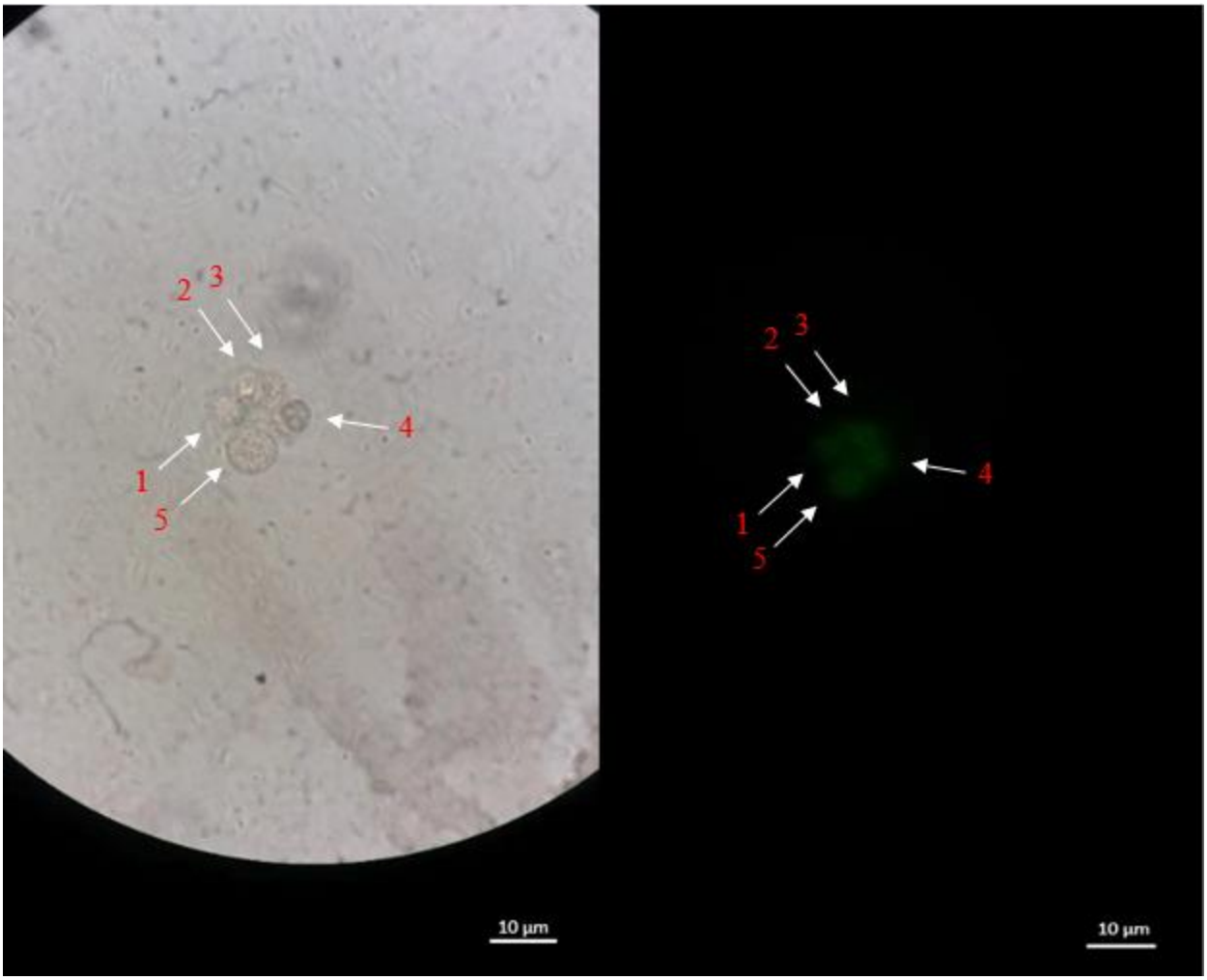


Figure 28. XFECT polymer transfected Pan 2-16 with pDidy 2.0 fluorescence observation. Pan 2-16 amoebae can be observed clumped together and expressing a faint fluorescence. Debris can be observed surrounding the cells. The image was taken 22 hours after FuGENE HD and plasmid exposure. Arrows 1-5 point to individually fluorescing cells.

Comparison of transfection methods

Table X summarizes the results of the transfection methods and construct combinations. The pDicty plasmid did not result in fluorescent cells with any method. The pPhys construct gave a weak fluorescence signal when introduced by electroporation, but not with any other methods. Fluorescent cells were observed with pDidy 1.0 with all three transfection methods. These rare fluorescent cells found using pDidy 1.0 ranged from a mildly to strongly fluorescence. The pDidy 2.0 construct only gave positive results with lipofection and the XFECT method.

Table X. Summary of fluorescence results

Plasmid	**Transfection method**		
	Lipofection	**Electroporation**	**XFECT**
pDicty	--	--	--
pPhys	--	+	--
pDidy 1.0	+	+	+
pDidy 2.0	+	--	+

DISCUSSION

All of the constructed myxomycete plasmids, pDidy 1.0, pDidy 2.0 and pPhys, had some degree of GFP expression. The plasmid pDicty was not successful with any transfection method used. This could be due to Dictyostelium regulatory elements not being recognized in Didymium or the method of introduction not being efficient. Dictyostelium and Didymium belong to the same phylum, Mycetozoa, but different classes. This may be too great of a distance for cross recognition. The pPhys construct was weakly expressed when introduced by electroporation. Although weakly expressed, this is evidence for recognition of Physarum regulatory elements in Didymium amoebae. The transfection efficiency was extremely low using electroporation, which consistent with an earlier study in Physarum (Burland and Pallotta**,** 1995). The pDidy 1.0 plasmid showed the highest degree of fluorescence (see figures 23, 25 and 27). Comparisons are difficult due to the weak and infrequent fluorescence observed. Given these results, the level of fluorescence was not quantified. Plasmid size may have been a factor in transfection efficiency, where larger plasmids may be harder to transfect. The pDicty plasmid is 6,736 bp compared to the smaller size of the successfully transfected pDidy1.0 (3,953 bp), 2.0 (3,895 bp), and pPhys (6,725 bp) plasmids.

Apart from size of the constructs, mutations were identified in our constructed plasmids which could have affected gene expression. Two constructs had mutations: pDidy 2.0 and pPhys. The pDidy 2.0 mutations were in less critical regions of the regulatory elements far from the coding region of *maxgfp*. On the other hand, pPhys had multiple mutations upstream and downstream of the *maxgfp* coding region. After sequencing the pPhys construct, we found some cloning vector sequence (about 290 bp) in the region we called *TardC*. We verified the location of the poly A adenylation site (see figure 19) within our *TardC* fragment using the NCBI database. *PardC* and *TardC*, including the contaminating vector sequence, worked in obtaining stable and transient

expression in Physarum (Burland et al., 1993). The low GFP expression levels observed with pPhys could possibly be attributed to the mutations introduced during pPhys construction. The pDidy 1.0 plasmid had no mutations in the profilin A regulatory elements used to drive *gfp* and resulted in the strongest fluorescent signal.

This project focused on transient expression of a reporter gene *gfp*. Given the low transfection efficiency, an alternative approach would be to try for integration paired with a selectable marker. Modifying our plasmids to include an antibiotic resistance gene would allow for selection of rare transformants. A novel transfection method successful in Dictyostelium is restriction enzyme-mediated integration, or REMI (Kuspa and Loomis, 1992). This method uses restriction enzymes to cut specific restriction sites on both a plasmid and the genome of the organism of interest. The plasmid and enzyme are introduced by electroporation. A disadvantage associated with this method is the randomness of insertion and the potential to cause many cuts in the genome rendering the cell nonviable.

An unusual feature of the myxomycetes that might be exploited in transfection studies is their minichromosomes. Minichromosomes are naturally occurring small chromosomes that can autonomously replicate and could be modified to carry specific selectable markers. The nuclear ribosomal genes in Didymium (Silliker and Collins, 1988) and Physarum (Campbell et al., 1979) are present on numerous autonomously replicating minichromosomes. In Physarum, the minichromosomes are 60 kb (Ferris and Vogt, 1982), but in Didymium the minichromosomes are as small as 20 kb (Johansen et al., 1992). If a selectable marker is added to a minichromosome it might be transfected by our methods, and once introduced stably maintained.

Another approach to be considered is performing transfections in a diploid Didymium plasmodium by microinjections. However, since our Didymium constructs have the *ProA*

regulatory elements, expressed only in the haploid amoebal phase, *ProP* regulatory elements would have to be used to drive expression in the diploid phase. Unlike amoebae, plasmodia may be more be more difficult to deal with since they are thicker due layers of slime. This could potentially cause issues when testing exogenous DNA introduction. However, transfection into diploid Physarum plasmodia has been shown to be possible when using electroporation (Liu et al, 2009).

Finally in its natural habitat Didymium is a microscopic predator, feeding on fungi, bacteria and anything else that is smaller than itself. Introducing exogenous DNA into Didymium using natural mechanisms is a possibility as well. The method of natural transfection by bacterial feeding has been successful in the model organism *Caenorhabditis elegans* (Lezzerini et al., 2015). Providing Didymium with transformed bacteria could potentially lead to both a transient or stable expression of our constructs.

This project tested DNA constructs with regulatory elements from close relatives of Didymium and Didymium, by introducing them into Pan 2-16 cells using electroporation, FuGENE HD lipofection and XFECT polymer transfection. We demonstrated that regulatory elements from either Physarum or Didymium could drive the expression of a foreign reporter gene in Didymium. Consistent with earlier studies in Physarum, myxomycete amoebae appear resistant to conventional transfection methods. Successful myxomycete transfection appears to require the development of novel methodologies.

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APPENDICES

Appendix A: pPhys sequence

Color coded to match figure 17.
Size: **6,725 bp**

PardC* (promoter)** ***maxgfp ***TardC* (terminator)**

```
   1 AGCGCCCAAT ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA
  51 TGCAGCTGGC ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA
 101 CGCAATTAAT GTGAGTTAGC TCACTCATTA GGCACCCCAG GCTTTACACT
 151 TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT
 201 CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTTG GTACCGAGCT
 251 CGGATCCACT AGTAACGGCC GCCAGTGTGC TGGAATTCGG CTTGAGCTCG
 301 GTACGGATCT CCACACTATT GCACATGCTA CCGTAATCAA TTATAGGCCG
 351 AAATCACTCT ATCAACTCAC CCCGAGCGGT GTACACTCAC TCCACTCACA
 401 ATCACACTTA ATCACATCTT CACCTTGTTC ATTTGTGCAT GTTTACTAGC
 451 GCATGTTCGC CCCACATGCT CACAATCATC ACTCCACTCA CTCTTGGCGC
 501 TAGCCATTAC ATACCTTGAA TTAGCAAAAC TTTACGCAAA TGTAGCTAGA
 551 GAATGAGTAC AAAGGGGAAA GGAAGTAATA AGTGAAAGTG GAATGGAAAA
 601 TGGGCAATGG GAAACGTGGT TAGGGATGTA TGTGGCTAGG ATATGGATAG
 651 GACGTATTGG GGAGGGAGTG ATAGGATAGA GGATATGGTA TGACAGTGCG
 701 AGCGTGATAT ATGAGACATG TAGCTAGTAA GCCGGAGGGA AGGATAAAAT
 751 CACAAGGGGA GTATAAAGGG AGGAAGTACA ACAACAATAC AACAAGAAAA
 801 CATGGACTAT GAGATGAAAA ACATTGTGTG CTTATGTAGA ACTAGTTAAA
 851 AACACGGACA AAAAGGTGAA CAAATACGAT TAGAACACAT ACAGAGGCGG
 901 TACAACAACA AAGCAACAGA GAAAATGTGA GATTCTGAAA TGAGAACGAG
 951 GAGAAGGACG ATCAGCTAAG TGATGGAGTG GAAAAGTTAT GATATGGGGA
1001 AAAAACACGG ATAGAAAGGT GAAGCTATAG AGACATGAAG GGAAAGCAAA
1051 AACAAAGAAA AAACATGCGG TTCTGAAATG AGAATGGGGG AGAAGGGCAA
1101 TCAGCTAAGT GATGGAGGAG AAGTTAGTGC TGGCACGTTG GTGAAGAGCG
1151 CCCCCAGACC TGTGGACCGG CATTTACGCA CGTTTTACGC ACGATTTACG
1201 CCAAATTTTC GGAGCGGTTG CGAAATTTGC CTTTTCGGGG TAAAATTTAC
1251 ACAACTTTTA CGCACTTTTT CGGTTCTGAT TTTTGCATTG GCAGGTGCGA
1301 AATGATTGGG GAGTGGAACA GATAAAAGGG TGAAACGCGT CGCTCTTCTT
1351 TGTGTTTAGC AAAACAACAA AACAAACAAA CCGCAATGGA AGGAGAAGAC
1401 GTTCAAGCTA TGCCCGCCAT GAAGATCGAG TGCCGCATCA CCGGCACCCT
1451 GAACGGCGTG GAGTTCGAGC TGGTGGGCGG CGGAGAGGGC ACCCCCGAGC
1501 AGGGCCGCAT GACCAACAAG ATGAAGAGCA CCAAAGGCGC CCTGACCTTC
1551 AGCCCCTACC TGCTGAGCCA CGTGATGGGC TACGGCTTCT ACCACTTCGG
1601 CACCTACCCC AGCGGCTACG AGAACCCCTT CCTGCACGCC ATCAACAACG
1651 GCGGCTACAC CAACACCCGC ATCGAGAAGT ACGAGGACGG CGGCGTGCTG
1701 CACGTGAGCT TCAGCTACCG CTACGAGGCC GGCCGCGTGA TCGGCGACTT
1751 CAAGGTGGTG GGCACCGGCT TCCCCGAGGA CAGCGTGATC TTCACCGACA
1801 AGATCATCCG CAGCAACGCC ACCGTGGAGC ACCTGCACCC CATGGGCGAT
1851 AACGTGCTGG TGGGCAGCTT CGCCCGCACC TTCAGCCTGC GCGACGGCGG
1901 CTACTACAGC TTCGTGGTGG ACAGCCACAT GCACTTCAAG AGCGCCATCC
1951 ACCCCAGCAT CCTGCAGAAC GGGGGCCCCA TGTTCGCCTT CCGCCGCGTG
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2001 GAGGAGCTGC ACAGCAACAC CGAGCTGGGC ATCGTGGAGT ACCAGCACGC
2051 CTTCAAGACC CCCATCGCCT TCGCCAGATC TCGAGCTCGA TGAAGTAGAT
2101 GCCGACCGAA CAAGAGCTGA TTTCGAGAAC GCCTCAGCCA GCAACTCGCG
2151 CGAGCCTAGC AAGGCAAATG CGAGAGAACG GCCTTACGCT TGGTGGCACA
2201 GTTCTCGTCC ACAGTTCGCT AAGCTCGCTC GGCTGGGTCG CGGGAGGGCC
2251 GGTCGCAGTG ATTCAGGCCC TTCTGGATTG TGTTGGTCCC CAGGGCACGA
2301 TTGTCATGCC CACGCACTCG GGTGATCTGA CTGATCCCGC AGATTGGAGA
2351 TCGCCGCCCG TGCCTGCCGA TTGGGTGCAG ATCCCGACGC ACCCGTAGTC
2401 GCTCCCATTG CTTAAGTTGT TTGCTACTCG CCCGTCAATG AAGTGTCCCT
2451 TTACTTCCTT GTTGTAATTG CATCCTTCAA CCACTCTCGG CTAGATAGTG
2501 TTTCTTGCCG TTTGTGTTTG TTGGGGCTGT AAATATATAC AAGTTAAACA
2551 AAATTTTTGT TCTACCCTCC CACAAGCCAA GTCTTGATTT TCTCGCCTTT
2601 CGTGAACTAA AAAGCCAAGT CTCAATTTTC TCGCCTTTAA AGTAATCTAA
2651 AATGCTATGT TGATGTTCTC ACTTTTCGTA CACGACGGGG CTACACTTTG
2701 TTTTTATAAA AAAACTAGTT TTTACGCTTA CCGTAGCTGA TAGTGAATGC
2751 CCCCTCCCCC TCGGATTAGA TCCTCGCTCC CCAACCTCCT TGAGTTGGTG
2801 ATGCGCGTGT CTTTTGATTT TTTGATTCAT TTATTTATTG GCTTTTTTAA
2851 TTTTTTATAT ATTTATTTAT TTATTTTTTT TTATACAAGT TTGCGAGGAC
2901 GCGAAAATAG GAGCAGGGGG GGGGGTAAAT AATTGCATTG TATGGAGCAT
2951 GCATTGTGCA TTGTATTATT GAAATGTCTA AAGCCGATGT CGCGGCTATA
3001 TTGTGTGGGG AGAATGTGAA TTCCGGTACC GAATTCCTCG AGTCTAGAGG
3051 AGCATGCGAC GTCTACCGCG AGACCCGGCA TGCAAGCTTA AGCCGAATTC
3101 TGCAGATATC CATCACACTG GCGGCCGCTC GAGCATGCAT CTAGAGGGCC
3151 CAATTCGCCC TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC
3201 AACGTCGTGA CTGGGAAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA
3251 GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA
3301 TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG ACGCGCCCTG
3351 TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT GGTTACGCGC AGCGTGACCG
3401 CTACACTTGC CAGCGCCCTA GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC
3451 TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT CAAGCTCTAA ATCGGGGGCT
3501 CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC CCCAAAAAAC
3551 TTGATTAGGG TGATGGTTCA CGTAGTGGGC CATCGCCCTG ATAGACGGTT
3601 TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT
3651 CCAAACTGGA ACAACACTCA ACCCTATCTC GGTCTATTCT TTTGATTTAT
3701 AAGGGATTTT GCCGATTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA
3751 CAAAAATTTA ACGCGAATTT TAACAAAATT CAGGGCGCAA GGGCTGCTAA
3801 AGGAAGCGGA ACACGTAGAA AGCCAGTCCG CAGAAACGGT GCTGACCCCG
3851 GATGAATGTC AGCTACTGGG CTATCTGGAC AAGGGAAAAC GCAAGCGCAA
3901 AGAGAAAGCA GGTAGCTTGC AGTGGGCTTA CATGGCGATA GCTAGACTGG
3951 GCGGTTTTAT GGACAGCAAG CGAACCGGAA TTGCCAGCTG GGGCGCCCTC
4001 TGGTAAGGTT GGGAAGCCCT GCAAAGTAAA CTGGATGGCT TTCTTGCCGC
4051 CAAGGATCTG ATGGCGCAGG GGATCAAGAT CTGATCAAGA GACAGGATGA
4101 GGATCGTTTC GCATGATTGA ACAAGATGGA TTGCACGCAG GTTCTCCGGC
4151 CGCTTGGGTG GAGAGGCTAT TCGGCTATGA CTGGGCACAA CAGACAATCG
4201 GCTGCTCTGA TGCCGCCGTG TTCCGGCTGT CAGCGCAGGG GCGCCCGGTT
4251 CTTTTTGTCA AGACCGACCT GTCCGGTGCC CTGAATGAAC TGCAGGACGA
4301 GGCAGCGCGG CTATCGTGGC TGGCCACGAC GGGCGTTCCT TGCGCAGCTG
4351 TGCTCGACGT TGTCACTGAA GCGGGAAGGG ACTGGCTGCT ATTGGGCGAA
4401 GTGCCGGGGC AGGATCTCCT GTCATCCCAC CTTGCTCCTG CCGAGAAAGT
4451 ATCCATCATG GCTGATGCAA TGCGGCGGCT GCATACGCTT GATCCGGCTA
4501 CCTGCCCATT CGACCACCAA GCGAAACATC GCATCGAGCG AGCACGTACT
4551 CGGATGGAAG CCGGTCTTGT CGATCAGGAT GATCTGGACG AAGAGCATCA
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4601 GGGGCTCGCG CCAGCCGAAC TGTTCGCCAG GCTCAAGGCG CGCATGCCCG
4651 ACGGCGAGGA TCTCGTCGTG ACCCATGGCG ATGCCTGCTT GCCGAATATC
4701 ATGGTGGAAA ATGGCCGCTT TTCTGGATTC ATCGACTGTG GCCGGCTGGG
4751 TGTGGCGGAC CGCTATCAGG ACATAGCGTT GGCTACCCGT GATATTGCTG
4801 AAGAGCTTGG CGGCGAATGG GCTGACCGCT TCCTCGTGCT TTACGGTATC
4851 GCCGCTCCCG ATTCGCAGCG CATCGCCTTC TATCGCCTTC TTGACGAGTT
4901 CTTCTGAATT GAAAAAGGAA GAGTATGAGT ATTCAACATT TCCGTGTCGC
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5001 AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG TGCACGAGTG
5051 GGTTACATCG AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTTCG
5101 CCCCGAAGAA CGTTTTCCAA TGATGAGCAC TTTTAAAGTT CTGCTATGTG
5151 GCGCGGTATT ATCCCGTATT GACGCCGGGC AAGAGCAACT CGGTCGCCGC
5201 ATACACTATT CTCAGAATGA CTTGGTTGAG TACTCACCAG TCACAGAAAA
5251 GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT GCTGCCATAA
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5351 CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG
5401 CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC
5451 GTGACACCAC GATGCCTGTA GCAATGGCAA CAACGTTGCG CAAACTATTA
5501 ACTGGCGAAC TACTTACTCT AGCTTCCCGG CAACAATTAA TAGACTGGAT
5551 GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC CTTCCGGCTG
5601 GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG GTCTCGCGGT
5651 ATCATTGCAG CACTGGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT
5701 CTACACGACG GGGAGTCAGG CAACTATGGA TGAACGAAAT AGACAGATCG
5751 CTGAGATAGG TGCCTCACTG ATTAAGCATT GGTAACTGTC AGACCAAGTT
5801 TACTCATATA TACTTTAGAT TGATTTAAAA CTTCATTTTT AATTTAAAAG
5851 GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA ATCCCTTAAC
5901 GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA GATCAAAGGA
5951 TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA
6001 AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA GAGCTACCAA
6051 CTCTTTTTCC GAAGGTAACT GGCTTCAGCA GAGCGCAGAT ACCAAATACT
6101 GTTCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA ACTCTGTAGC
6151 ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG GCTGCTGCCA
6201 GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG ATAGTTACCG
6251 GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG
6301 CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG CGTGAGCTAT
6351 GAGAAAGCGC CACGCTTCCC GAAGGGAGAA AGGCGGACAG GTATCCGGTA
6401 AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC CAGGGGGAAA
6451 CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC TGACTTGAGC
6501 GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG GAAAAACGCC
6551 AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC CTTTTGCTCA
6601 CATGTTCTTT CCTGCGTTAT CCCCTGATTC TGTGGATAAC CGTATTACCG
6651 CCTTTGAGTG AGCTGATACC GCTCGCCGCA GCCGAACGAC CGAGCGCAGC
6701 GAGTCAGTGA GCGAGGAAGC GGAAG

Appendix B: pDidy 1.0 sequence

Color coded to match figure 8.
Size: **3,953 bp**

ProAup (promoter) ***gfp*** **ProAdown (terminator)**

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   1 CCCCGTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA TCTGGCCCCA
  51 GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA
 101 GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC
 151 TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA
 201 GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC
 251 ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC
 301 CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG
 351 TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG
 401 TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC
 451 ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT
 501 GAGAATAGTG TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG
 551 GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA TCATTGGAAA
 601 ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA
 651 GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT
 701 TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA
 751 AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT
 801 TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC
 851 ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC CGCGCACATT
 901 TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT ATCATGACAT
 951 TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTCT CGCGCGTTTC
1001 GGTGATGACG GTGAAAACCT CTGACACATG CAGCTCCCGG AGACGGTCAC
1051 AGCTTGTCTG TAAGCGGATG CCGGGAGCAG ACAAGCCCGT CAGGGCGCGT
1101 CAGCGGGTGT TGGCGGGTGT CGGGGCTGGC TTAACTATGC GGCATCAGAG
1151 CAGATTGTAC TGAGAGTGCA CCATATGCGG TGTGAAATAC CGCACAGATG
1201 CGTAAGGAGA AAATACCGCA TCAGGCGCCA TTCGCCATTC AGGCTGCGCA
1251 ACTGTTGGGA AGGGCGATCG GTGCGGGCCT CTTCGCTATT ACGCCAGCTG
1301 GCGAAAGGGG GATGTGCTGC AAGGCGATTA AGTTGGGTAA CGCCAGGGTT
1351 TTCCCAGTCA CGACGTTGTA AAACGACGGC CAGTGAATTC GAGCTCGGTA
1401 CAAATTGACC CAAAGGTAAC TTTCAACGTG TCAATCAGAC GTAACGGCTC
1451 TCACACACTT TTTTTGCATT GGGAGCCAAT CGAAAAGCCA TTCTTAACCG
1501 GTTAAGAATG GGTTAAAAAT GGGTGACCCT AACCGGTCCG ACCGTGTAAA
1551 AGGCTCAATT TATTGCGTTT TTGCAGTAAC CGTCTTACCA CAACACCACC
1601 AATCAAAGGT AACTTTCTCT CAGAAATATA TTGCTGATCA CTCCCTTTCT
1651 TTTGTGATCC TCACTCCCAC TTGACCAACC ACTCATCCTT TAATCACTAT
1701 AGATGGCTAG CAAAGGAGAA GAACTTTTCA CTGGAGTTGT CCCAATTCTT
1751 GTTGAATTAG ATGGTGATGT TAATGGGCAC AAATTTTCTG TCAGTGGAGA
1801 GGGTGAAGGT GATGCTACAT ACGGAAAGCT TACCCTTAAA TTTATTTGCA
1851 CTACTGGAAA ACTACCTGTT CCATGGCCAA CACTTGTCAC TACTTTCTCT
1901 TATGGTGTTC AATGCTTTTC CCGTTATCCG GATCATATGA AACGGCATGA
1951 CTTTTTCAAG AGTGCCATGC CCGAAGGTTA TGTACAGGAA CGCACTATAT
2001 CTTTCAAAGA TGACGGGAAC TACAAGACGC GTGCTGAAGT CAAGTTTGAA
2051 GGTGATACCC TTGTTAATCG TATCGAGTTA AAAGGTATTG ATTTTAAAGA
2101 AGATGGAAAC ATTCTCGGAC ACAAACTCGA GTACAACTAT AACTCACACA
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2151 ATGTATACAT CACGGCAGAC AAACAAAAGA ATGGAATCAA AGCTAACTTC
2201 AAAATTCGCC ACAACATTGA AGATGGATCC GTTCAACTAG CAGACCATTA
2251 TCAACAAAAT ACTCCAATTG GCGATGGCCC TGTCCTTTTA CCAGACAACC
2301 ATTACCTGTC GACACAATCT GCCCTTTCGA AAGATCCCAA CGAAAAGCGT
2351 GACCACATGG TCCTTCTTGA GTTTGTAACT GCTGCTGGGA TTACACATGG
2401 CATGGATGAG CTCTACAAAT AAATTATTGT CTATTTAGTA AATAATTTCT
2451 GTAAAATTTA AAAAAATATA ATATAAAAAC ATCAGTACAT AATTTCAGTC
2501 TTGCACTTTT TAGTTATATT AATAGATCCA AGGGGAATAA AATGGAGATA
2551 AAGAGATTAG AAGAAGAATT GGGATAGCAA AGCCTTTTTC CGTGGCAGGT
2601 GGTAATTTTT TCTCATGTCA AAAGTAAAGG TTATATTAAT ATTATCATAA
2651 ATAATAATAA AAAAAGACTT TTTGGCATGC AAGCTTGGCG TAATCGGCAT
2701 GCAAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT GAAATTGTTA
2751 TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG
2801 CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC GTTGCGCTCA
2851 CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT
2901 CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC TCTTCCGCTT
2951 CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA
3001 TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA
3051 CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA
3101 AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG
3151 CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT
3201 ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG
3251 TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA
3301 AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA GTTCGGTGTA
3351 GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG
3401 ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA
3451 CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC
3501 GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG
3551 GCTACACTAG AAGAACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT
3601 ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC
3651 TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA
3701 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG
3751 TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG
3801 GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT
3851 AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT
3901 GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG
3951 ACT
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Appendix C: pDidy 2.0 sequence

Color coded to match figure 12.

Size: **3,895 bp**

ProAup (promoter) ***maxgfp*** **ProAdown (terminator)**

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   1 TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG
  51 ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT
 101 CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC
 151 TAGAGTAAGT AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG
 201 CTACAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC
 251 TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA
 301 AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG
 351 CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT
 401 GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA
 451 GTCATTCTGA GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCGGCGT
 501 CAATACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC
 551 ATTGGAAAAC GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
 601 GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT
 651 CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT
 701 GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT
 751 CTTCCTTTTT CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA
 801 GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG
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 901 CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTCG
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1051 GGGCGCGTCA GCGGGTGTTG GCGGGTGTCG GGGCTGGCTT AACTATGCGG
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1401 AACGGCTCTC ACACACTTTT TTTGCATTGG GAGCCAATCG AAAAGCCATT
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1501 CGTGTAAAAG GCTCAATTTA TTGCGTTTTT GCAGTAACCG TCTTACCACA
1551 ACACCACCAA TCAAAGGTAA CTTTCTCTCA GAAATATATT GCTGATCACT
1601 CCCTTTCTTT TGTGATCCTC ACTCCCACTT GACCAACCAC TCATCCTTTA
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1751 CAGGGCCGCA TGACCAACAA GATGAAGAGC ACCAAAGGCG CCCTGACCTT
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1851 GCACCTACCC CAGCGGCTAC GAGAACCCCT TCCTGCACGC CATCAACAAC
1901 GGCGGCTACA CCAACACCCG CATCGAGAAG TACGAGGACG GCGGCGTGCT
1951 GCACGTGAGC TTCAGCTACC GCTACGAGGC CGGCCGCGTG ATCGGCGACT
2001 TCAAGGTGGT GGGCACCGGC TTCCCCGAGG ACAGCGTGAT CTTCACCGAC
2051 AAGATCATCC GCAGCAACGC CACCGTGGAG CACCTGCACC CCATGGGCGA
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2401 ACATCAGTAC ATAATTTCAG TCTTGCACTT TTTAGTTATA TTAATAGATC
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3501 ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC
3551 TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA
3601 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG
3651 TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG
3701 GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT
3751 AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT
3801 GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG
3851 ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA CCATC
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